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Amphoterin Stimulates Myogenesis and Counteracts the Antimyogenic Factors Basic Fibroblast Growth Factor and S100B via RAGE Binding

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The receptor for advanced glycation end products (RAGE), a multiligand receptor of the immunoglobulin superfamily, has been implicated in the inflammatory response, diabetic angiopathy and neuropathy, neurodegeneration, cell migration, tumor growth, neuroprotection, and neuronal differentiation. We show here that (i) RAGE is expressed in skeletal muscle tissue and its expression is developmentally regulated and (ii) RAGE engagement by amphoterin (HMGB1), a RAGE ligand, in rat L6 myoblasts results in stimulation of myogenic differentiation via activation of p38 mitogen-activated protein kinase (MAPK), up-regulation of myogenin and myosin heavy chain expression, and induction of muscle creatine kinase. No such effects were detected in myoblasts transfected with a RAGE mutant lacking the transducing domain or myoblasts transfected with a constitutively inactive form of the p38 MAPK upstream kinase, MAPK kinase 6, Cdc42, or Rac-1. Moreover, amphoterin counteracted the antimyogenic activity of the Ca^{2+}-modulated protein S100B, which was reported to inhibit myogenic differentiation via inactivation of p38 MAPK, and basic fibroblast growth factor (bFGF), a known inhibitor of myogenic differentiation, in a manner that was inversely related to the S100B or bFGF concentration and directly related to the extent of RAGE expression. These data suggest that RAGE and amphoterin might play an important role in myogenesis, accelerating myogenic differentiation via Cdc42–Rac-1–MAPK kinase 6–p38 MAPK.

Myogenesis is a multistep process in which myoblasts cease to proliferate, express genes responsible for differentiation, and fuse into multinucleated cells, the myotubes, which finally build up the myofibrils (1, 2, 18, 31, 33, 39, 59). Several extracellular factors have been identified that participate in the regulation of myogenesis, some of which promote myoblast differentiation and/or myotube formation, while other factors inhibit these processes. Insulin, insulin-like growth factors (IGF I and IGF II), neuregulin, and nerve growth factor belong to the first category of agents (13–15, 28, 45), while tumor necrosis factor alpha (TNF-α), basic fibroblast growth factor (bFGF), and transforming growth factor β belong to the second category (12, 29, 30, 35, 37, 40, 42, 50, 56). However, IGF I and IGF II were reported to promote or inhibit myogenic differentiation depending on the absence or presence of TNF-α, respectively (16), and down-regulation of nerve growth factor low-affinity receptor was shown to be required for myoblast terminal differentiation (12). Signaling pathways implicated in the transduction of the effects of these agents acting on myoblasts include (i) the mitogen-activated protein (MAP) kinase (MAPK) p38 and Akt, the activation of which is required for myogenesis (5, 9, 10, 17, 32, 44, 55, 57, 62, 66); (ii) an NF-κB-dependent pathway activated by cytokines such as TNF-α, which interferes with myogenesis (30); (iii) a PW1-dependent, NF-κB-independent activation of caspases in the absence of apoptosis (8); (iv) the Ras–MEK–extracellular signal-regulated kinase (ERK) pathway, which suppresses myogenesis (4, 42, 43, 61) but is required at a later stage of muscle differentiation (4); and (v) activation of inducible nitric oxide synthase via NF-κB, which results in stimulation of myogenesis (25).

Recently, we found that S100B, a member of a multigenic family of Ca^{2+}-modulated proteins of the EF-hand type with both intracellular and extracellular regulatory activities (11, 19), inhibited myoblast differentiation and myotube formation when administered to the rat myoblast cell line L6 (51). Inhibition of myogenesis was registered at picomolar doses of S100B and was reversible, pointing to S100B binding to a cell surface receptor with a relatively high affinity. S100B was shown to bring about these inhibitory effects by inactivating p38 MAPK. While in other cell types responsive to S100B such as neurons and microglia the receptor for advanced glycation end products (RAGE) was demonstrated to bind S100B and to transduce the regulatory effects of S100B (20, 23) and while myoblasts were observed to express RAGE (mRNA and protein) constitutively (51), no such role for RAGE could be documented in the case of the inhibitory effects of S100B on myoblasts. In fact, S100B inhibited myoblast differentiation and myotube formation in L6 myoblasts stably overexpressing either full-length RAGE (L6/RAGE myoblasts) or a RAGE mutant lacking the cytoplasmic and transducing domain (L6/RAGEΔcyto myoblasts) (51). Collectively, these data suggested that S100B negatively regulated myogenesis in vitro by binding to a receptor other than RAGE. While the cell surface receptor transducing the effects of S100B on myoblasts remains to be identified, these data indicate that RAGE may not transduce the activity of myoblast differentiation inhibitory factors. Actually, in the course of these studies (51) we ob-

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served that overexpression of RAGE, but not of RAGEΔcyto, enhanced myoblast differentiation and myotube formation, suggesting the possibility that RAGE, likely activated by a factor contained in the culture medium, might play a role in myogenesis, transducing a promyogenic signal.

RAGE, a multiligand receptor belonging to the immunoglobulin superfamily (48, 49), has been shown to transduce inflammatory stimuli and effects of neurotrophic and neurotoxic factors and to have a role in tumor growth (20–23, 34, 54, 64, 65). As a member of the immunoglobulin superfamily RAGE is a potential candidate implicated in neuronal and myogenic differentiation. In fact, other members of the immunoglobulin superfamily have been shown to play a role in neuronal and skeletal muscle development (6, 27, 41, 46). Besides transducing effects of AGEs and β-amyloid (34, 60, 65), RAGE has been shown to transduce the effects of amphoterin (HMGB1), a protein with both intracellular and extracellular regulatory activities (38, 58), on neurons and astrocytes (21–23, 54), and S100A12, another member of the S100 protein family (11, 19), on inflammatory cells (20). It is known that RAGE is expressed in several cell types during development, is repressed or down-regulated at completion of development, and is reexpressed in adulthood in the course of certain pathological conditions, including neurodegeneration, inflammation, and cancer (48, 49). We show here that RAGE is expressed in rat skeletal muscle fibers in a developmentally regulated manner, RAGE engagement in myoblasts results in stimulation of myogenesis via activation of p38 MAPK, and overexpression of RAGE results in a further acceleration of myogenic differentiation. In contrast, these effects are negated in L6/RAGEΔcyto myoblasts as well as L6/RAGE myoblasts transfected with a constitutively inactive form of the p38 MAPK upstream kinase, MAPK kinase 6 (MKK6); Rac-1; or Cdc42. We also show that amphoterin activates RAGE, thereby promoting myogenic differentiation via p38 MAPK signaling and up-regulation of myogenin expression, and that amphoterin can override the antimyogenic activity of S100B and bFGF in myoblasts overexpressing RAGE.

MATERIALS AND METHODS

Cell culture conditions, transfections, and luciferase assay. Rat L6 myoblasts (clone L6C31) were cultured for 24 h in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U of penicillin/ml, and 100 μg of streptomycin/ml, in an H_2O-saturated 5% CO_2 atmosphere at 37°C before decreasing FBS to 2% to induce myoblast differentiation and myotube formation. L6 myoblast clones stably overexpressing RAGE or RAGEΔcyto were selected and characterized as described previously (51). Experiments were performed on wild-type (WT), mock-transfected, L6/RAGE, and L6/RAGEΔcyto myoblasts in 2% FBS unless stated otherwise. Recombinant S100B was expressed, purified, and characterized as described previously (23). bFGF was from Sigma Chemical.

Transient transfections were carried out using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Briefly, myoblasts cultured in 10% FBS without antibiotics were transfected with reporter gene MCK-luc (muscle creatine kinase-luc); expression plasmid for MKK6AA, N17Rac-1, N17Cdc42, or N17Ras (constitutively inactive forms of MKK6, Rac-1, Cdc42, and Ras, respectively); or empty vector. After 6 h in the cases of MCK, N17Rac-1, N17Cdc42, and N17Ras and 24 h in the case of MKK6AA, cells were cultivated in 10, 2, or 0.5% FBS containing no additions or amphoterin, S100B, and bFGF in various combinations, as indicated in the figure legends. After another 24 h cells were harvested to measure luciferase activity. MCK promoter transcriptional activity was normalized for transfection efficiency by cotransfecting cells with a cDNA encoding green fluorescent protein. The percentage of green fluorescent protein-positive cells (20 to 25%) was determined by fluorescence-activated cell sorter analysis.

Immunofluorescence and immunocytochemistry. Immunofluorescence analyses of L6 myoblasts were carried out as described previously (52) with a polyclonal anti-RAGE antibody (1:25, N16; Santa Cruz Biotechnology). Rat fetuses (embryonic day 18 [E18]) and skeletal muscle tissue from 11- and 60-day-old rat posterior limbs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 16 to 18 h at 4°C, rinsed several times with PBS for 24 h, and cryoprotected in 10 and 30% sucrose in PBS, each for 24 h. The tissues were then immersed in embedding medium (O.C.T. compound; BDH Italy), frozen in liquid nitrogen, and stored at −70°C. Ten-micrometer sections were cut at −26°C in a Cryocut 1800 (Leica) cryostat and placed on glass slides coated with Vectabound reagent (Vector Laboratories Inc.). Sections were stored dried at −70°C until staining. The cryostat sections were sequentially incubated for 1 h with 3% bovine serum albumin–1% glycine in PBS to block nonspecific sites and with the primary antibodies (see below), washed three times in 0.1% Tween 20 in PBS and two times in PBS, incubated with the appropriate fluorochrome-labeled secondary antibody for 1 h, and washed as described above. The samples were mounted in 80% glycerol, containing 0.02% NaN_3 and *p*-phenylenediamine (1 mg/ml) in PBS to prevent fluorescence fading. The antibody incubations were done in a humid chamber at room temperature. Immunofluorescence microscopy was performed on a Leica DM Rb fluorescence microscope equipped with a digital camera. Polyclonal anti-RAGE (M20; Santa Cruz Biotechnology) and monoclonal anti-sarcomeric α-actinin (Sigma Chemical) antibodies were diluted 1:25 and 1:2,000, respectively, in 3% bovine serum albumin in PBS. Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) and tetramethyl rhodamine isocyanate-conjugated goat anti-rabbit IgG (both from Sigma Chemical). In control experiments, the primary antibody was omitted. No fluorescence signal was detected under these conditions.

