Atypical Rho GTPases Have Roles in Mitochondrial Homeostasis and Apoptosis*

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The human genomic sequencing effort has revealed the presence of a large number of Rho GTPases encoded by the human genome. Here we report the characterization of a new family of Rho GTPases with atypical features. These proteins, which include Miro-1 and Miro-2 (for mitochondrial Rho), have tandem GTP-binding domains separated by a linker region containing putative calcium-binding EF hand motifs. Genes encoding Miro-like proteins were found in several eukaryotic organisms from Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila melanogaster to mammals, indicating that these genes evolved early during evolution. Immunolocalization experiments, in which transfected NIH3T3 and COS 7 cells were stained for ectopically expressed Miro as well as for the endogenous Miro-1 protein, showed that Miro was present in mitochondria. Interestingly, overexpression of a constitutively active mutant of Miro-1 (Miro-1/Val-13) induced an aggregation of the mitochondrial network and resulted in an increased apoptotic rate of the cells expressing activated Miro-1. These data indicate a novel role for Rho-like GTPases in mitochondrial homeostasis and apoptosis.

Small GTPases have been shown to be pivotal signaling intermediates in cell growth, cell cycle progression, cell survival, cell transformation, and cell trafficking (1–3). The potential of small GTPases to function as signaling switches resides in their ability to cycle between active, GTP-bound states, and inactive, GDP-bound states. This cycling is regulated in a precise manner by guanine nucleotide exchange factors (4), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (GDIs)1 (5, 6). Guanine nucleotide exchange factors stimulate the replacement of GDP by GTP, whereas GTPase-activating proteins stimulate the intrinsic GTP hydrolysis of the GTPase. GDIs act by blocking GDP dissociation, and in resting cells, Rho GTPases are thought to reside in an inactive, GDP-bound state. GDIs act by blocking GDP dissociation, and in resting cells, Rho GTPases are thought to reside in an inactive, GDP-bound state. This cycling is regulated in a precise manner by guanine nucleotide exchange factors (4), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (GDIs)1 (5, 6). Guanine nucleotide exchange factors stimulate the replacement of GDP by GTP, whereas GTPase-activating proteins stimulate the intrinsic GTP hydrolysis of the GTPase. GDIs act by blocking GDP dissociation, and in resting cells, Rho GTPases are thought to reside in an inactive, GDP-bound state. GDIs act by blocking GDP dissociation, and in resting cells, Rho GTPases are thought to reside in an inactive, GDP-bound state. This cycling is regulated in a precise manner by guanine nucleotide exchange factors (4), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors (GDIs)1 (5, 6). Guanine nucleotide exchange factors stimulate the replacement of GDP by GTP, whereas GTPase-activating proteins stimulate the intrinsic GTP hydrolysis of the GTPase. GDIs act by blocking GDP dissociation, and in resting cells, Rho GTPases are thought to reside in an inactive, GDP-bound state.
EXPERIMENTAL PROCEDURES

DNA Work—Miro-1 and Miro-2 were identified by searching the public DNA and protein data bases for novel members of the Rho GTPases employing the Blast search motor. The translated amino acid sequence for Miro-1 and Miro-2 were then used to search EST data bases for the presence of EST clones encoding putative full-length Miro-1 and Miro-2, and two such clones were obtained from the UK Human Genome Mapping Project Resource Centre in Hinxton, Cambridge, UK. The nucleotide sequences of Miro-1 and -2 have been deposited in the GenBankTM data base with accession numbers AJ517412 (Miro-1) and AJ517413 (Miro-2). DNA fragments encoding full-length Miro-1 and Miro-2 were generated by PCR and subcloned into the pRK5Myc vector. The Miro-1/Val-13 and Miro-1/Asn-18 mutants were generated employing the QuickChange protocol (Stratagene) according to the procedure provided by the manufacturer. The fidelity of all DNA constructs was confirmed by DNA sequencing employing the ABI Prism 310 Genetic Analyzer.

