Different roles of H-ras for regulation of myosin heavy chain promoters in satellite cell-derived muscle cell culture during proliferation and differentiation

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Scholz ME, Meissner JD, Scheibe RJ, Umeda PK, Chang KC, Gros G, Kubis HP. Different roles of H-ras for regulation of myosin heavy chain promoters in satellite cell-derived muscle cell culture during proliferation and differentiation. Am J Physiol Cell Physiol 297: C1012–C1018, 2009. First published July 22, 2009; doi:10.1152/ajpcell.00567.2008.—The effect of constitutively activated proto-oncogene H-ras (H-rasQ61L) on the regulation of myosin heavy chain (MHC) promoter activities was investigated in rabbit satellite cell-derived muscle cell culture during the proliferation stage and early and later stages of differentiation, respectively. During proliferation, overexpression of H-rasQ61L did not affect basal level of activity of the slow MHC/β or the fast MHCII/d/x promoter luciferase reporter gene construct in transient transfection assays. By contrast, H-rasQ61L affected both MHC promoter activities during differentiation, and this effect changes from inactivation after 2 days to activation after 4 days of differentiation. The activating effect of H-rasQ61L on both MHC promoters after 4 days of differentiation was significantly reduced by LY-294002, a specific inhibitor of the PI3k-Akt signaling pathway is involved in the activation of MHC/β and MHCII/d promoters in a later stage of differentiation of muscle cells, presumably by a known inhibiting effect of activated Akt on the Raf/MEK1/2-ERK1/2/ MAPK pathway. The experiments demonstrate that during differentiation of muscle cells activated H-ras is an important regulator of MHC isoform promoter function with opposite effects during early and later stages.

extracellular signal regulated protein kinase 1/2; mitogen-activated protein kinase; phosphosinositol-3-kinase; Akt; mammalian target of rapamycin

SATELLITE CELLS are undifferentiated mononuclear myogenic cells of the adult muscle (12). Their primary function is to mediate postnatal growth and repair (25). Moreover, satellite cells are activated by muscle injury resulting for example from mechanical trauma or exercise (19), but they are also involved in the hypertrophic response following increased muscle load (1). Regenerative processes provoked by muscle damage involve the activation of satellite cells, their proliferation, withdrawal from cell cycle for fusion, and differentiation into myofibers (12).

Although satellite cells are present in all skeletal muscles, differences between fiber types exist (20). A higher number of satellite cells is found in slow compared with fast fibers of the same muscle. Moreover, the number of satellite cells is kept constant within a specific muscle even after multiple rounds of injury and regeneration but decreases during aging in addition to a lower differentiation capacity (16, 21). A reduction of the regenerative capacity is associated with severe myopathic diseases such as Duchene Muscular Dystrophy (39).

After fusion of myoblasts and differentiation into myotubes, cells start to express a variety of muscle-specific gene products like myosin heavy chains (MHC) (5). The expression of MHC isoforms I and II is a hallmark of differentiation into fiber types (10). The muscle regulatory factor (MRF) MyoD has been shown to be crucial to initiate differentiation of myotubes (15). However, skeletal myoblast differentiation can be inhibited by proto-oncogenic Ras by downregulating the expression of MyoD (24, 27, 33). The Ras proto-oncogenes belong to a family of small 21-kDa molecular mass GTP-binding proteins, which have critical regulatory functions in nearly all eukaryotic cells. They are essential for cell growth and, when constitutively activated, cause cellular transformation (17).