To detect myosin heavy chain (MHC) by immunocytochemistry, L6/RAGE myoblasts cultivated in 2% FBS for 3 days were fixed in cold methanol at −20°C for 7 min and subjected to immunocytochemistry with a monoclonal anti-developmental MHC antibody (Biogenesis) at a 1:1,000 dilution. The immune reaction product was visualized using the Vectastain Elite ABC kit (Vector Laboratories Inc.).

Western blot analyses. To detect MHC, myogenin, phosphorylated and total p38 MAPK, RAGE, and tubulin in myoblast extracts by Western blotting, myoblasts were cultivated as detailed in the legend of pertinent figures, washed twice with PBS, and solubilized with 2.5% sodium dodecyl sulfate–10 mM Tris-HCl (pH 7.4)–0.1 M dithiothreitol–0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone protease inhibitor (Roche). Detection of phosphorylated and total p38 MAPK was performed on myoblast extracts by Western blotting with a polyclonal antibody specific to phosphorylated (Thr180/Tyr182) p38 MAPK (1:1,000) and a polyclonal anti-p38 antibody (1:2,000), respectively (both from New England BioLabs). Amphoterin, MHC, myogenin, RAGE, and tubulin were detected by Western blotting with a monoclonal anti-RAGE antibody (1:1,000; Chemicon), a polyclonal antiamphoterin antibody (1:500; BD PharMingen), a monoclonal anti-developmental MHC antibody (1:300; Biogenesis), a monoclonal antimyogenin antibody (1:1,000; BD PharMingen), and a monoclonal anti-α-tubulin antibody (1:10,000; Sigma Chemical), respectively. The immune reaction was developed by enhanced chemiluminescence (SuperSignal West Femto Maximum for RAGE and SuperSignal West Pico for amphoterin, MHC, myogenin, and tubulin, both from Pierce). FBS was depleted of amphoterin by absorption to immobilized antiamphoterin antibody.

RT-PCR. Total cytoplasmic RNA was isolated from L6 myoblasts by the Trizol reagent method. Subsequent steps were done as described previously (51). After amplification, samples (10 μl) of each PCR mixture were electrophoresed on a 1.2% agarose gel and reverse transcription-PCR (RT-PCR) products were revealed by ethidium bromide staining.

RESULTS

RAGE is expressed in skeletal muscle tissue in a developmentally regulated manner. By immunofluorescence, in E18 rat fetuses RAGE was detected at the periphery of skeletal muscle fibers (identified by sarcomeric α-actinin immunostaining of Z disks), where it was associated with and restricted to the sarcolemma in both transverse and longitudinal sections irrespective of whether axial or limb muscles were analyzed (Fig. 1A, B, A′, and B′). In immature muscle fibers, recognized

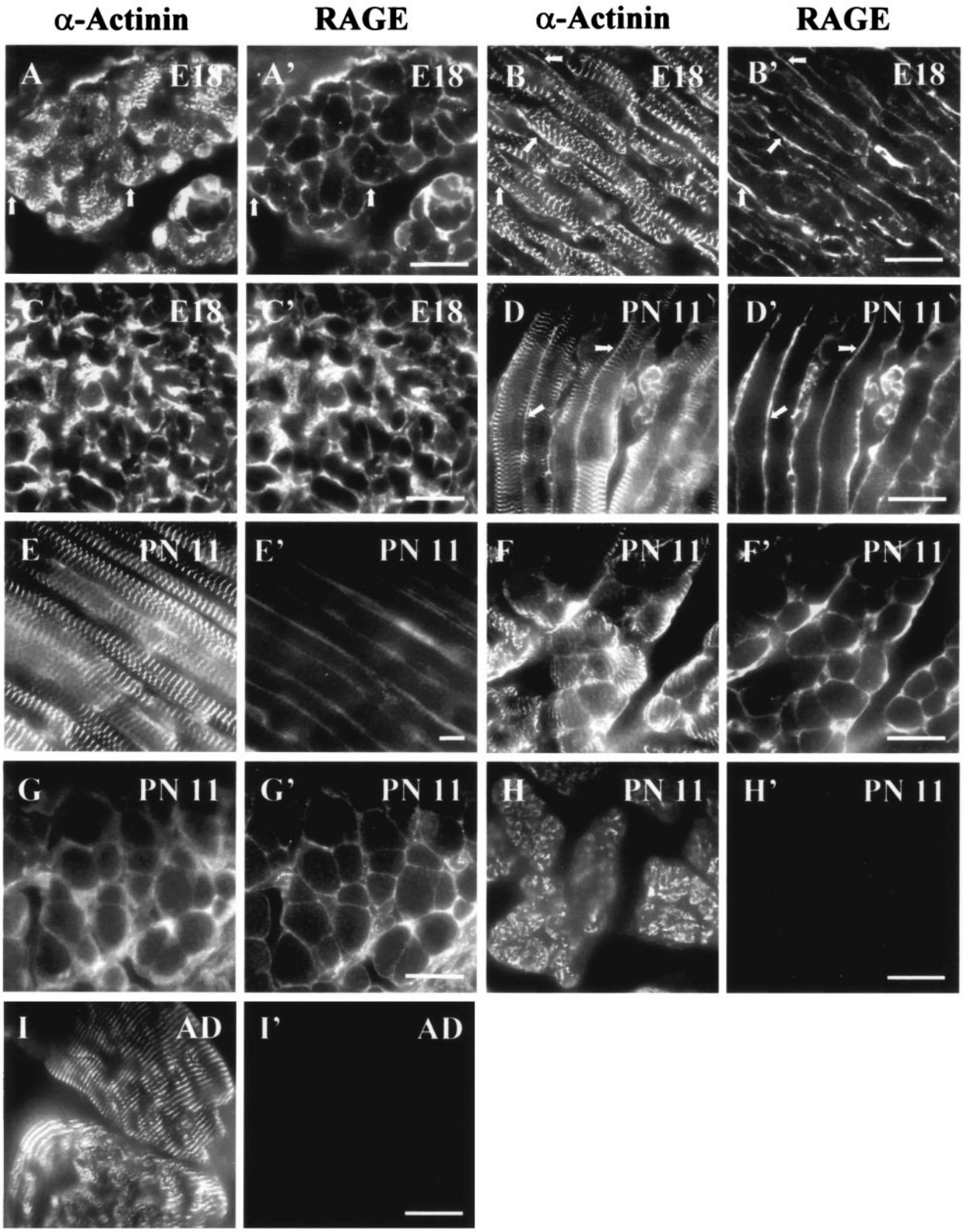


FIG. 1. Immunofluorescence detection of RAGE in developing and adult rat skeletal muscles. (A to C′) E18 rat. RAGE immunofluorescence is restricted to the sarcolemma in transverse (A′) and longitudinal (B′) sections, while α-actinin is found localized to Z disks and the sarcolemma (A and B). Arrows in panels A, A′, B, and B′ point to codistribution of RAGE and α-actinin at the sarcolemma. In some fibers α-actinin immunofluorescence is restricted to Z disks (A and B). RAGE is also found at the sarcolemma of immature fibers (C′) recognized by codistributed sarcomeric α-actinin (C). (D to H′) Eleven-day-old (PN 11) rat skeletal muscle tissue (posterior limb). RAGE localizes to the sarcolemma in both

by the presence of sarcomeric α-actinin at the sarcolemma and absence of Z-disk-associated α-actinin (Fig. 1C), RAGE co-distributed with α-actinin (Fig. 1C′). Immature skeletal muscle fibers were mostly found beneath the derma and immediately peripheral to bone tissue. In 11-day-old rats both mature skeletal muscle fibers, in which α-actinin was found restricted to Z disks (Fig. 1D and F), and fibers exhibiting α-actinin immunostaining at both Z disks and the sarcolemma (Fig. 1E) were found. These latter were taken as nearly mature fibers. RAGE immunostaining was again detected on and restricted to the sarcolemma in 11-day-old rats, in both mature and nearly mature muscle fibers (Fig. 1D′ to F′). Moreover, at this postnatal stage immature fibers could be found displaying sarcomeric α-actinin immunoreactivity at the sarcolemma only (Fig. 1G); these fibers also showed an intense RAGE immunofluorescence at the sarcolemma (Fig. 1G′). However, some fibers in which α-actinin immunostaining was restricted to Z disks (Fig. 1H) showed no RAGE immunostaining (Fig. 1H′). In contrast, while an intense α-actinin immunostaining that was restricted to Z disks was detected in 60-day-old rat skeletal muscles (Fig. 1I), no RAGE immunofluorescence signal could be registered (Fig. 1I′). Thus, RAGE was expressed in skeletal muscle fibers and its expression was found to be developmentally regulated, being completely absent in adult muscle tissue. This pattern of expression, which is typical of RAGE (48, 49), suggested that RAGE might play a role in skeletal muscle development. The presence of both RAGE-positive and RAGE-negative muscle fibers in 11-day-old rats suggested that the switch from RAGE expression to RAGE repression occurred around postnatal day 11.