Northern Blot Analysis—cDNA probes of the open reading frame of Miro-1 and Miro-2 were used to search EST data bases for the presence of EST clones encoding putative full-length Miro-1 and Miro-2, and two such clones were obtained from the UK Human Genome Mapping Project Resource Centre in Hinxton, Cambridge, UK. The nucleotide sequences of Miro-1 and -2 have been deposited in the GenBankTM data base with accession numbers AJ517412 (Miro-1) and AJ517413 (Miro-2). DNA fragments encoding full-length Miro-1 and Miro-2 were generated by PCR and subcloned into the pRK5Myc vector. The Miro-1/Val-13 and Miro-1/Asn-18 mutants were generated employing the QuickChange protocol (Stratagene) according to the procedure provided by the manufacturer. The fidelity of all DNA constructs was confirmed by DNA sequencing employing the ABI Prism 310 Genetic Analyzer.

Cell Cultivation and Cell Transformation—NIH3T3 cells and COS 7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS and penicillin/streptomycin at 37 °C in an atmosphere of 5% CO₂. For immunostaining purposes, cells were seeded on coverslips and transfected by LipofectAMINE or LipofectAMINE Plus (Invitrogen) according to the protocols provided by the manufacturer. Transfections of COS 7 cells for immunoprecipitation purposes were performed by the DEAE-dextran method essentially as described before (22). Metabolic 35S labeling of Miro-1 was performed as described before (22). Miro-1 immunoprecipitates were subjected to SDS-PAGE, and the gels were fixed for 15 min in 25% methanol and 7.5% acetic acid. The gels were thereafter dried and exposed on a PhosphorImager (Fujix BAS 2000).

Antibodies and Immunocytochemistry—A Miro-1-specific antibody was produced by immunizing rabbits with a keyhole limpet hemocyanin-conjugated Miro-1-specific peptide representing amino acid residues 560–574 of human Miro-1. Mouse monoclonal anti-Myc (9E10) and rabbit polyclonal anti-Myc antibodies (Santa Cruz Biotechnology) were used to determine the subcellular localization of Myc-tagged Miro-1 and Miro-2 expression in the human tissues depicted in the panel.
Miro-2 as well as Miro-1 mutants. The mouse monoclonal M30 antibody (Roche Molecular Biochemicals) was used to stain apoptotic cells. MitoTracker Green FM (Molecular Probes) and a mouse monoclonal anti-cytochrome c antibody (Pharmingen) were used according to the protocol supplied by the manufacturer to visualize mitochondria, and 4',6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei. Filamentous actin was visualized by TRITC-conjugated phalloidin (Sigma). Microtubules were visualized by a mouse monoclonal anti-α-tubulin antibody (Sigma). The following secondary antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-mouse, FITC-conjugated anti-rabbit, TRITC-conjugated anti-rabbit (Dako), and a TRITC-conjugated anti-mouse (Jackson ImmunoResearch). The caspase inhibitors Z-VAD-FMK and Z-DEVD-FMK were obtained from Calbiochem. For the immunocytochemistry, transiently transfected NIH3T3 and COS 7 cells were grown on coverslips and fixed in 3% paraformaldehyde in PBS for 20 min at 37 °C. The cells were washed with PBS and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The cells were thereafter washed again and incubated in the presence of 5% FCS in PBS for 30 min. Primary as well as secondary antibodies were diluted in PBS containing 5% FCS. Cells were incubated with primary antibodies followed by secondary antibodies for intervals of 1 h with a washing step in between. The coverslips were mounted in Fluoromount-G (Southern Biotechnology Associates) on object slides. Cells were photographed by a Hamamatsu ORCA CCD digital camera employing the QED Imaging System software using a Zeiss Axioplan2 microscope.

