Perturbation of intracellular Ca\(^{2+}\) homeostasis has been shown to regulate the process of cell proliferation and apoptosis. Our previous studies show that mitsugumin 29 (MG29), a synaptophysin-related protein localized in the triad junction of skeletal muscle, serves an essential role in muscle Ca\(^{2+}\) signaling by regulating the process of store-operated Ca\(^{2+}\) entry. Here we report a functional interaction between MG29 and the ryanodine receptor (RyR)/Ca\(^{2+}\) release channel. The purified MG29 protein enhances activity of the RyR/Ca\(^{2+}\) release channel incorporated into the lipid bilayer membrane. Co-expression of MG29 and RyR in Chinese hamster ovary (CHO) cells leads to apoptotic cell death resulting from depletion of intracellular Ca\(^{2+}\) stores, despite neither protein expression alone exhibits any significant effect on cell viability. In transient expression studies, the presence of RyR in the endoplasmic reticulum leads to retention of MG29 from the plasma membrane into the intracellular organelles. This functional interaction between MG29 and RyR could have important implications in the Ca\(^{2+}\) signaling processes of muscle cells. Our data also show that perturbation of intracellular Ca\(^{2+}\) homeostasis can serve as a key signal in the initiation of apoptosis.

Ca\(^{2+}\) as a second messenger plays important roles in a variety of cellular processes, ranging from cell motility to enzymatic control, and to gene regulation and apoptosis. The ryanodine receptors (RyR)/Ca\(^{2+}\) release channels are present in the endo(sarco)plasmic reticulum of virtually every cell type, providing a pathway for fast Ca\(^{2+}\) release from intracellular stores (1–4). Mitsugumin 29 (MG29) is a novel membrane protein localized specifically to the triad junction of skeletal muscle (5). The primary amino acid sequence of MG29 shares ~45% homology to synaptophysin, a family of proteins with presumed roles in secretion and neurotransmitter release (6, 7). The MG29 protein appears to participate in the excitation-contraction coupling process of skeletal muscle, since mutant mice lacking the MG29 gene exhibit apparent reduction in contractile force, with altered structure of the triad junction (8), and increased susceptibility to fatigue of the skeletal muscle (9, 10).

Several studies have suggested a central role for the participation of endoplasmic reticulum (ER) and intracellular Ca\(^{2+}\) release in the initiation of apoptosis (11–13). Our previous studies have shown that depletion of intracellular Ca\(^{2+}\) stores via activation of the RyR/Ca\(^{2+}\) release channel could induce apoptosis (14, 15). Here we tested the hypothesis that overexpression of MG29 can influence the intracellular Ca\(^{2+}\) homeostasis through changes of the RyR/Ca\(^{2+}\) release function. We show that co-expression of MG29 and RyR in cultured cells leads to apoptotic cell death resulting from depletion of intracellular Ca\(^{2+}\) stores, and the purified MG29 protein can enhance activity of the RyR/Ca\(^{2+}\) release channel incorporated into the lipid bilayer membrane. Our data provide evidence for a functional interaction between the RyR and MG29 proteins, which adds insights into the function of MG29 in excitation-contraction coupling of muscle cells, and the cellular mechanism of Ca\(^{2+}\) signaling in apoptosis.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis—The cDNA encoding the green fluorescent protein (GFP) was ligated to the 5' end of the MG29 cDNA (5) and cloned into the pCMV3 vector (Invitrogen) to yield the GFP-MG29 fusion construct. The GFP-RyR construct was obtained by replacing the first 290 amino acids of the rabbit skeletal muscle RyR with GFP (16). The E4032A RyR mutant was generated using site-directed mutagenesis, as described in previous studies (14).

Cell Culture, Transfection, and DNA Laddering—c1148 and mg-5 are stable clones of Chinese hamster ovary (CHO) cells permanently expressing the RyR and MG29 proteins, respectively. The cells were grown at 37 °C and 5% CO\(_2\) in Ham’s F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.5 mg/ml G418. Transfections of GFP-MG29 into c1148 cells and GFP-RyR into mg-5 cells were mediated by the LipofectAMINE reagent (15–17). Genomic DNA were collected from CHO cells transfected with various plasmids, and electrophoresis was performed (14).