Characterization of L6/RAGE and L6/RAGEΔcyto myoblasts. Rat L6 myoblasts express RAGE (mRNA and protein) constitutively (51), suggesting the possibility that RAGE may be implicated in the transduction of some important signal in these cells. To investigate the role of RAGE in myoblasts and its potential implication in myogenesis, we generated L6 myoblast cell lines stably overexpressing either full-length human RAGE or RAGEΔcyto (L6/RAGE and L6/RAGEΔcyto myoblasts, respectively) (51). Expression of individual forms of human RAGE mRNA in stably transfected L6 myoblasts was verified by RT-PCR (51). U251 cells, a human astrocyte cell line, and rat L6 myoblasts transfected with empty vector were used as positive and negative controls, respectively (data not shown). Western blot analyses demonstrated that L6/RAGE and L6/RAGEΔcyto myoblasts expressed similar amounts of L6/RAGE and L6/RAGEΔcyto proteins, respectively, and that these amounts were much larger than the amount of endogenous RAGE (51). By immunofluorescence, an antibody specific to RAGE decorated myotubes and nonfused myoblasts in both L6/RAGE and L6/RAGEΔcyto myoblasts with similar fluorescence intensities (Fig. 2A). Mock-transfected and WT myoblasts (Fig. 2A) also displayed RAGE immunoreactivity, although with a less intense fluorescence signal, which was in

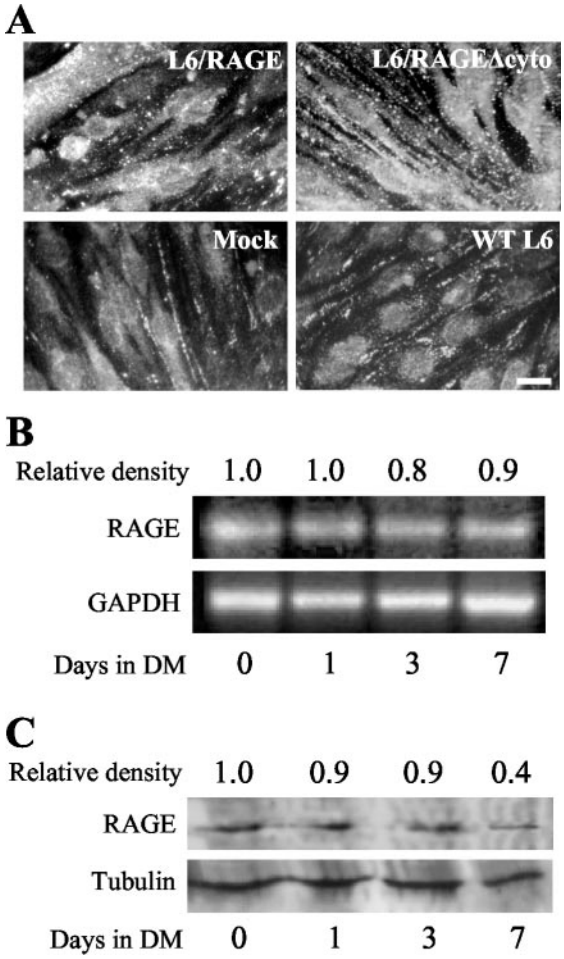


FIG. 2. Characterization of RAGE expression in rat L6 myoblasts by RT-PCR and immunofluorescence. (A) L6/RAGE, L6/RAGEΔcyto, mock-transfected, and WT myoblasts were cultivated for 2 days in 2% FBS (DM, differentiation medium) on glass coverslips, washed in PBS, and processed for indirect immunofluorescence as described previously (51) with a polyclonal anti-RAGE antibody. Note the presence of RAGE in both myoblasts and myotubes (these latter being detected in L6/RAGE myoblasts but not in L6/RAGEΔcyto, mock-transfected, and WT myoblasts, under the present experimental conditions). Also note the granular distribution of RAGE. Bar = 20 μm. (B) RT-PCR products for rat RAGE in rat L6 myoblasts under various culture conditions. WT myoblasts were cultivated for 24 h in 10% FBS and then for 1 to 7 days in 2% FBS. Shown are PCR products obtained with rat RAGE primers on day 0 (1 day after plating in 10% FBS) and 1, 3, and 7 days after the switch from 10 to 2% FBS (upper panel). The RAGE/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ratio is indicated as relative density above the panel. (C) Western blot analysis of RAGE under various culture conditions. Parallel WT myoblasts cultivated as described for panel B were processed for Western blotting with a monoclonal anti-RAGE antibody. The RAGE/tubulin ratio is indicated as relative density above the panel.

longitudinal (D′ and E′) and transverse (F′) sections, while α-actinin is mostly found associated with Z disks (D to F) and, in some fibers, the sarcolemma (arrows in panel D). Immature fibers exhibit both sarcomeric α-actinin (G) and RAGE (G′) immunofluorescence at the sarcolemma only. In contrast, completely mature fibers show α-actinin at the Z disks with no labeling of the sarcolemma (H), while no RAGE immunofluorescence can be detected (H′). (I and I′) Adult (AD) skeletal muscle tissue. α-Actinin immunofluorescence is restricted to Z disks in both longitudinal and transverse sections (I), while no RAGE immunofluorescence can be detected (I′). Bars = 25 μm (A to I′).

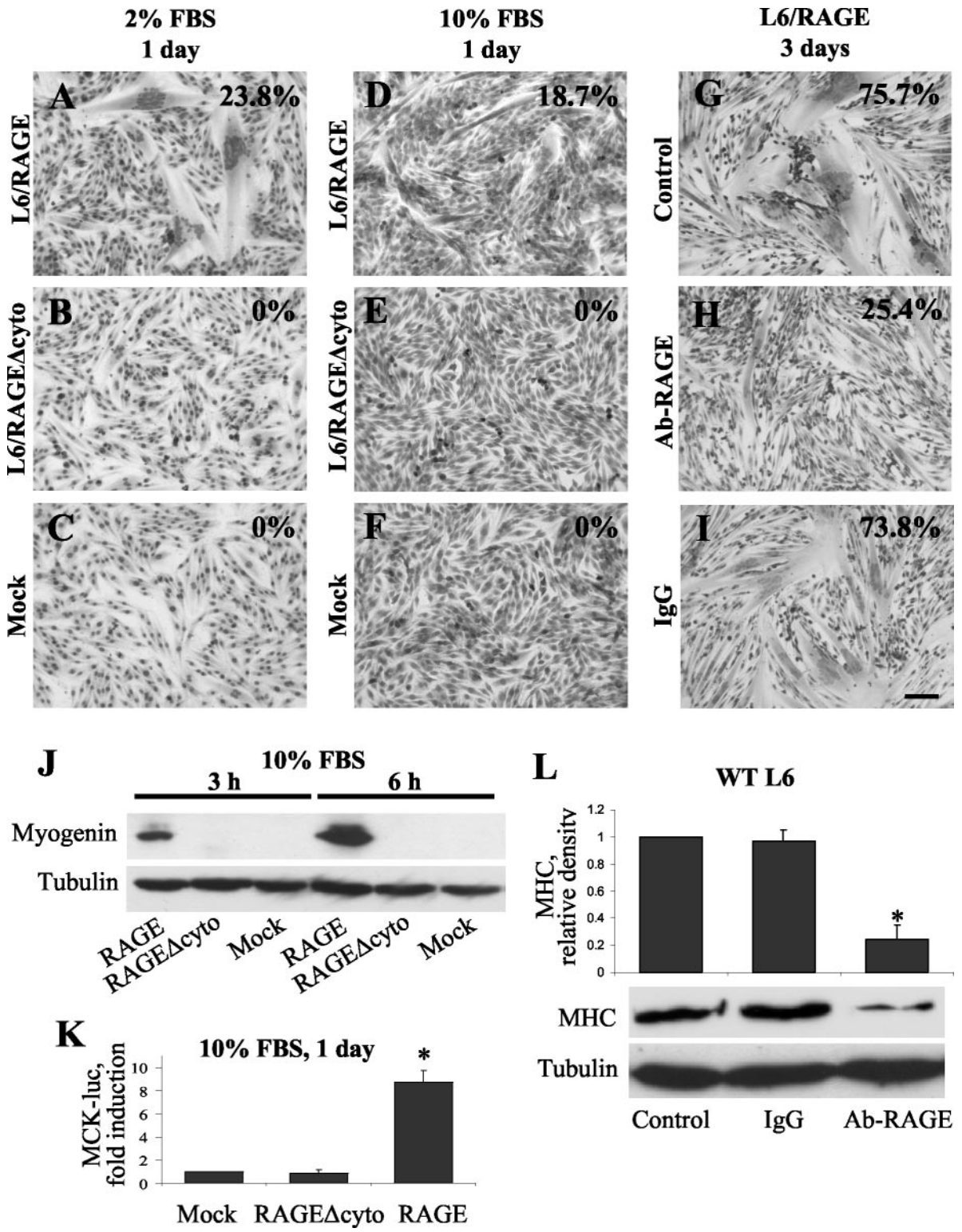


FIG. 3. RAGE-dependent acceleration of myogenic differentiation. (A to F) L6/RAGE, L6/RAGEΔcyto, and mock-transfected myoblasts were cultivated for 1 day in 2% FBS (A to C) or 10% FBS (D to F), fixed, and stained with May-Grünwald Giemsa stain (A and D, B and E, and C and F, respectively). (G to I) L6/RAGE myoblasts were cultivated for 3 days in 2% FBS containing 50 μg of a polyclonal anti-RAGE antibody/ml (H), 50 μg of nonimmune IgG/ml (I), or no additions (G); fixed; and stained with May-Grünwald Giemsa stain. In panels A to I, 20 random fields were analyzed to calculate the myogenic index (i.e., the fraction of nuclei residing in cells containing ≥3 nuclei after staining with May-Grünwald Giemsa stain) as a measure of myoblast fusion (see percentages in panels A to I). Shown in panels A to I are results from one experiment representative of three. Bar = 100 μm (A to I). (J) L6/RAGE, L6/RAGEΔcyto, and mock-transfected myoblasts were cultivated for 3 or 6 h as indicated, and cell extracts were analyzed for myogenin content by Western blotting. A Western blot of tubulin is included to show equal protein

agreement with previously reported RT-PCR and Western blotting data (51). Moreover, L6/RAGE myotubes were larger and more numerous than were L6/RAGEΔcyto and WT myotubes, in agreement with previous data (51). RAGE was mostly found in the form of focal and granular aggregates on the plasma membrane and, to a lesser extent, diffusely in the cytoplasm, similarly to immunolocalization data obtained using other cell types (7, 34).

RAGE mRNA and protein expression were analyzed in WT myoblasts in growth and differentiation medium (10 and 2% FBS, respectively). RAGE mRNA expression was shown to be nearly constant in proliferating and differentiating myoblasts in the time interval investigated (Fig. 2B). In contrast, RAGE protein levels were nearly constant in proliferating myoblasts and differentiating myoblasts for up to 3 days in differentiation medium and declined significantly between day 3 and day 7 after the switch (Fig. 2C). Thus, the maximal extent of RAGE protein expression was detected in proliferating WT myoblasts and during the initial stages of in vitro differentiation, while the lowest amounts were measured once ≥60% of myoblasts had fused into myotubes (see Fig. 4D). These data suggested that L6 myoblasts reduced RAGE mRNA translation and/or increased RAGE protein degradation during late phases of differentiation.