Major downstream targets of Ras are the Raf mitogen-activated protein kinase kinase 1/2 (MEK1/2) extracellular signal-regulated protein kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK) and the phosphoinositol-3-kinase (PI3K)-Akt (protein kinase B) pathways. Growth factor-mediated activation of the Raf-MEK1/2-ERK1/2/MAPK pathway via Ras provides a positive signal for proliferation in many cell types (13). Interestingly, both Ras and MEK1/2-ERK1/2 MAPK play a dual role in myogenic differentiation (7, 42). Whereas initiation of differentiation in C2C12 myoblasts is repressed by MEK1/2-ERK1/2 MAPK, later stages of differentiation are promoted by MEK1/2-ERK1/2 MAPK contributing to the activation of myogenic transcription. Activated MEK1/2 inhibited the switch from proliferation to differentiation of C2C12 myoblasts (34). By contrast, it has been demonstrated that MEK1/2-ERK1/2 MAPK positively regulates the activity of the muscle regulatory factor MyoD to initiate terminal differentiation of L8 myotubes (22). Furthermore, PI3K induces the transcriptional activity of myocyte enhancer factor-2 (MEF2) proteins during muscle differentiation.
tion (40). Members of the MEF2 transcription factor family are important interaction partners for MRFs (8, 36).

In addition, expression of an ERK MAPK pathway-activating Ras mutant during regeneration of denervated rat soleus muscle induced the expression of slow MHC isoform I without affecting muscle fiber size. On the contrary, a PI3K-activating Ras mutant positively affected muscle fiber growth but not the fiber type (32). Activation of the protein kinase Akt (protein kinase B), a target of PI3K, leads to increases in protein synthesis and muscle mass (6).

The present paper investigates the effect of inactivated and constitutively activated proto-oncogene H-ras on the regulation of MHCI and MHCIId/x promoter activities in rabbit satellite cell-derived muscle cell culture during proliferation and different stages of differentiation. Here we demonstrate that activated H-ras affects MHCI promoters during early and later stages of differentiation differently but independent of the type of myosin isoform promoter. H-ras represses type I and II MHCI promoters during an early stage of differentiation. During a later stage of muscle cell differentiation, the Ras-PI3K-Akt signaling pathway is shown to be involved in the activation of MHCI/β and MHCIId/x promoters, presumably by inhibiting the MEK1/2-ERK1/2 MAPK signaling pathway. The experiments demonstrate no effect of activated H-ras for MHCI isoform promoter regulation during proliferation but a changing role during early and later stages of differentiation of rabbit satellite cell-derived muscle cells.

MATERIALS AND METHODS

Plasmids. The luciferase reporter gene vectors pMHCI/β-Luc and pMHCIId/x-Luc were cloned by introducing proximal 780 bp fragments of the rabbit MHCI/β (41) or the porcine MHCIId/x (31) promoters into the pTAL-LUC vector (Clontech), from which TATA minimal promoter sequences were deleted. A 781-bp promoter region, including 5'-untranslated sequences, has previously been shown to be sufficient for both tissue- and developmental stage-specific transcription of the MHCI/β gene in primary skeletal muscle culture (14). For constitutively active expression of Ras, the vector pUSEAmp-H-ras(Q61L) (Upstate) was used. A dominant negative Ras expression plasmid pRas(S17N) (18) was a gift from M. Dangers (Hannover Medical School, Hannover, Germany).

Cell culture. Rabbit (New Zealand White) muscle satellite cells, a gift from Dr. Eva Margreiter, Innovacell Biotechnologie, Innsbruck, Austria, were isolated from a muscle biopsy as described by Blau and Webster (9). Satellite cell-derived myoblasts were grown in monolayer cultures using the skeletal muscle cell growth medium kit (Promocell, Heidelberg, Germany) to maintain a proliferative state and specifically enhance myoblast proliferation. Cells were passaged when the cultures reached 80% confluence. To induce differentiation the cells were grown to 100% confluence and then the medium was changed to differentiation medium (DM), a low-glucose Dulbecco’s modified Eagle medium (DMEM) with 5% newborn calf serum (NCS).

Transfection and reporter gene assays. Satellite cell-derived myoblasts were transfected with a total amount of 2 μg of plasmid DNA with the Effectene transfection reagent (Qiagen, Hilden, Germany). After incubation for 8 h, the cells were washed and covered with proliferation medium. For the analysis of an early stage of differentiation, GM was replaced by DM and myotubes were harvested after further 2 days. To investigate a later stage of differentiation, cells were cultivated for 4 days in DM.