Confocal Microscopy—For subcellular localization of GFP-MG29 and GFP-RyR transiently expressed in CHO cells, the cells were grown on glass-bottomed microwell dishes and visualized with a Bio-Rad Radiance 2100 or Zeiss LSM510 laser scanning confocal microscope (18). For immunolocalization of RyR stably expressed in the c1148 cells or caveolin in CHO cells, the cells were first treated with antibody against RyR (mAb 34C) or caveolin-1 (pAb C2327, Sigma) and then labeled with an appropriate secondary antibody conjugated with Texas Red. To monitor the cell death process, 10 μM Hoechst 33342 (Molecular Probes) was added to the culture medium for 10 min at 37 °C (14). Separate filter sets for GFP (485 nm excitation, a 500–550 nm band pass emission),
FIG. 1. Expression and subcellular localization of MG29 in CHO cells. a, Western blot of MG29 and RyR1. The proteins were separated on a 3–15% gradient SDS-polyacrylamide gel. The top portion of the gel was blotted with monoclonal antibody 34C against the RyR, and the bottom portion of the gel was blotted with mAb1007. MG29 is present in SR membrane of rabbit skeletal muscle (lane 2). mg-5 and c1148 are stable clones of CHO cells that permanently express the MG29 and RyR proteins (lane 3 and 4). The parental CHO cells do not express endogenous MG29 or RyR proteins (lane 1). b, confocal microscopic images of GFP-MG29 transiently expressed in parental CHO cells. GFP-MG29 is distributed in a punctuated fashion at the plasma membrane (left). Red fluorescence represents immunostaining of caveolin-1 (middle). A certain degree of colocalization between GFP-MG29 and caveolin-1 can be observed in the merged image (right). c, the GFP-MG29 pattern becomes predominantly perinuclear when expressed in c1148 cells (left). Red fluorescence represents staining of RyR expressed in the ER membrane (middle). A certain degree of overlap between GFP-MG29 and RyR could be detected in the merge image (right). d, the distribution of caveolin-1 in c1148 cells remains similar to that of the parental cells (b), whereas the overlapping pattern of GFP-MG29 and caveolin-1 is mainly present at the plasma membrane. The calibration bars represent 10 μm.

Texas Red (543 nm excitation, 560 nm long pass emission), and Hoechst (351 nm excitation, 385–470 emission) were used for image acquisitions.

Intracellular Ca2+ Measurement—The different clones of CHO cells (parental, c1148, mg-5, and after transient transfection with GFP-MG29 or GFP-RyR) were loaded with 2 μM Fura 2-AM for 30 min at 37 °C, in a balanced salt solution (14). The release of intracellular Ca2+ in individual cells was measured following exposure to either caffeine or ATP in a Ca2+-free solution by rapid solution exchange. Due to the fast interaction process, the Ca2+ measurements with c1148 cells transfected with GFP-MG29 or mg-5 cells transfected with GFP-RyR were performed 14–17 h after transfection. GFP fluorescence was used to select for transfected cells that are in the pre-apoptotic stage.

Vesicle Isolation and Protein Purification—Microsomal membrane vesicles containing MG29 or RyR proteins were isolated from the mg-5 or c1148 cells following the procedure of Bhat et al. (17). For purification of the MG29 proteins, the vesicles were solubilized with 1% CHAPS in a buffer containing 0.5 M NaCl, 0.5 M sucrose, 20 mM Tris-HCl (pH 7.4). The solubilized proteins were loaded onto a CNBr-activated Sepharose column that had the anti-MG29 antibody (mAb 1007) bound to it. The column was washed, and the MG29 protein was eluted with 0.3% CHAPS, 0.5 M NaSCN, 20 mM Tris-Cl (pH 7.4). The NaSCN was removed from the final buffer through dilution dialysis.

Electrophysiology—Lipid bilayer membranes were formed with a mixture of phosphatidylserine-phosphatidylethanolamine:cholesterol (6:6:1). Incorporation of the Ca2+ release channel in bilayers was achieved by addition of microsomal membrane vesicles isolated from the c1148 cells to the cis solution, under a concentration gradient of 200 mM (cis)/50 mM (trans) cesium gluconate (17). The purified MG29 was added to the cis solution to study the interaction with the Ca2+-release channel.

RESULTS AND DISCUSSION

Using monoclonal antibodies against triad junctional proteins from rabbit skeletal muscle, we have identified MG29, a membrane protein specifically localized to the triad junction of skeletal muscle. The primary structure deduced by cDNA cloning revealed that MG29 is a novel member of the synaptophysin family, with ~45% identity in amino acid sequence to synaptophysin (5). The cDNAs encoding either MG29 or RyR from rabbit skeletal muscle were introduced into the CHO cells using the Ca2+-phosphate precipitation method (19). Following selection with G418, individual clones of CHO cells stably expressing either MG29 (mg-5) or RyR (c1148) were selected (Fig. 1a). The levels of protein expression in these cells were maintained in multiple passages, suggesting that the CHO cells can take up well these exogenous MG29 or RyR proteins.