Overexpression of RAGE results in enhanced myogenesis. L6/RAGE myoblasts displayed a high fusogenic potential compared with L6/RAGEΔcyto or mock-transfected myoblasts, with myogenic indices of ~81, 15, and 17%, respectively, 3 days after switching FBS from 10 to 2% (51). One day after the FBS switch the myogenic index was ~23% in the case of L6/RAGE myoblasts and 0% in the cases of L6/RAGEΔcyto and mock-transfected myoblasts (Fig. 3A to C). Notably, L6/RAGE myoblasts started to differentiate (i.e., to express myogenin) and to form myotubes 3 and 24 h after plating in 10% FBS, respectively, i.e., under conditions in which WT L6 myoblasts (data not shown), L6/RAGEΔcyto myoblasts, and mock-transfected myoblasts did not differentiate or fuse (Fig. 3D to F and J). Administration of an anti-RAGE antibody reduced the extent of myotube formation in WT and mock-transfected myoblasts (data not shown) and L6/RAGE myoblasts (Fig. 3G to I). Stimulation of myogenic differentiation in L6/RAGE myoblasts in 10% FBS was also documented by the higher extent of induction of the early myogenic differentiation marker MCK compared with that for mock-transfected myoblasts (Fig. 3K). Importantly, little, if any, MCK induction was detected in parallel L6/RAGEΔcyto myoblasts, indicating that the myogenic differentiation detected in myoblasts in 10% FBS was strictly dependent on RAGE overexpression and activation and that RAGE engagement in myoblasts cultivated under these conditions was crucial for MCK induction. MCK induction in the myoblast cell line C2C12 has been shown to be under the control of the transcription factor MyoD, the activity of which is positively regulated by activated p38 MAPK (44). L6 myoblasts, which do not express MyoD (14), were observed to express myogenin in a p38 MAPK-dependent manner (36). As up-regulation of myogenin expression and MCK induction were detected in L6/RAGE myoblasts, while virtually no MCK induction could be seen in L6/RAGEΔcyto and mock-transfected myoblasts cultivated in 10% FBS (Fig. 3J and K, respectively), we speculated that RAGE engagement in L6 myoblasts might cause myogenin expression and MCK induction via activation of p38 MAPK (see below). Also, anti-RAGE antibody reduced the expression of the late differentiation marker MHC when administered to WT myoblasts (Fig. 3L), pointing to an important role of endogenous RAGE in myoblast differentiation.

Together, these data suggested that RAGE engagement was important for myogenesis and that myoblast RAGE was engaged by a serum factor with the ability to promote and sustain myoblast differentiation and myotube formation.

Amphoterin stimulates myogenic differentiation via RAGE engagement. Amphoterin is a recognized RAGE ligand (21, 22, 54). We detected amphoterin in FBS (Fig. 4G) and measured ~360 ng (~12 nM) of amphoterin/ml in FBS (data not shown). Administration of an antiamphoterin antibody to myoblasts in 2% FBS resulted in reduced myotube formation (Fig. 4A to F) and reduced induction of MCK (Fig. 4H) in both WT and L6/RAGE myoblasts. From these data we inferred that FBS amphoterin was able to engage myoblast RAGE, thereby promoting myogenic differentiation. Incidentally, data in Fig. 4A to F suggested that amphoterin-RAGE accelerated the process of myoblast differentiation, in accordance with data in Fig. 3J, given that similar myogenic indices were measured in L6/RAGE myoblasts at 2 days and WT myoblasts at 4 days. Thus, the promyogenic activity of amphoterin-RAGE appeared to add to that of the serum factors-receptors shown to promote myoblast differentiation (see the introduction). Conversely, administration of amphoterin resulted in up-regulation of myogenin expression in L6/RAGE but not L6/RAGEΔcyto or mock-transfected myoblasts cultivated for 6 h in 0.5% FBS (Fig. 4I). To confirm the role of FBS amphoterin as a stimulator of myogenic differentiation, FBS was depleted of amphoterin by absorption to immobilized antiamphoterin antibody, and the amphoterin-depleted FBS was used to cultivate L6/RAGE myoblasts. L6/RAGE myoblasts cultivated in amphoterin-depleted FBS expressed a smaller amount of myogenin and MHC than did parallel myoblasts cultivated in mock-absorbed FBS (Fig. 4J), and adding amphoterin back to am-

loading in each lane. (K) L6/RAGE, L6/RAGEΔcyto, and mock-transfected myoblasts in 10% FBS without antibiotics were transiently transfected with reporter gene MCK-luc, further cultivated in 10% FBS, and processed as described in Materials and Methods to measure luciferase activity. Under these culture conditions, L6/RAGE myoblasts show ~9-fold-more MCK induction than do L6/RAGEΔcyto and mock-transfected myoblasts. Thus, overexpression of RAGE in L6 myoblasts cultivated in 10% FBS (i.e., a condition usually referred to as "growth condition") triggers a differentiation program resulting in MCK induction, and this differentiation relies nearly entirely on RAGE activity. Values are averages of three determinations ± standard deviations. (L) Extracts from WT myoblasts treated as described for panels G to I were analyzed for MHC content by Western blotting. A Western blot of tubulin is included to document equal protein loading in each lane. Values are averages of three determinations ± standard deviations. *, significantly different from control ($P < 0.001$). Blockade of RAGE by a specific anti-RAGE antibody reduces myogenesis in both L6/RAGE and WT myoblasts (G to I and L), and RAGE overexpression results in accelerated myogenic differentiation (J and K).

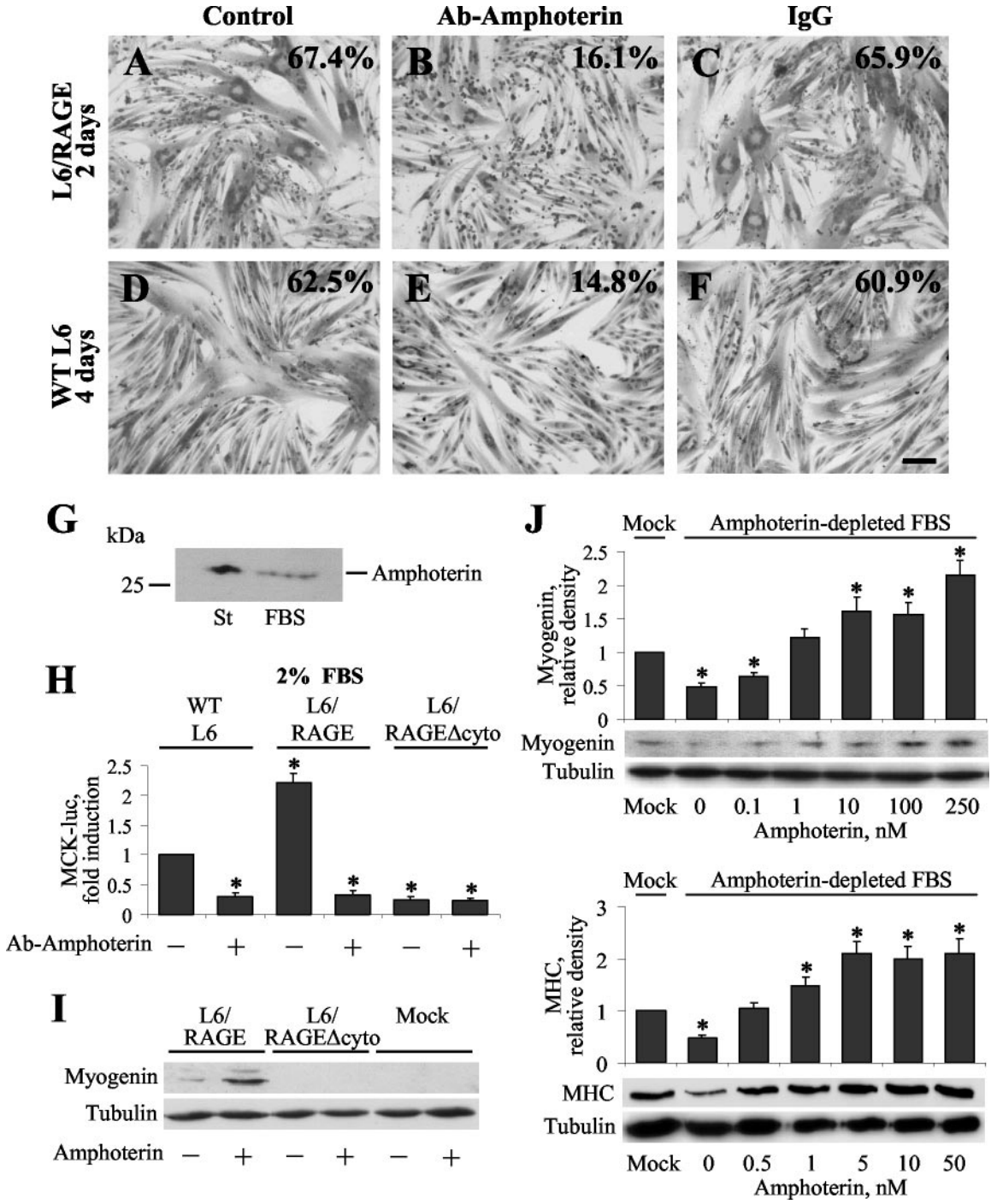


FIG. 4. Amphoterin stimulates myogenic differentiation via RAGE engagement. (A to F) L6/RAGE (A to C) and WT (D to F) myoblasts were cultivated for 2 and 4 days, respectively, in 2% FBS containing 5 μg of a polyclonal antiamphoterin antibody/ml (B and E), 5 μg of nonimmune IgG/ml (C and F), or no additions (A and D); fixed; and stained with May-Grünwald Giemsa stain to calculate the myogenic index (percentages in panels A to F). Shown are results of one experiment representative of three. Bar = 100 μm (A to F). (G) Detection of amphoterin in FBS by Western blotting. St refers to purified amphoterin. (H) WT, L6/RAGE, and RAGEΔcyto myoblasts were cultivated in the absence or presence of 5 μg of polyclonal antiamphoterin antibody/ml and analyzed for MCK induction. Neutralization of FBS amphoterin results in a decrease in MCK induction in both WT and L6/RAGE myoblasts. Similar (and low) extents of MCK induction are detected in L6/RAGEΔcyto myoblasts irrespective of the absence or presence of antiamphoterin antibody. (I) L6/RAGE, L6/RAGEΔcyto, and mock-transfected myoblasts were cultivated for 6 h in 0.5% FBS in the absence or presence of 300 nM amphoterin. Cell extracts were analyzed for myogenin expression by Western blotting. (J) L6/RAGE myoblasts cultivated in amphoterin-depleted FBS express lower levels of myogenin (upper panel) and MHC (lower panel) than do myoblasts cultivated in mock-absorbed FBS (Mock), and adding amphoterin back to amphoterin-depleted FBS results in a dose-dependent increase in myogenin and MHC expression, as investigated by Western blotting. Values are averages of three determinations ± standard deviations. *, significantly different from control ($P < 0.001$).