For lysis the cells were washed with PBS and then incubated with lysis buffer (25 mM Tris, 2 mM EDTA, 10% glycerol, 1% Triton X-100, pH 7.8) for 10 min at ambient temperature. The cell debris was removed by centrifugation and the supernatant was used for the assays. The supernatant (100 μl) was used to measure luciferase activity in a Berthold 9502 tube luminometer (Berthold Technologies, Bad Wildbad, Germany). Cells were cotransfected with pcMV-Gal, a CMV-promoter-controlled expression vector for β-galactosidase, as an internal reference. The β-galactosidase activity was estimated in a standard assay (38) by adding 100 μl of 2 μM ortho-nitrophenyl-galactopyranoside to 40 μl cell lysate. The normalized luciferase activity is expressed as relative light units per β-galactosidase activity (RLU/β-Gal).

Inhibitors. LY-294002 (Merck Biosciences, Schwalbach/Ts.) was added in a concentration of 10 μM to the medium to block PI3K activity. PD-98059 (Sigma, Taufkirchen, Germany) was used in a concentration of 20 μM to block MKK1/2, the direct upstream kinase of ERK1/2 MAPK (p42/p44). The protein kinase mammalian target of rapamycin (mTOR) was inhibited by 2 ng/ml rapamycin (Merck Biosciences).

Western blot analysis. Satellite cell-derived myoblasts or myotubes, respectively, were washed once with PBS and collected by immediate cell lysis and protein denaturation adding 200 μl SDS-PAGE-loading-buffer (95°C) to the culture flasks. Lysed cells were further denatured for 3 min at 95°C. Sample protein was measured by the modified Lowry method (11), and same amounts of protein were loaded to a 10% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane and blocked with 5% fat-free dry milk in PBS, 0.2% Tween. Immunodetection of Akt, phospho-Akt, phospho-p42/44, and Ras was performed using anti-Akt (No. 9272), anti-phospho-Thr308-Akt (No. 9275), anti-phospho-p42/44 (No. 4370), and anti-Ras (No. 3339) from Cell Signaling Technology (Danvers, MA) and anti-rabbit horseradish-peroxidase linked secondary antibody (Sigma). Enhanced chemiluminescence detection was performed using Supersignal Pico ECL kit (Pierce, Rockford, IL).

Statistics. The statistical significance of differences between mean values was estimated by one-way ANOVA followed by the Newman-Keuls multiple comparison test using GraphPAD Prism software version 3.00.

RESULTS

Effect of H-ras on MHCI promoter activities in satellite cell-derived muscle cell culture depends on the state of differentiation. Undifferentiated myoblasts derived from adult rabbit skeletal muscle satellite cells were transiently transfected with a slow-fiber-type 780-bp MHCI/β or a fast-fiber-type 780-bp MHCIId/x promoter luciferase reporter construct. In proliferation medium (PM), the fast MHC promoter showed a higher level of basal activity than the slow MHC promoter (compare Fig. 1, A and B). Two days after change to differentiation medium (DM), increased levels of activity were found for both promoter constructs (Fig. 1), indicating their activation at the myotube versus myoblast stage. A higher level of activity of the MHCIId/x promoter is compatible with a fast-fiber-type of the satellite cell-derived myotubes in terms of MHC promoter expression after 2 days in DM.

To examine the effect of the proto-oncogene Ras in both developmental stages, the satellite cell-derived myoblasts were transiently transfected with a constitutively active Ras (pUSEAmp-H-ras(Q61L)) expression plasmid. The activity of both MHC promoter constructs was not altered by pUSEAmp-H-ras(Q61L) during proliferation (Fig. 1). By contrast, activation of both MHC promoters was prevented when pUSEAmp-H-ras(Q61L) was overexpressed under DM-induced differentiation. In addition, a dominant negative Ras expression plasmid pRas(S17N) neither changed the level of MHC pro-
The formation of multinucleated myotubes is a multistep process, requiring several days in vitro (5). Therefore, after 2 days in DM the cells have not reached a state of fully differentiated myotubes. From day 2 to day 4 of culture, increases of myotube fusion were observed, indicating ongoing differentiation of myotubes. Around day 7 of culture, myotubes start to contract, leading to detachment from the culture plates. In addition, the activity of creatine kinase, a muscle-specific marker, increased from day 2 to 4 in DM (data not shown).