RESULTS
Identification of Miro Genes—Searches in the public genomic DNA sequence data bases generated by the human genome projects implicated the presence of several novel members of the Rho GTPases (8, 9). Expressed sequence tag (EST) clones, encoding two putative Rho GTPases, were obtained. Sequence analysis demonstrated that these clones encoded two related Rho GTPases, which were named Miro-1 and Miro-2, respectively. Miro-1 and Miro-2 were found to be 60% identical to each other, and both proteins encoded polypeptides of 618 amino acid residues with predicted molecular masses of 70 kDa (Fig. 1, A and B). The amino acid sequence of Miro-1 and -2 revealed an unusual domain organization; the N-terminal part encoded a GTPase domain, which is related to the Rho GTPases (Fig. 1 C). This domain was followed by two EF hands (20, 21), a type of domain that confers binding to calcium ions. Surprisingly, a second potential GTP-binding domain, but in this case without homology to the Rho GTPases, was found in the C terminus (Fig. 1 E). In addition, Miro lacks a so-called CAAX motif, a domain usually found in the C termini of small GTPases. The CAAX motif undergoes post-translational isoprenylation, a modification that confers membrane targeting of the protein. The unusual domain organization of Miro implicates that the proteins are regulated in a manner distinct from the archetypal Rho GTPases.

Interestingly, data base searches demonstrated the presence of genes, orthologous to Miro, in several eukaryotic organisms including Saccharomyces cerevisiae, Schizosaccharomyces pombe, Caenorhabditis elegans, Arabidopsis thaliana, and D. melanogaster (Fig. 1 F). This observation indicated that the Miro genes evolved early during evolution. Northern blot analysis, employing human multiple tissue Northern blots, demon-
strated the presence of Miro mRNA in most human tissue (Fig. 1G). Miro-1 mRNA of a size of 3 kb was abundant in heart and skeletal muscle, and Miro-2 mRNA of 2.4 kb was abundant in heart, liver, skeletal muscle, kidney, and pancreas (Fig. 1G).

Miro Are Localized to Mitochondria—The Rho GTPases have been shown to have a major impact on the organization of the actin cytoskeleton (1–3). For this reason the effect on the cytoskeletal organization by ectopic expression of Miro-1 and -2 was studied. Plasmids encoding Myc-tagged Miro-1 and Miro-2 were transiently transfected into NIH3T3 cells after which Miro expression was visualized by a Myc-specific antibody, whereas filamentous actin was visualized with TRITC-conjugated phalloidin. Microtubules were visualized by an antibody against β/H9251-tubulin. Neither Miro-1 nor Miro-2 expression visibly affected the organization of the actin filament system (Fig. 2, A and C) or the microtubules (Fig. 2, B and D). Interestingly, it was noticed that Miro staining followed a very distinct pattern; both Miro-1 and Miro-2 were organized in worm-like structures in a manner reminiscent of the mitochondrial network (Fig. 2). A Miro construct expressing only the N-terminal GTPase domain did not localize into such worm-like structures, instead this deletion mutant was dispersed evenly in the cytoplasm of transfected cells (Fig. 2E). In order to test if the localization of Miro coincided with mitochondria, NIH3T3 cells were transiently transfected with Myc-Miro-1 and co-stained with the mitochondria marker MitoTracker Green FM. Miro-1 overlapped to a large extent with the MitoTracker staining, suggesting that Miro-1 is present at the mitochondria (Fig. 2F, detail). Transiently transfected Miro-2 colocalized with mitochondrial marker in a similar fashion (data not shown). In addition, Myc-Miro-1 staining colocalized with the mitochondria protein cytochrome c, further emphasizing the notion of Miro as a mitochondria-associated protein (Fig. 2G).