photerin-depleted FBS caused a dose-dependent stimulation of myogenin and MHC expression with maximal effect at a 5 to 10 nM concentration (Fig. 4J). About 0.5 to 1 nM amphoterin was sufficient to restore the extent of myogenin and MHC expression in L6/RAGE myoblasts cultivated in amphoterin-depleted FBS (Fig. 4J). Thus, a positive correlation was found between the amphoterin concentration in 2% FBS (~0.24 nM) and the amphoterin concentration (0.5 to 1 nM) required to restore myogenin and MHC expression in L6/RAGE myoblasts cultivated in amphoterin-depleted FBS.

Exposure of WT myoblasts for 24 h in 0.5% FBS to increasing doses of amphoterin resulted in a dose-dependent stimulation of MCK induction with half-maximal effect at ~50 nM and maximal effect at ~250 nM (~500% stimulation) (Fig. 5A). Similar results were obtained with mock-transfected myoblasts (data not shown). Under the same conditions L6/RAGE myoblasts showed a ~10-times-higher MCK induction in the absence of added amphoterin than did WT myoblasts, and administration of amphoterin resulted in a dose-dependent stimulation of MCK induction with half-maximal effect at ~10 nM and maximal effect at ~250 nM (~300% stimulation) (Fig. 5A). The robust MCK induction in L6/RAGE myoblasts in the absence of added amphoterin, compared with that in WT myoblasts, could be explained by the fact that cells had been exposed to ~1.2 nM amphoterin (i.e., the amphoterin concentration in 10% FBS) for 24 h before the switch to 0.5% FBS for another 24 h. During this time FBS amphoterin likely bound to and stimulated RAGE, thereby up-regulating myogenin expression (Fig. 3J), inducing MCK (Fig. 3K and 5A), and promoting myotube formation (Fig. 3D to F). Increasing the amphoterin concentration to 5 nM did not cause significant changes in the extent of MCK induction; however, a further increase in the amphoterin concentration did cause a further (approximately threefold) increase in MCK induction. A possible explanation for this latter result might be that, as RAGE ligands can up-regulate RAGE expression (48, 49), added amphoterin could have up-regulated RAGE expression. Alternatively, amphoterin might have induced a larger extent of receptor clustering, thereby increasing RAGE transducing activity. Amphoterin-induced RAGE clustering with consequent RAGE hyperactivation has been recently proposed (24). Considering the much smaller extent of MCK induction in WT myoblasts under the same experimental conditions (Fig. 5A), a similar situation could apply to WT myoblasts.

We repeated the experiments in Fig. 5A using WT and L6/RAGE myoblasts that had been exposed to increasing doses of amphoterin for 72 h. In the absence of added amphoterin L6/RAGE myoblasts displayed only a twofold-higher MCK induction than that of WT myoblasts under the same conditions (Fig. 5B). Administration of amphoterin to L6/RAGE myoblasts caused half-maximal stimulation of MCK induction at ~25 nM and maximal stimulation (~300%) at ~250 nM (Fig. 5B). Administration of amphoterin to WT myoblasts also determined a dose-dependent stimulation of MCK induction, with half-maximal effect at ~25 nM and maximal effect at ~250 nM (~450% stimulation), and the extent of MCK induction tended to equal that detected in L6/RAGE myoblasts as amphoterin doses were increased (Fig. 5B). Combined with data in Fig. 5A that pointed to a robust stimulation of MCK induction by amphoterin-RAGE at an early time point (i.e., 24 h), data in Fig. 5B suggested that amphoterin-RAGE actually accelerated myogenic differentiation, its promyogenic activity adding to that of other factors-receptors, such that at later time points (e.g., 3 to 4 days) differences between WT and L6/RAGE myoblasts were reduced and at relatively high amphoterin concentrations these differences proved to be negligible. Amphoterin exerted its promyogenic effect in the same range of concentrations in which the protein was reported to affect other cell types via RAGE binding (21–23, 48, 49).

Collectively, these data suggested that amphoterin may be a factor implicated in myogenesis, acting via RAGE binding, and that RAGE engagement by amphoterin in myoblasts results in an acceleration of myogenic differentiation.

Amphoterin counteracts S100B and bFGF antimyogenic activities via RAGE binding. As mentioned above, the Ca^{2+}-modulated protein S100B was shown to inhibit myogenic differentiation in a RAGE-independent manner (51). Thus, in a another set of experiments myoblasts were exposed to increasing doses of S100B and amphoterin in various combinations. S100B caused a dose-dependent inhibition of MCK induction in WT myoblasts with half-maximal effect at ~50 pM and maximal effect, i.e., ~60% inhibition, at 5 nM (Fig. 5C), in agreement with previous data (51). When WT myoblasts were exposed to increasing doses of amphoterin in the presence of 50 pM S100B, amphoterin completely reversed the S100B inhibitory effect at ~100 nM and caused a >200% stimulation of MCK induction at ~500 nM (Fig. 5D). With 50 nM S100B present, reversal of the S100B inhibitory effect was observed at ~250 nM amphoterin and increasing the amphoterin concentration to 500 nM resulted in no further changes (Fig. 5D). Thus, amphoterin was able to override the inhibitory effect of S100B on MCK induction with a potency that was inversely proportional to the S100B concentration and, conversely, S100B was able to override the stimulatory effect of amphoterin. However, at equimolar doses of amphoterin and S100B the inhibitory effect of S100B appeared to prevail over the stimulatory effect of amphoterin likely due to the high affinity (K_d of approximately 40 pM) of the binding of S100B to a thus far unidentified receptor, transducing its antimyogenic activity (51).

However, in L6/RAGE myoblasts as little as 10 nM amphoterin was able to override the inhibitory effect of 50 nM S100B on MCK induction, and maximal stimulatory effect of amphoterin was detected at ~100 nM (~300% stimulation) (Fig. 5E). Thus, overexpression of RAGE made myoblasts less sensitive to the antimyogenic activity of S100B, provided that sufficient amphoterin was present in the culture medium. Finally, with L6/RAGEΔcyto myoblasts no effects of amphoterin on MCK induction could be observed (Fig. 5F), while S100B reduced the extent of MCK induction irrespective of the absence or presence of amphoterin (Fig. 5F).

Results similar to those described for Fig. 5A to F were obtained using bFGF, a known inhibitor of myogenic differentiation (29), instead of S100B; bFGF caused a smaller reduction of MCK induction in L6/RAGE myoblasts than in WT myoblasts, and smaller doses of amphoterin were required to override the antimyogenic effect of bFGF on L6/RAGE myoblasts than were required for WT myoblasts (Fig. 5G and H).