Therefore, the effect of constitutively activated Ras on MHC promoter activity was investigated in myotubes grown for 2 more days in DM. A further marked increase in MHCIId/x promoter activity was found after 4 days compared with 2 days in DM (Fig. 2), also indicating ongoing differentiation of the myotubes. Again, a higher level of MHCIId/x promoter activity after 4 days in DM indicated a fast-fiber type of the satellite cell-derived muscle cells in terms of MHC promoter expression. The activity of the MHCI/β promoter was slightly decreased after 4 days compared with 2 days in DM. These findings might indicate development of the myotubes to a more pronounced fast-fiber type in terms of MHC promoter activity. However, the activity of both MHC promoter constructs was increased by overexpression of pUSEAmp-H-ras(Q61L) but decreased by dominant negative pRas(S17N) after 4 days in DM (Fig. 3). The data indicate that in contrast to myotubes at an early stage of differentiation (2 days in DM, see Fig. 1), Ras can activate both MHC promoter at a later stage of differentiation (4 days in DM). Therefore, the effect of Ras on MHC promoter activity has changed from inactivation to activation in the course of transition from onset to later stages of differentiation.

**PI3K pathway is involved in the Ras-mediated increase of MHC promoter activities, whereas the MKK1/2-ERK1/2 MAPK pathway inhibits the promoters in satellite cell-derived myotubes at a later stage of differentiation.** The PI3K-Akt-mTor and the Ras-Raf-MKK1/2-ERK1/2 MAPK pathways are important
downstream mediators of Ras in skeletal muscle (37) and are important for muscle cell differentiation and growth (6, 7, 42). Therefore, we investigated a possible role of both pathways in mediating the activating effect of Ras on MHC promoters after 4 days of differentiation. For this purpose PI3K was inhibited specifically by the addition of LY-294002 (10 μM) to the culture medium. Moreover, Akt-dependent activation of mTOR was inhibited by rapamycin at a final concentration of 2 ng/ml. In a further set of experiments, the specific inhibitor of MKK1/2 (PD-98095, 20 μM), was used. MKK1/2 is the direct upstream kinase of ERK1/2 MAPK.

The PI3K inhibitor LY-294002 (10 μM) did not affect the level of basal activity of the MHCI/β promoter activity of myotubes cells grown for 4 days in DM but reduced the activating effect of pUSEAmp-H-ras(Q61L) by around 50% (Fig. 3). A comparable level of reduction was found for the basal level of activity of the MHCIId/x promoter activity and for the activating effect of pUSEAmp-H-ras(Q61L). These experiments indicate that the Ras/PI3K pathway is involved in the regulation of the basal level of activity of the fast MHC promoter but not of the slow MHC promoter in a later stage of differentiation. In addition, the activating effect of Ras on both MHC promoter activities is at least in part mediated via PI3K.

We next investigated the MKK1/2-ERK1/2 MAPK pathway in the later stage of differentiation. In myotubes grown for 4 days in DM both the MHCI/β and IId/x promoter activities were increased by addition of PD-98095 (20 μM) to levels similar to the activation by constitutive active Ras (Fig. 4), indicating that the ERK1/2-MAPK pathway has an inhibitory effect on the MHC promoters. By contrast, the activating effect of pUSEAmp-H-ras(Q61L) on the MHC promoters was not altered (Fig. 4). In turn, pUSEAmp-H-ras(Q61L) did not affect the activation of the promoters by PD-98095, no additive effect was found. These results indicate that the positive effect of activated Ras on the fast and the slow MHC promoter activities in maturing satellite cell-derived myotubes is not mediated by the MKK1/2-ERK1/2 MAPK pathway. Furthermore, the negative effect of MKK1/2-ERK1/2 MAPK on both MHC promoter activities is not mediated by Ras.