For subcellular localization of MG29 expressed in CHO cells, GFP-MG29 fusion construct was generated. Confocal microscopic images indicate that GFP-MG29 can be clearly visualized at the cell surface membrane in a punctuated fashion 2 days after transfection (Fig. 1b). A similar punctated-vesicular labeling of MG29 was also observed in mg-5 cells, with a monoclonal antibody against MG29 (mAb 1007; data not shown). In co-localization studies, a certain degree of overlap in the membrane distribution of MG29 and caveolin-1, an endogenous caveolea-specific protein present in CHO cells, could be observed in CHO cells (Fig. 1b). Such characteristic patterns of MG29 expression in CHO cells suggest that as yet unknown cellular mechanisms probably result in concentration of MG29 at certain subdomains of the plasma membrane (e.g. rafts or caveolae). These data are also consistent with the specific localization of MG29 at the transverse-tubule membrane of matured skeletal muscle cells (20). Interestingly, transient expression of GFP-MG29 in c1148 cells revealed a different pattern of fluorescence distribution (Fig. 1c). The c1148 cells are bigger than the parental CHO cells, as has been reported in our previous studies (16). It appears that the presence of RyR in the ER membrane facilitates the retention of GFP-MG29 to the perinuclear regions and also causes apparent aggregation of GFP-MG29 (Fig. 1c). The distribution of caveolin-1 in c1148 cells, however, did not appear to be different from that of

![FIG. 2. Apoptosis of cells co-expressing MG29 and RyR. a, confocal microscopic images of c1148 cells transiently transfected with GFP-MG29. Left, measurement of GFP fluorescence; middle, Hoechst dye staining of the nuclear chromatin; right, merge of GFP and Hoechst staining. 12 h after transfection, expression of GFP-MG29 could be observed and most of the cells appear to be healthy. 30 h after transfection, the cells started to undergo apoptosis, as shown by the distinct labeling of the fragmented nucleus by the Hoechst dye. The images shown were representative of five other experiments. The bars represent 10 μm. b, laddering of genomic DNA following transfection of MG29 cDNA into CHO cells. Lane 1, c1148 cells; lane 2, E4032A cells; lane 3, parental cells.](http://www.jbc.org/Downloaded from)
caffeine (4.84 
ATP (0.31 
changes in cytoplasmic [Ca2+
]
parental CHO cells (Fig. 1a). As shown in Fig. 
loaded with a Hoechst 33342 dye, which labeled the nuclear 
process. To differentiate apoptosis from necrosis, the cells were 
RyR in 
E4032A 
cells transfected with the MG29 cDNA (Fig. 2b). Since the E4032A RyR mutant did not respond to caffeine. c1148 cells (14), the MG29 overexpression-induced apoptosis of 
DNA isolated from c1148 cells transfected with MG29 revealed 
characteristic pattern of DNA laddering, a hallmark of cells 
undergoing apoptosis (Fig. 2c). In contrast, the parental CHO 
cells and those cells stably expressing the E4032A mutant of 
RyR did not exhibit apparent DNA laddering following identi-
cal treatment with the MG29 cDNA (Fig. 2b). Since the E4032A RyR mutant is know to have defective Ca2+ release channel function (21), and we have shown that stable expression of E4032A did not affect the proliferation and apoptosis of CHO 
cells (14), the MG29 overexpression-induced apoptosis of c1148 cells is probably related to the RyR-mediated Ca2+ release from the ER membrane.

Further studies show that co-expression of MG29 and RyR in 
the ER membrane leads to drastic changes in the intracellular 
Ca2+ homeostasis in CHO cells (Fig. 3). The release of Ca2+ from the ER membrane was measured in individual CHO cells...
following stimulation with 10 µM caffeine or 200 µM ATP. Caffeine is a known activator of the RyR/Ca2⁺ release channel, and ATP is an agonist of the purinergic receptor on the plasma membrane, which activates the IP₃ receptor on the ER membrane via generation of IP₃ (22). As shown in Fig. 3a, the parental CHO cells do not respond to caffeine but contain active IP₃ receptors as indicated by the rapid release of Ca²⁺ triggered by ATP (n = 30). Both caffeine and ATP caused rapid release of intracellular Ca²⁺ in the c1148 cells (Fig. 3b, n = 66/67). Expression of MG29 in CHO cells alone did not affect the ER Ca²⁺ pool, since the response of mg-5 cells to ATP was similar to the parental CHO cells (Fig. 3c, n = 8). 14–17 h after transfection of GFP-MG29 into c1148 cells, the amount of Ca²⁺ in the ER was diminished, since the cells showed significantly reduced responses to either caffeine or ATP (Fig. 3c). This was likely due to the activation of RyR, because CHO cells stably transfected with the E4032A mutant did not show changes of their ER Ca²⁺ store upon introduction of GFP-MG29 (Fig. 3d).