Collectively, these data suggested that amphoterin stimu-

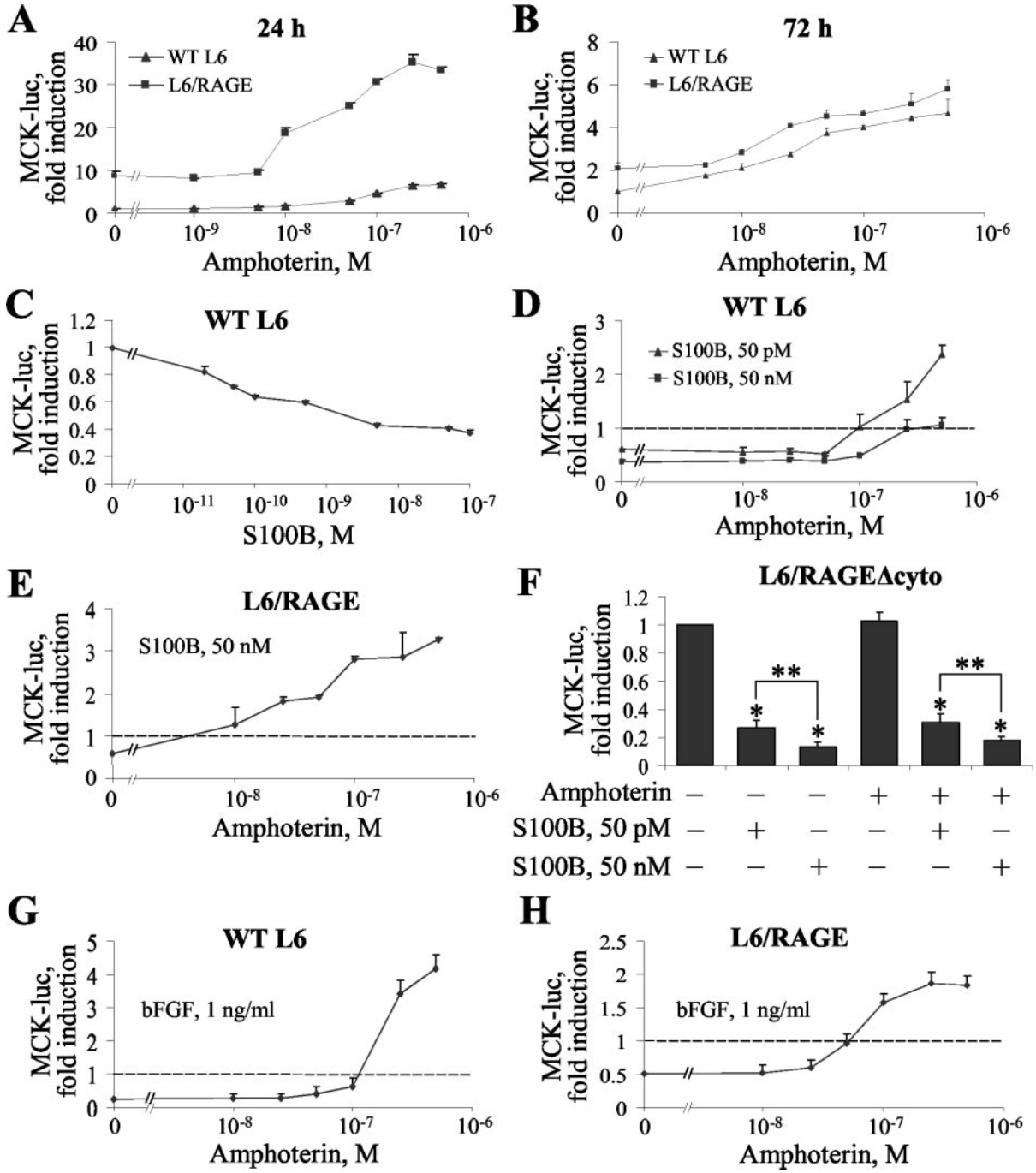


FIG. 5. Administration of amphoterin causes a dose-dependent increase in MCK induction in WT and L6/RAGE myoblasts and counteracts the antimyogenic activity of S100B protein and bFGF. (A and B) MCK induction in WT and L6/RAGE myoblasts cultivated in 0.5% FBS in the presence of increasing amphoterin concentrations for 24 (A) or 72 (B) h. Amphoterin causes a dose-dependent increase in MCK induction. (C) WT myoblasts were cultivated in 2% FBS in the presence of increasing S100B concentrations. S100B causes a dose-dependent reduction of MCK induction. (D) WT myoblasts were cultivated in 0.5% FBS containing 50 pM or 50 nM S100B plus increasing amphoterin concentrations, as indicated. Amphoterin overrides the inhibitory effect of S100B with a potency that is inversely proportional to the S100B concentration. The dashed line indicates MCK fold induction in myoblasts cultivated in the absence of added amphoterin and S100B. (E) L6/RAGE myoblasts in 0.5% FBS containing 50 nM S100B were cultivated in the presence of increasing amphoterin concentrations. Smaller concentrations of amphoterin are required for reversal of the inhibitory effect of S100B in the case of L6/RAGE myoblasts than in the case of WT myoblasts (compare panels D and E), suggesting that RAGE expression and amphoterin confer resistance to the antimyogenic activity of S100B. The dashed line indicates MCK fold induction in myoblasts cultivated in the absence of added amphoterin and S100B. Also note the smaller extent of inhibition of MCK induction by S100B (50 nM) in L6/RAGE myoblasts (E) than in WT myoblasts (D). (F) MCK induction in L6/RAGEΔcyto myoblasts cultivated in 0.5% FBS in the absence or presence of amphoterin or S100B. While amphoterin (500 nM) is without effect, S100B inhibits MCK induction irrespective of the absence or presence of amphoterin. (G and H) Conditions were as in panels D and E except that bFGF was used instead of S100B. The dashed line indicates MCK fold induction in myoblasts cultivated in the absence of added amphoterin and bFGF. Values are averages of three determinations ± standard deviations (A to E). *, significantly different from control ($P < 0.001$); **, significantly different ($P < 0.01$).

lated myogenic differentiation via RAGE binding and that the density of RAGE molecules on the myoblast surface and the relative concentration of amphoterin and S100B or bFGF dictated whether myoblasts did or did not undergo differentiation under the simultaneous action of the amphoterin and S100B or bFGF under our experimental conditions. Thus, amphoterin-RAGE appeared to effectively promote and sustain myoblast differentiation, thereby countering the activity of antimyogenic factors.

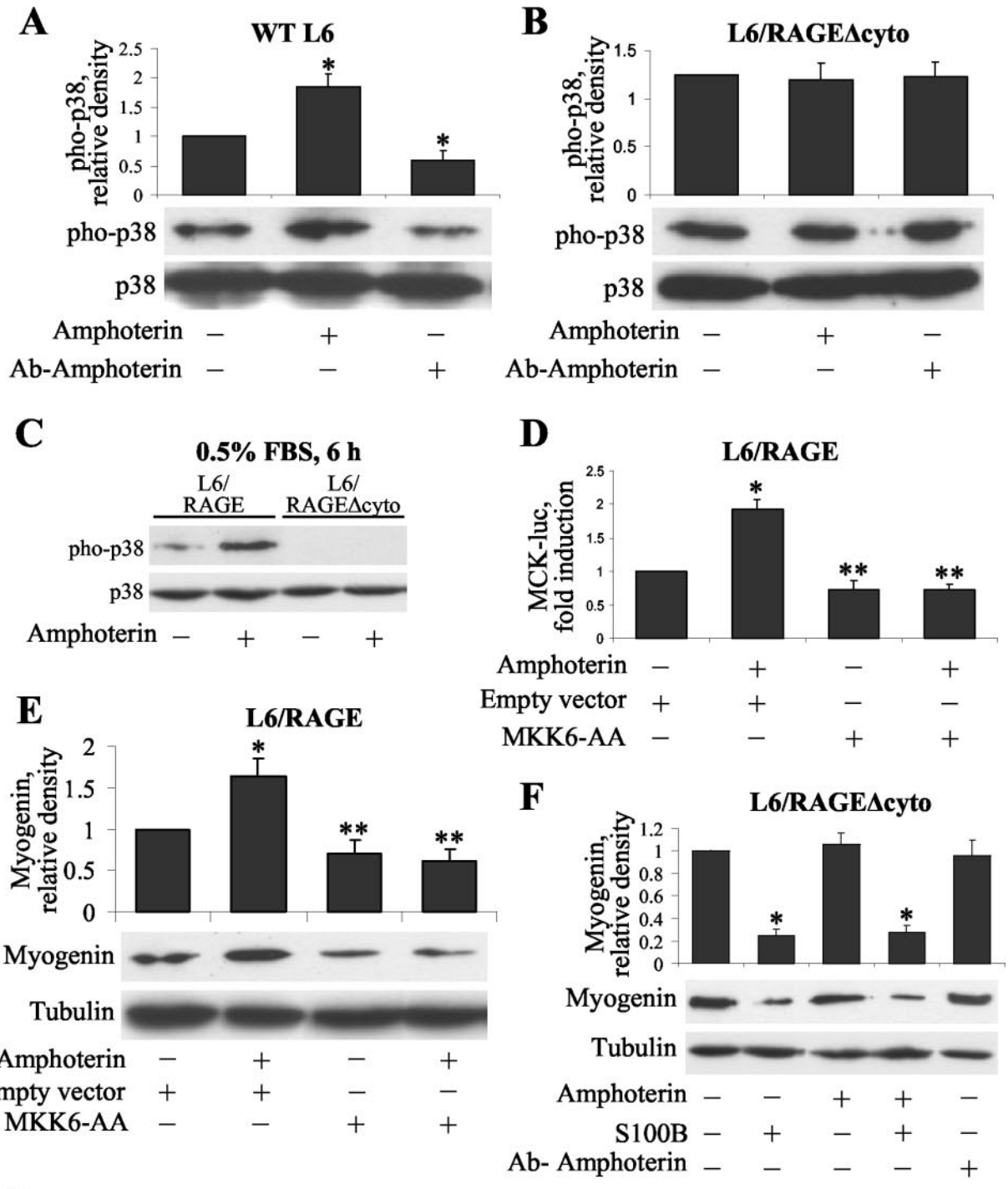
RAGE engagement on myoblasts activates p38 MAPK via Cdc42–Rac-1–MKK6. Myogenic differentiation and myotube formation were shown to depend on activation of p38 MAPK and Akt (5, 10, 17, 55, 62, 66). Treatment of WT myoblasts and L6/RAGE, L6/RAGEΔcyto, and mock-transfected myoblasts with either SB203580 (an inhibitor of p38 MAPK) or LY294002 (an inhibitor of the Akt-activating kinase phosphatidylinositol 3-kinase) resulted in the blockade of myogenic differentiation and myotube formation (data not shown), in accordance with the crucial role played by these kinases in myogenesis. Amphoterin increased the extent of p38 MAPK phosphorylation (activation) in WT myoblasts, and neutralization of FBS amphoterin with antiamphoterin antibody resulted in a reduction of p38 MAPK activation (Fig. 6A). In contrast, no changes in the extent of p38 MAPK phosphorylation were observed in L6/RAGEΔcyto myoblasts irrespective of whether amphoterin or antiamphoterin antibody was added to the cultures (Fig. 6B). Also, at a rather early time point (i.e., 6 h) L6/RAGE myoblasts exhibited p38 MAPK activation while L6/RAGEΔcyto myoblasts did not (Fig. 6C), and addition of amphoterin caused a ~10-fold activation of p38 MAPK in L6/RAGE myoblasts compared with untreated matched cells (Fig. 6C). In contrast, no p38 MAPK activation could be detected in L6/RAGEΔcyto myoblasts irrespective of the absence or presence of added amphoterin (Fig. 6C). Thus, the extent of amphoterin-induced phosphorylation of p38 MAPK (Fig. 6C) correlated positively with amphoterin-induced up-regulation of myogenin expression (Fig. 4I) and MCK induction (Fig. 5A) in L6/RAGE myoblasts, and overexpression of a dominant-negative form of RAGE (RAGEΔcyto) resulted in a reduction of the extent of p38 MAPK phosphorylation (Fig. 6C), myogenin expression (Fig. 4I), and MCK induction (Fig. 4H). In contrast, in L6/RAGEΔcyto myoblasts S100B reduced the extent of p38 MAPK phosphorylation, in accordance with previous data showing that S100B inhibits myogenic differentiation independently of RAGE via inhibition of p38 MAPK activity (51), and S100B was equally effective in the presence of amphoterin under these conditions (data not shown). Moreover, upon transient transfection of L6/RAGE myoblasts with MKK6AA, a constitutively inactive form of the p38 MAPK upstream kinase MKK6, a small extent of MCK induction, low levels of myogenin and MHC expression, and a reduced number of MHC-positive cells were detected in the absence of added factors, and administration of amphoterin was without effect (Fig. 6D, E, G, and H). Moreover, while amphoterin or antiamphoterin antibody did not affect myogenin expression in L6/RAGEΔcyto myoblasts, S100B inhibited myogenin expression irrespective of the absence or presence of amphoterin under the same conditions (Fig. 6F). No amphoterin-induced changes in the extent of Akt phosphorylation could be detected in WT or L6/RAGE myoblasts (data not shown).