A peptide derived from the C terminus of Miro-1 was synthesized and used to raise a Miro-1-specific antiserum in rabbits. The antiserum was affinity-purified over a column of Sulfolink-conjugated Miro-1-specific peptide. The antiserum, as well as the affinity-purified antibody, was tested in immunoprecipitation experiments employing lysates from 35S-labeled COS 7 cells transiently transfected with Myc-Miro-1. The Myc antibody, the Miro-1 antiserum and the affinity-purified antibody, precipitated a band of 70 kDa. This band was neither precipitated by pre-immune serum nor with the Miro-1 antiserum pre-blocked with the antigenic Miro-1-specific peptide (Fig. 3A). Next, a number of cell lines were tested for expression of Miro-1 by Western blotting. Human hepatocellular carcinoma Hep G2 cells and human embryonic kidney HEK293T cells contained high levels of Miro-1, whereas NIH3T3 fibroblasts and COS 7 cells expressed lower levels of Miro-1 (Fig. 3B). Hep G2 and HEK293T cells grow in a fashion that made visualization of their mitochondria difficult. For this reason, despite the lower expression of Miro-1, COS 7 cells...
overlapped to a large extent with cytochrome protein was localized to mitochondria. The staining pattern used to stain COS 7 cells in order to examine if endogenous turned out to be more useful for immunocytochemical analysis of the mitochondrial network. The Miro-1-specific antibody was used to stain COS 7 cells in order to examine if endogenous protein was localized to mitochondria. The staining pattern overlapped to a large extent with cytochrome c; moreover, the Miro-1-specific staining was quenched by pre-treatment with the blocking peptide. Collectively, these observations strongly suggested that endogenous Miro-1 is indeed a mitochondrial protein (Fig. 3C).

Activation of Miro-1 Induces Collapse of the Mitochondrial Network—Studies employing constitutive active and dominant negative mutants of Rho GTPases have been extensively used to examine the involvement of these proteins in numerous signal transduction pathways (1, 3). In analogy with the mutation employed for studies of RhoA, Rac1, and Cdc42, we made a constitutive active mutant Miro-1 in which the amino acid residue at position 13 was replaced with a valine. This mutation locks the GTPase in a GDP-bound conformation. Myc-Miro-1/Asn-18 or Myc-Miro-1/Val-13 overexpression from 35S-labeled COS 7 cells demonstrated a colocalization between cytochrome c and Miro-1 (data not shown). Myc-Miro-1/Val-13 overexpression resulted again in a loss of the mitochondrial network. Miro-1/Val-13 was present in the mitochondrial assemblies, but it was also dispersed into the cytoplasm (Fig. 4C). The cytochrome c staining was present in the mitochondrial assemblies, but an increased number of cells with cytoplasmic cytochrome c staining were seen after 48 h (Fig. 4C, arrowhead).

Ectopic Expression of Miro-1 Induces Apoptosis—Disturbances of mitochondrial homeostasis have been shown to result in an increased rate of apoptosis (24). We noticed a clear reduction of Miro-1/Val-13-expressing cells over time, compared with the amount of wt-Miro-1-expressing cells, which could implicate an increased rate of apoptosis in this cell population. For this reason we stained COS 7 cells transiently transfected with Myc-Miro-1, Myc-Miro-1/Val-13, or Myc-Miro-1/Asn-18 with a Myc-specific antibody as well as with the apoptosis marker antibody M30 (which specifically recognizes the caspase-cleaved cytokeratin 18). We calculated the amount of M30 positive cells that was also transfected with the Miro-1 mutants. A clear increase in M30 positive cells was noticed in the population of cells expressing Miro-1/Val-13. After 24 h the M30 positive and Myc-Miro-1/Val-13 expressing cells often showed a network of cytokeratin filaments (Fig. 5A). However, after a longer time the M30 staining was confined to dot-like structures, in particular in the frequent rounded up Myc-Miro-1/Val-13-expressing cells (Fig. 5B). The Miro-1/Val-13 and M30 positive cells also had fragmented nuclei, which could be visualized by DAPI staining (Fig. 5C). We counted the ratio between M30 positive cells and Miro-1-expressing cells. In the population of Miro-1/Val-13 33.0% of the cells were apoptotic. In contrast in cells expressing Miro-1 wild type or Asn-18 only 10.7 or 9.2%, respectively, of the cells were undergoing apoptosis (Fig. 5D). The Miro-1/Val-13 induced apoptosis was drastically reduced from 33 to 7.5% when the cells were treated with the caspase inhibitor Z-DEVD-FMK (Fig. 5D). Treatment of the cells with caspase-3 inhibitor Z-DEV-FMK also reduced the Miro-1/Val-13-induced apoptosis, from 33 to 14.5% (Fig. 5D), indicating that this response is dependent on the
caspase cascade. Collectively, these data implicate that Miro proteins are mitochondrial constituents and that the activity of Miro affects the homeostasis of mitochondria leading to an increased rate of apoptosis.