PI3K can phosphorylate and therefore activate the protein kinase Akt, Akt in turn activates mTOR, an important Akt downstream target also in skeletal muscle (6). Western blot analysis demonstrated an increase in phosphorylated Akt (p-Akt) relative to fairly unchanged total Akt after 4 days in DM compared with the level of p-Akt after 2 days in DM (Fig. 5, lanes 2 and 4), demonstrating increased activation of Akt in a later stage of differentiation of the myotubes cells. In addition, inhibition of mTOR by 2 ng/ml rapamycin failed to affect the MHC promoter basal activities and did not change the effect of constitutively active Ras on the promoter activities (Fig. 6). The data indicate that despite an activation of Akt, the effect of Ras on the MHC promoter activities in satellite cell-derived myotubes is not mediated by mTOR but might be explained by the known ability of activated Akt to inhibit ERK1/2 MAPK in nonmuscle as well as in muscle cells (37, 43). Therefore, in a later stage of differentiation, the ERK1/2 MAPK pathway might be downregulated in myotubes overexpressing H-rasQ61L by the PI3K-Akt pathway. In accordance, a decrease in level of phosphorylated ERK1/2 MAPK (P-p42/44) was found after 4 days in DM compared with 1 day in DM in Western blot analysis (Fig. 5, lanes 1 and 4).
DISCUSSION

The effect of constitutively active and dominant negative Ras on the activity of slow MHC/β and fast MHCII/d promoter luciferase reporter constructs was investigated in rabbit satellite cell-derived muscle cells growing in PM or DM for different durations. MHC expression is a marker of ongoing myogenic differentiation (5). During proliferation, Ras is not involved in the regulation of the low level of both MHC promoter activities. By contrast, after induction of differentiation by DM, activated Ras abolishes promoter activation of both MHC promoters (33). Ras can inhibit myogenic differentiation by downregulating the expression of the MRF MyoD (24, 27), an important transcription factor initiating differentiation of myotubes (15). The activity of an MHC/β promoter construct has recently been shown to be increased by MyoD (31).

The higher level of basal activity of the fast MHCII/d compared with the slow MHC/β promoter indicates that the differentiating rabbit satellite cell-derived myotubes express a fast-fiber type in terms of MHC promoter activity. Similarly, primary rabbit myotubes also express a fast-fiber type on the level of MHC protein and mRNA (26, 30). In addition, a fast-fiber type was also found in C2C12 myotubes on the level of endogenous MHC protein expression and of transfected MHC promoter activities (28, 31). The fast promoter activity increases after 4 days compared with 2 days in DM, whereas slow promoter activity decreases. Expression of fast and some slow MHC promoter activity in the early stage of differentiation is followed by a pronounced shift toward fast MHC expression in the later stage. This observation made here for the satellite-derived cell culture was also made in a primary rabbit muscle cell culture on the level of endogenous MHC expression (26, 30).

In vivo, expression of slow MHC/β depends on innervation by slow motoneuron, with denervation of slow muscle fibers leading to a slow-to-fast fiber transformation (35). Interestingly, attempts to induce a fast-to-slow transformation on the level of MHC expression in differentiating rabbit satellite-derived muscle cells by using Ca²⁺-ionophore were not successful (data not shown). Ca²⁺-ionophore A23187 has been used previously to induce the transformation in primary rabbit skeletal muscle cells (26, 29, 30) as well as in C2C12 myotubes (28, 31). The reason for the missing plasticity of the differentiating satellite cells is not clear so far. Longer cultivation in DM might lead to further differentiation and a level of plasticity as found in other skeletal muscle cell culture systems. The missing plasticity of the rabbit satellite cell-derived myotubes regarding MHC expression in vitro might be an explanation for discrepancies to experiments in vivo. After 4 days in DM, Ras induces both fast MHCII/d and slow MHC/β promoter activity in the satellite cells. By contrast, in a model of regeneration after denervation, activated Ras was found to increase slow MHC/β but not fast MHCII expression in rat soleus muscle (32).