Inactivation of Rac and Cdc42 has been shown to result in inhibition of myogenesis via inactivation of p38 MAPK (36), and Rac and Cdc42, but not Ras activation, by amphoterin-RAGE has been reported to induce differentiation in neuronal cell lines (22). Thus, we analyzed effects of transient transfection of L6/RAGE and L6/RAGEΔcyto myoblasts with constitutively inactive mutants of the small GTPases of the Rho family, Rac-1, Cdc42, and Ras (N17Rac-1, N17Cdc42, and N17Ras, respectively). Transfection of L6/RAGE myoblasts with either N17Rac-1 or N17Cdc42 resulted in a decreased extent of amphoterin-RAGE-dependent induction of MCK, while transfection with N17Ras was without effect (Fig. 7). In contrast, similar and small extents of MCK induction were noted in L6/RAGEΔcyto myoblasts irrespective of the small GTPase transfected and the absence or presence of amphoterin (Fig. 7). These data suggested that RAGE engagement by amphoterin resulted in the recruitment of Cdc42–Rac-1 with resulting activation of MKK6-p38 MAPK and p38 MAPK-dependent stimulation of myogenin and MHC expression and MCK induction.

Collectively these data suggested that (i) myogenin expression was positively regulated by p38 MAPK activity in L6 myoblasts, in agreement with previous work (36), and amphoterin-RAGE was able to enhance this event; (ii) a positive correlation could be drawn among p38 MAPK activation, myogenin and MHC expression, and MCK induction in L6/RAGE myoblasts; and (iii) amphoterin was able to engage RAGE, thereby triggering p38 MAPK activation, myogenin and MHC expression, and MCK induction via Cdc42–Rac-1–MKK6.

DISCUSSION

RAGE, a multiligand receptor of the immunoglobulin superfamily, plays an important role in a variety of activities-conditions such as the inflammatory response, diabetic angiopathy and neuropathy, neurodegeneration, cell migration, tumor growth, neuroprotection, and neuronal differentiation (20–23, 34, 48, 49, 54, 60, 64, 65). The pleiotropic and, in a way, contradictory activities of this receptor might depend on the variety of ligands able to engage it, the signaling pathways operating downstream of it, the nature of the intermediate(s) acting upstream of RAGE-activated signaling pathways, the ability of certain RAGE ligands (e.g., amphoterin) to functionally cross-link RAGE to extracellular matrix molecules, and, likely, the intensity and duration of stimuli acting on it. AGEs, β-amyloid, amphoterin, S100A12, S100B, and, recently, S100P have been shown to bind and activate RAGE in a variety of cell types with an affinity in the nanomolar range, thereby stimulating the activity of signaling pathways such as Ras-MEK-ERK, Cdc42–Rac-1, p38 MAPK, SAPK-JNK MAPK, and/or phosphatidylinositol 3-kinase–Akt (3, 20–23, 34, 48, 49, 54, 60, 63–65). While there is evidence that molecules recognized by RAGE might share a consensus sequence (26), and N-glycans on RAGE might play an important role in amphoterin-dependent activation of RAGE (53), the structural requirements of binding of individual ligands to RAGE are not fully elucidated at present. Interestingly, RAGE is expressed during development, repressed at completion of development, and reexpressed in adulthood in the course of certain pathological conditions (48, 49). Thus, aside from the role of this receptor


A
WT L6
pho-p38, relative density
pho-p38
p38
Amphoterin
Ab-Amphoterin
B
L6/RAGEΔcyto
pho-p38, relative density
pho-p38
p38
Amphoterin
Ab-Amphoterin
C
0.5% FBS, 6 h
L6/ RAGE
L6/ RAGEΔcyto
pho-p38
p38
Amphoterin
D
L6/RAGE
MCK-luc, fold induction
Amphoterin
Empty vector
MKK6-AA
E
L6/RAGE
Myogenin, relative density
Myogenin
Tubulin
Amphoterin
Empty vector
MKK6-AA
F
L6/RAGEΔcyto
Myogenin, relative density
Myogenin
Tubulin
Amphoterin
S100B
Ab- Amphoterin
G
Control
MKK6AA
H
MHC, relative density
MHC
Tubulin
Control
MKK6AA

in inflammation and atherosclerosis, for which there is growing and convincing evidence (see reference 47 and references therein), RAGE might play a role during development in a number of tissues. On the other hand, its belonging to the immunoglobulin superfamily makes RAGE a potential candidate involved in, e.g., neuronal and myogenic differentiation (6, 27, 41, 46).

Based on our earlier observation that rat L6 myoblasts express RAGE (mRNA and protein) constitutively and display a higher myogenic index and MHC immunoreactivity upon stable transfection with RAGE (51), we sought to investigate whether RAGE is actually expressed in skeletal muscle tissue and to determine the role of RAGE in myogenesis in vitro and obtain information about its mechanism of action. Immunofluorescence analyses suggest that RAGE is indeed expressed in skeletal muscle tissue, where it is restricted to the sarcolemma, and that its expression is developmentally regulated. Actually, RAGE can be detected in fetal and 11-day-old rat skeletal muscles while no RAGE immunofluorescence signal can be detected in adult skeletal muscles (Fig. 1). Our data also show that the anti-RAGE antibody uniformly decorates the sarcolemma in nearly mature skeletal muscle fibers and that RAGE codistributes with sarcomeric α-actinin at the sarcolemma in immature fibers. This pattern of expression is typical of RAGE, which, as mentioned above, is expressed during development in a variety of cell types and is normally repressed in their adult counterparts (48, 49).

To obtain information about the role of RAGE in myogenesis and its mechanism of action, WT L6 myoblasts, myoblasts stably transfected with either full-length RAGE or RAGEΔcyto (a RAGE mutant lacking the cytoplasmic and transducing domain), and mock-transfected myoblasts were used. Our present data suggest that RAGE and its ligand, amphoterin, might play a role in myogenesis, stimulating myogenic differentiation in rat L6 myoblasts by activating p38 MAPK via Cdc42–Rac-1–MKK6. Several lines of evidence support this conclusion. Firstly, an anti-RAGE antibody decorates WT myoblasts and myotubes as investigated by immunofluorescence, the intensity of the immunofluorescence signal is higher in L6/RAGE and L6/RAGEΔcyto myoblasts than in mock-transfected and WT myoblasts in agreement with RT-PCR and Western blot data (51), and RAGE is mostly found in the form of focal aggregates as described for other cell types (7, 34) (Fig. 2). Secondly, the myogenic index, the expression of the muscle-specific transcription factor myogenin and the late muscle differentiation marker MHC, and the induction of the early muscle differentiation marker MCK are all enhanced in L6/RAGE myoblasts compared with L6/RAGEΔcyto and mock-transfected myoblasts; treatment with an anti-RAGE antibody strongly reduces the extent of myotube formation and MHC expression; overexpression of full-length RAGE results in myoblast fusion, myogenin expression, and MCK induction even under culture conditions (i.e., in the presence of 10% FBS) in which WT, L6/RAGEΔcyto, and mock-transfected myoblasts do not differentiate; and L6/RAGEΔcyto myoblasts show a much lower induction of MCK than do WT myoblasts and L6/RAGE myoblasts (Fig. 3 and 4H). Thirdly, neutralization of FBS amphoterin with an antiamphoterin antibody reduces myogenic differentiation, lower levels of myogenin and MHC are detected in myoblasts cultivated in amphoterin-depleted FBS than in myoblasts cultivated in mock-absorbed FBS, adding amphoterin back to amphoterin-depleted FBS causes a dose-dependent enhancement of myogenin and MHC expression, administration of amphoterin to both WT and L6/RAGE myoblasts under differentiation conditions accelerates myogenic differentiation, and no such effects can be seen in L6/RAGEΔcyto myoblasts (Fig. 4 to 6). Lastly, amphoterin increases and antiamphoterin antibody reduces the extent of activation of p38 MAPK in WT and L6/RAGE myoblasts while no changes can be seen in L6/RAGEΔcyto myoblasts (Fig. 6), amphoterin cannot up-regulate myogenin and MHC or induce MCK in myoblasts transfected with a constitutively inactive form of MKK6 (Fig. 6), and no MCK induction can be registered in L6/RAGE myoblasts transfected with a constitutively inactive form of either Rac-1 or Cdc42 irrespective of the absence or presence of amphoterin (Fig. 7). Collectively, our data indicate that amphoterin-RAGE signals to MKK6 via Cdc42–Rac-1 and operates via a kinase, i.e., p38 MAPK,