Similarly, expression of GFP-RyR in mg-5 cells also resulted in reduction of the ER Ca²⁺ pool (Fig. 3f). The traces shown in Fig. 3, e and f, were representative of 13/17 and 8/9 experiments performed, i.e. over 75% of the transfected cells had an ER Ca²⁺ content that was nearly empty. These fluorescent cells, expressing both GFP-MG29 and RyR or both GFP-RyR and MG29, generally died within 24–36 h after transfection. On the other hand, non-transformed cells expressing either RyR or MG29 showed intact ER Ca²⁺ pools and remained viable. Thus it appears that there is a close correlation between cellular apoptosis and Ca²⁺ homeostasis, which has been observed in our previous studies (14). It is possible that the interaction between RyR and MG29 enhances the activity of the Ca²⁺ release channel or alters the trafficking and function of MG29, which leads to the depletion of Ca²⁺ from the ER membrane.

We next purified the MG29 protein from the mg-5 cells and tested its effect on the individual RyR/Ca²⁺ release channel using the lipid bilayer reconstitution system (Fig. 4). Microsomal membrane vesicles were isolated from mg-5 cells and solubilized with the CHAPS detergent, from which the MG29 protein was purified to homogeneity using a monoclonal antibody affinity column (Fig. 4a). A single RyR/Ca²⁺ release channel was incorporated into the lipid bilayer by fusing vesicles isolated from the c1148 cells (Fig. 4b). The RyR channel had an ohmic conductance of ~400 picosiemens, which exhibited fast transitions between the open and closed states (16). At a test potential of ~50 mV with free [Ca²⁺]m buffered at 220 µM and 5 mM MgCl₂ added to the cytosolic solution, the channel had an average open probability of \( P_O = 0.007 \pm 0.002 \) (n = 4). Following addition of the purified MG29 protein (0.05–0.2 µM/ml) to the cytosolic solution, open probability of the Ca²⁺ release channel was increased significantly (\( P_O = 0.052 \pm 0.006, n = 4, p < 0.001 \)), whereas the single channel current amplitude remained unchanged (Fig. 4c). This result supports a direct functional interaction between MG29 and RyR.

Taken together, our data show that a functional interaction between MG29 and RyR in CHO cells enhances opening of the Ca²⁺ release channel, which likely causes depletion of the intracellular Ca²⁺ pool, leading the cells to undergo apoptosis. We also showed that the presence of RyR in the ER membrane could influence the processing and trafficking properties of MG29, causing retention of MG29 from plasma membrane to the intracellular organelles. This result is interesting but not surprising. We know that MG29 exists as an oligomer in the transverse-tubule membrane of matured skeletal muscle (23). We also know that a significant portion of MG29 co-localize with the SR membrane in the developing skeletal muscle (20). In addition, knock-out of MG29 has been shown to cause fragmentation of the SR membrane (8). Thus, there appears to be a close association between the expression pattern of MG29 and the ultrastructure of the membrane cross-talk in skeletal muscle. Perhaps, a functional interaction between MG29 and RyR could have a significant role in the overall Ca²⁺ signaling process in muscle cells, at least in the regulation of store-operated Ca²⁺ entry (9). It is interesting that although both MG29 and RyR are essential for the normal contractile function of the skeletal muscle, co-expression or overexpression of MG29 and RyR in CHO cells leads to eventual cell death due to the leakage of the ER Ca²⁺ store. Therefore, to maintain the closed state of the RyR channel at the resting state of the skeletal muscle, other cellular mechanisms must be involved. For example, the voltage sensor located on the transverse-tubule membrane has been shown to play an inhibitory role on the RyR function with muscle cells at a polarized state (24).

Other cytosolic proteins, such as FKBP12 or calmodulin, have been shown to associate with RyR and to modulate the active state of the Ca²⁺ release channel (25–27). It remains to be examined how the acute absence of MG29 (e.g. through small interfering RNA-mediated knockdown mechanism) in the adult skeletal muscle affects the overall Ca²⁺ signaling process in muscle contraction, such as store-operated Ca²⁺ entry, voltage-induced Ca²⁺ release, and Ca²⁺-induced Ca²⁺ release.

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Co-expression of MG29 and Ryanodine Receptor Leads to Apoptotic Cell Death: EFFECT MEDIATED BY INTRACELLULAR Ca2+ RELEASE

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