Fig. 5. Time course of ERK1/2 MAPK and Akt expression in differentiation medium. Western blot analysis of phosphorylated ERK1/2 MAPK (P-p42/44) in rabbit satellite cell-derived muscle cells grown from 1 to 4 days in DM (lanes 1 to 4) by using an anti-ERK1/2 MAPK antibody and of phosphorylated (activated) Akt (p-Akt) and total Akt as demonstrated by probing the blot with anti-p-Akt and reprobing with anti-Akt antibodies (top). Expression of p-Akt relative to total Akt expression was shown in the bottom.

Fig. 6. Effect of mammalian target of rapamycin (mTOR) inhibition on MHC/β and MHCII/d promoter activities in rabbit satellite cell-derived muscle cell culture. Satellite cell-derived myoblasts were transfected with a slow −800 bp MHC/β or a fast −800 bp MHCII/d promoter luciferase reporter gene construct and cotransfected with an expression vector coding for constitutively active Ras (Ras⁺), or empty expression vector, respectively, as indicated. Transfected cells were grown for 4 days in DM with or without 2 ng/ml rapamycin, as indicated. Results are expressed as RLU/β-Gal. Each column in the figure represents measurements from three equally treated culture dishes. All bars are means ± SD. *Significantly different, P < 0.05.
In a later stage of differentiation, 4 days after the onset of differentiation, inhibition of PI3K decreases the positive Ras effect on MHC promoter activities in satellite cell-derived myotubes, indicating that the activating effect of Ras is PI3K dependent. It has been shown that PI3K can induce the transcriptional activity of MEF2 transcription factors during muscle differentiation (40), and MEF2 isoforms activate fast and slow MHC isoform promoters in C2C12 myotubes (2, 3, 28, 31) and in vivo (4, 23). By contrast to PI3K, the MKK1/2-ERK1/2 MAPK pathway has an inhibitory effect on the MHC promoter activities but not on the Ras effect, indicating Ras-independent signaling. The protein kinase Raf is a target of Ras and can in turn activate the MKK1/2-ERK1/2 MAPK pathway (13). Interestingly, Akt as a target of PI3K can inhibit Raf in myotubes but not in myoblasts (37). Increased activation of Akt and a decreased level of activated ERK1/2 MAPK (P-p42/44) are found in the satellite cell-derived myotubes after 4 days in DM. Therefore, the ERK1/2 MAPK pathway might be downregulated in myotubes overexpressing activated Ras in a later stage of differentiation by activated Akt. Myogenic differentiation is a multistep process in vitro (5). Our data do not exclude a possible activating effect of MKK1/2-ERK1/2 MAPK on MHC promoter activities in satellite cell-derived myotubes grown longer than 4 days in DM. This promotion of myogenic transcription during terminal differentiation as found in C2C12 myotubes (22) would be in accordance with the dual role of the pathway during differentiation (7, 34, 42).

In cells overexpressing constitutively active Ras for 4 days in DM, we observed moderately accelerated myotube formation, presumably correlated with increased expression of adult MHC. Murgia et al. (32) used a different approach by cotransfecting double mutants with constitutively active Ras bearing additional mutation in the effector-site domain. They found increases in cell size by overexpression of Ras double mutants activating PI3K and Raf but not ERK MAPK. We did not observe significant changes in morphology when using the PI3K inhibitor LY-294002 (10 μM). Nevertheless, despite some differences in the outcome of Ras activation in both models, our data support findings of Murgia et al. that Ras is an important regulator of muscle cell differentiation.

Taken together, the experiments demonstrate a different role of activated H-ras during proliferation and early and later stages of differentiation of satellite cell-derived myotubes, respectively. The effect of Ras on MHC promoter activity changes from inactivation during an early to activation during a later stage of differentiation. In addition, the Ras-PI3K-Akt signaling pathway mediates the activation of MHC/β and IId/x promoters in a later stage of differentiation of myotubes presumably by counteracting a repressive effect of the MKK1/2-ERK1/2 MAPK pathway. The data indicate that activated H-ras is an important regulator of MHC isoform promoter activity during differentiation of rabbit satellite cell-derived myotubes with a changing role during early and later stages.

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