FIG. 6. RAGE engagement by amphoterin activates p38 MAPK in L6 myoblasts. (A and B) WT (A) and L6/RAGEΔcyto (B) myoblasts were seeded in 10% FBS, left undisturbed for 24 h, and then cultivated for 24 h in 0.5% FBS in the presence of 5 μg of nonimmune IgG/ml (first lane from left in panels A and B), 5 μg of polyclonal antiamphoterin antibody/ml (third lane from left in panels A and B), or 300 nM amphoterin (second lane from left in panels A and B). Cell extracts were then analyzed for phosphorylated (activated) and total p38 MAPK content by Western blotting. Neutralization of FBS amphoterin results in a reduced activation of p38 MAPK in WT myoblasts (A) and no effects in L6/RAGEΔcyto myoblasts (B). Amphoterin stimulates p38 MAPK phosphorylation in WT but not L6/RAGEΔcyto myoblasts. (C) Conditions were as described for panels A and B except that myoblasts were cultivated for 6 h in 0.5% FBS in the absence or presence of 300 nM amphoterin. Cells were lysed and analyzed for phosphorylated and total p38 MAPK levels by Western blotting. Amphoterin determines a ~10-fold increase in the extent of stimulation of p38 MAPK phosphorylation, compared with untreated cells, in L6/RAGE but not L6/RAGEΔcyto myoblasts. (D) L6/RAGE myoblasts were transiently transfected with MKK6AA and MCK-luc, cultivated in 2% FBS for 24 h in the absence or presence of 250 nM amphoterin, harvested, and analyzed for luciferase activity. Amphoterin does not stimulate MCK induction in L6/RAGE myoblasts overexpressing a constitutively inactive form of MKK6 (MKK6AA). (E) L6/RAGE myoblasts were transiently transfected with MKK6AA or empty vector in 10% FBS, cultivated in 2% FBS for 3 h in the absence or presence of 300 nM amphoterin, lysed, and analyzed for myogenin content by Western blotting. Functional inactivation of p38 MAPK results in a reduced expression of myogenin irrespective of the absence or presence of amphoterin. (F) Conditions were as described for panels A and B except that myoblasts were cultivated for 3 days in 0.5% FBS in the absence or presence of amphoterin (300 nM), antiamphoterin (5 μg of polyclonal antiamphoterin antibody/ml), or S100B (50 nM); lysed; and analyzed for myogenin content by Western blotting. While amphoterin and antiamphoterin antibody are without effects, S100B down-regulates myogenin expression in accordance with the RAGE independence of S100B effects on myoblasts (51). (G) L6/RAGE myoblasts were transiently transfected with either MKK6AA or empty vector (control) and cultivated for 3 days in 2% FBS before fixation and immunocytochemistry with a monoclonal anti-MHC antibody. Bar = 100 μm. (H) Conditions were as in panel G except that myoblasts were analyzed for MHC content by Western blotting. Transfection with MKK6AA strongly reduces MHC expression (G and H). *, significantly different from control ($P < 0.001$); **, significantly different ($P < 0.01$).

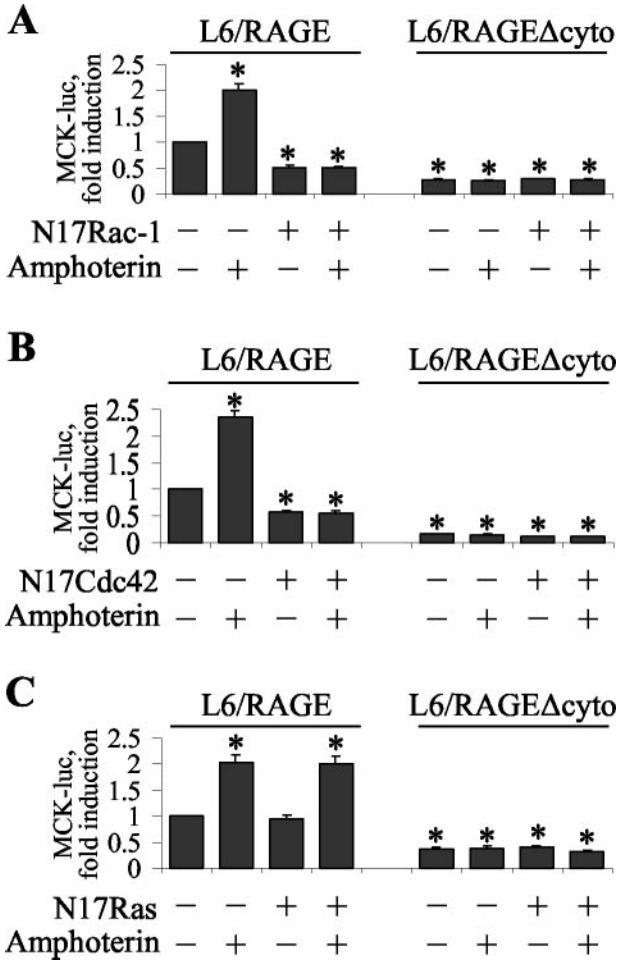


FIG. 7. Amphoterin-RAGE operates via Cdc42–Rac-1. (A) L6/RAGE and L6/RAGEΔcyto myoblasts were transiently transfected with N17Rac-1 and MCK-luc, cultivated in 2% FBS for 24 h in the absence or presence of 250 nM amphoterin, harvested, and analyzed for luciferase activity. Amphoterin does not stimulate MCK induction in L6/RAGE myoblasts overexpressing a constitutively inactive form of Rac-1 (N17Rac-1). No effects can be seen in parallel L6/RAGEΔcyto myoblasts. (B) Conditions were as for panel A except that cells were transfected with N17Cdc42. Again, amphoterin does not stimulate MCK induction in L6/RAGE myoblasts overexpressing a constitutively inactive form of Cdc42 (N17Cdc42), and no effects can be seen in parallel L6/RAGEΔcyto myoblasts. (C) Conditions were as for panel A except that cells were transfected with a constitutively inactive form of Ras (N17Ras). In this case, amphoterin was able to stimulate MCK induction in L6/RAGE, but not L6/RAGEΔcyto myoblasts, indicating that amphoterin-RAGE does not stimulate myogenesis via Ras signaling. Values in all panels are averages of three determinations ± standard deviations. *, significantly different from control ($P < 0.001$).

shown to be crucial for myogenesis (5, 9, 10, 32, 44, 62, 66). Both inactivation and hyperactivation of Cdc42 or Rac-1 result in inhibition of myogenesis (reference 36 and references therein), pointing to a complex regulation of myogenic differentiation and a delicate equilibrium between promyogenic and antimyogenic signaling pathways. Our data lend support to the notion that a certain extent of Cdc42–Rac-1 activation is required for p38 MAPK to induce the expression of myogenic transcription factors and for myogenic differentiation to take place (36) and suggest that RAGE engagement by amphoterin is one event playing a role in this process.

Our data suggest that amphoterin-RAGE acts to accelerate myogenic differentiation in vitro and that the amphoterin-RAGE promyogenic activity adds to that of other factors-receptors known to promote myogenesis. This is evidenced by the fact that, for example, similar myogenic indices are measured in L6/RAGE myoblasts at 2 days and WT myoblasts at 4 days (Fig. 3A to F); amphoterin-RAGE-dependent MCK induction is 10 times higher at day 1 and only 2 times higher at day 3 in L6/RAGE myoblasts than in WT myoblasts (Fig. 5A and B); and myogenin expression, MCK induction, and myotube formation can be detected in L6/RAGE myoblasts after 6 h (for myogenin expression) and 24 h (for MCK induction and myotube formation) of cultivation under growth conditions (10% FBS) (Fig. 3D to F, J, and H).

We also show that the Ca^{2+}-modulated protein S100B, which is another RAGE ligand (20, 23), which, however, inhibits L6 myoblast differentiation in a RAGE-independent manner (51), counteracts the stimulatory activity of amphoterin (Fig. 5). Low doses of S100B are sufficient to counteract the promyogenic effect of amphoterin likely due to the high affinity of S100B binding to its receptor on myoblasts (51). However, amphoterin can override the antimyogenic effects of S100B at lower doses in L6/RAGE myoblasts than in WT myoblasts. This suggests that the density of RAGE molecules on myoblasts and the relative concentrations of amphoterin and S100B might dictate the final fate of L6 myoblasts. Likewise, the promyogenic activity of amphoterin-RAGE can also override the antimyogenic effect of bFGF and vice versa (Fig. 5G and H), which adds to the ability of amphoterin-RAGE to promote myoblast differentiation and stresses the notion that skeletal muscle development is the net result of the coordinated activity of promyogenic and antimyogenic factors-receptors. That amphoterin and RAGE might act to promote myogenic differentiation is also documented by the observation that the rhabdomyosarcoma cell line TE671, which is characterized by a high proliferation rate, displays no ability to differentiate or form myotubes under differentiation conditions (44, 45), and does not express RAGE constitutively (unpublished data), can be induced to express myogenin, MCK, and MHC upon transfection with full-length RAGE in an amphoterin-regulated manner (G. Sorci, F. Riuzzi, and R. Donato, unpublished data). Thus, RAGE, activated by amphoterin, can be viewed as a receptor involved in the transduction of a myogenic signal in the in vitro cell system used here as represented schematically in Fig. 8.

Our immunofluorescence analysis of RAGE in rat skeletal muscle tissue (Fig. 1) and the biochemical characterization of RAGE signaling in rat L6 myoblasts (Fig. 2 to 7) strongly support the possibility that RAGE might be physiologically involved in skeletal muscle development. In particular, this receptor and amphoterin might cooperate with other factors-receptors in the regulation of prenatal and postnatal myogenesis and, potentially, skeletal muscle regeneration-repair. While the manuscript was in preparation evidence was presented that RAGE-null mice developed normally and showed no obvious phenotype (47). While no in-depth analysis of skeletal muscle development and morphology was carried out in the cited work, these data (47) suggest that RAGE is

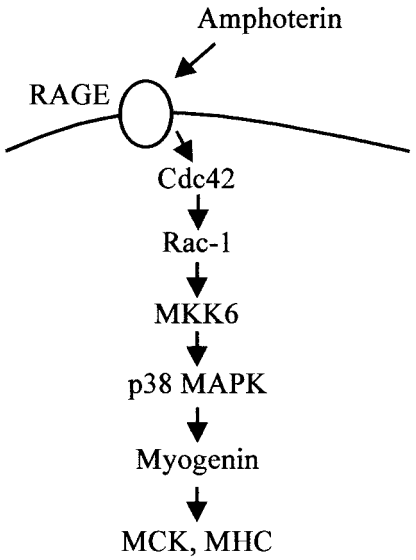


FIG. 8. Schematic representation of the putative mechanisms by which amphoterin regulates myogenic differentiation via RAGE engagement. Signals generated by amphoterin binding to RAGE converge on Cdc42–Rac-1–MKK6–p38 MAPK to up-regulate myogenin and MHC expression, induce MCK in L6 myoblasts, and accelerate myotube formation.

neither a vital gene nor indispensable for muscle development. However, it is known that muscle development is regulated by several factors-receptors including insulin and IGF and their receptors and novel receptors belonging to the immunoglobulin superfamily (6, 13–15, 27, 41, 46). Thus, RAGE knockout may not result in obvious muscular disturbances, which is in accordance with the present data that show that in our in vitro system amphoterin-RAGE accelerates, but is not indispensable for, myogenic differentiation (for example, Fig. 4A to F and 5A and B). Future analyses should establish whether RAGE might play a role in regeneration-repair of skeletal muscles.

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