**DISCUSSION**

The roles of Cdc42, Rac, or Rho in cell migration and morphogenesis are well documented. A majority of the studies involving Rho GTPases are still focused to this archetypal
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Fig. 5. Ectopic expression of the mutant Miro-1/Val-13 induced an increased apoptotic rate. A–C, apoptotic cells were detected by the M30 antibody, which specifically recognizes the caspase-cleaved cytokeratin 18, followed by FITC (TRITC in C)-conjugated anti-mouse antibody, and by DAPI staining (C) to visualize degraded nuclei in the apoptotic cells. Miro-1 was detected by a Myc-specific rabbit antibody followed by a TRITC (FITC in C)-conjugated anti-rabbit antibody. Bar represents 20 μm. D, the ratio of Miro-1-expressing cells and M30 positive, apoptotic cells, was determined by counting cells under the microscope. For each experiment, at least 200 cells were scored using the 63× immersion oil objective, and the values shown in the diagram represent the means from at least 3 independent transfection experiments. Miro-1/Val-13-transfected cells were treated with 100 μM Z-VAD-FMK or 100 μM Z-DEVD-FMK, which was added 24 h post-transfection. After addition of the inhibitors the cells were incubated for an additional 24 h and then scored for apoptosis.

Mitochondrial homeostasis is a vital cellular process, and alterations in the intracellular environment that affect mitochondrial function normally triggers the apoptosis program (23). In this respect, the mitochondria are functioning as cellular sensors that, upon the receipt of proapoptotic signals, release factors such as apoptosis-inducing factor and cytochrome c into the cytoplasm, which assist in inducing the caspase cascade (23, 24). Rho GTPases have been implicated in various aspects of apoptosis (reviewed in Ref. 13). For example, Cdc42 is necessary for the nerve growth factor withdrawal-induced apoptosis of neuronal cells (33). In addition, Cdc42 is a caspase substrate, and mutation of the caspase cleavage site results in apoptosis of neuronal cells (33). In this respect, the mitochondria are functioning as cellu-

of cellular processes require a tightly balanced regulated activation of different Rho GTPases.

Miro proteins are unique not only by means of their size, because they are bigger than the classical small GTPases, but also by the presence of additional protein domain structures. Another group of atypical Rho-like proteins, the RhoBTBs, has also been implicated in mitochondrial homeostasis. Work in budding yeast, D. melanogaster and C. elegans, as well as in mammalian cells has identified three conserved GTPases in this process: Fzo/mitofusin, Dnm1/Drp1/Dlp1, and Mgm1/Opa1/Msp1 (30, 31). Fzo/mitofusin is a transmembrane protein residing on the outside of mitochondria where it regulates fusion of the outer mitochondrial membrane during mitochondrial fusion (29). The converse process is regulated by Dnm1/Drp1/Dlp1. This protein bears resemblance to dynamins and regulates outer mitochondrial membrane fission (31). Yet another dynamin-like molecule, Mgm1/Opa1/Msp1, seems to regulate several aspects of inner mitochondrial membrane remodeling. Interestingly, overexpression of a constitutively active mutant form of the mitofusin Mfn2 in COS 7 cells resulted in the formation of a continuous mitochondrial network, presumably caused by a shift in the balance of the mitochondrial dynamics favoring an over activity of mitochondrial fusions (32). This observation may relate to the mitochondrial aggregates seen in cells expressing constitutively active Miro-1/Val-13. Thus, these aggregates might be caused by an increase in mitochondrial membrane fusion; however, the exact mechanisms for remains to be studied.

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balance between mitochondrial fusion and fission for cell viability.

We have identified an evolutionarily conserved family of Rho GTPases, Miro, with atypical domain organization and unique cellular properties. Experiments employing NIH3T3 fibroblasts and COS 7 cells indicated that Miro is a constituent of mitochondria and that the protein is involved in the regulation of mitochondrial homeostasis and apoptosis.

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