p38 Mitogen-activated protein kinase expression and activation in smooth muscle

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Recent investigations in nonmuscle cells suggest that p38 mitogen-activated protein kinases (MAPKs) are part of a "stress-response" pathway coupled to phosphorylation of the low-molecular-mass heat shock protein (HSP27) (8, 14, 28). Four isoforms of p38 MAPKs have been cloned and sequenced from mammalian cells (13, 16, 17, 23). Analysis of the cDNA sequences shows they are homologues of yeast HOG1 MAPK, which is important for growth of yeast in high-osmolarity media as well as the formation of buds (12). The first mammalian p38 MAPK described is activated by physical and chemical stressors as well as by more physiological conditions (27), such as the proinflammatory cytokines, interleukins-1 (3) and -6 (4, 5). The p38 MAPK pathway is thought to be coupled to these diverse stimuli via upstream kinases PAK1 and MKK3 (7, 28, 35). Some of the important downstream events coupled to p38 MAPK activation include phosphorylation of MAPK-activated protein (MAPKAP) kinases 2 and 3, which in turn phosphorylate HSP27 (19, 21, 25, 29).

Although the precise function of HSP27 in smooth muscle is unknown, several reports suggest that HSP27 may modulate actin filament dynamics in vivo. This modulation of actin dynamics may be regulated by phosphorylation, since recombinant HSP27 (rHSP27) mutated at the MAPKAP kinase phosphorylation sites is unable to promote F-actin polymerization normally seen in control cells transfected with wild-type rHSP27 (21). A functional role for HSP27 phosphorylation in smooth muscle was suggested by Bitar et al. (6), who showed bombesin-induced contraction of isolated, permeabilized rectosigmoid smooth muscle cells was blocked by preincubation with a monoclonal antibody against HSP27. We recently showed that muscarinic receptors are coupled in airway smooth muscle to activation of MAPKAP kinase activity and phosphorylation of HSP27 and that both events are blocked by SB-203580, an inhibitor of p38 MAPKs (20). Therefore phosphorylation and activation of HSP27 may be linked to several cellular functions, including proliferation, locomotion, and smooth muscle contraction, possibly via actin remodeling.

Another potentially important function for the p38 MAPK in smooth muscles is phosphorylation of h-caldesmon. Adam and Hathaway (2) showed that h-caldesmon is a substrate for the extracellular-regulated protein kinase (ERK) MAPKs and that caldesmon is phosphorylated in vivo at proline-directed sites similar to MAPK consensus sequences (24). Because the amino acid sequences of the ERK MAPKs and the p38 MAPKs are >50% homologous and can both phosphorylate myelin basic protein in vitro (16), we tested the notion that caldesmon might be a substrate for p38 MAPKs.

Because there is relatively little known about expression of p38 MAPKs in mammalian smooth muscle and little evidence for activation through G protein-linked, seven-transmembrane-spanning (STM) receptors, we cloned and sequenced p38 MAPK expressed in canine smooth muscles. We find that the canine p38 MAPK is expressed in colonic, tracheal, and vascular smooth muscles. We also identified a p38 immunoreactive protein in canine smooth muscles and underwent increased tyrosine phosphorylation in response to motor neurotransmitters, acetylcholine (ACh) and neurokinin A (NKA), in colonic smooth muscle. There was an eightfold increase in p38 MAPK phosphorylation after a 10-min incubation with ACh and a threefold increase with NKA. We also identified a p38 immunoreactive kinase activity isolated from colonic smooth muscle homogenate by Mono Q chromatography. Partially purified p38 MAPK and activated recombinant p38 MAPK (Mpk2) phosphorylated both the known p38 MAPK substrate ATF2, as well as porcine stomach h-caldesmon in vitro. The results suggest that elements of the "stress-response" pathway may be coupled to transcriptional control as well as to cytoskeletal and possibly contractile protein phosphorylation in mammalian smooth muscle.
MATERIALS AND METHODS

Materials. Adult mongrel dogs of either sex were killed by barbiturate overdose. The colon was removed and placed in cold physiological salt solution (PSS) composed of 2 mM MOPS (pH 7.4), 140 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM Na2HPO4, 0.02 mM EDTA, and 5.6 mM d-glucose. Colonic smooth muscle of the circular smooth muscle layer was dissected free of the longitudinal smooth muscle and mucosa. Colonic smooth muscle cells were dispersed and grown to 100% confluence in medium 199 (GIBCO BRL) containing 10% fetal bovine serum. ^32P was purchased from ICN Biomedicals. ERK1-CT anti-MAPK polyclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-p38 MAPK antibody (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific p38 MAPK antibodies were purchased from New England Biolabs (Beverly, MA). Anti-rabbit IgG alkaline phosphatase-conjugated antibody was purchased from Promega (Madison, WI). Activated p38 MAPK was purchased from Upstate Biotechnology. The preparation is an NH2-terminal fusion protein containing MAL-E and amino acid residues 4–361 of Xenopus Mpk2 in a reaction volume of 50 µl in buffer containing 10 µl of 5× PCR buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, and 15 mM MgCl2; GIBCO BRL), 6 µl of 0.1 M dithiothreitol (DTT), 15 µl of 5 mM dNTPs (Invitrogen), 200 units of RNase inhibitor (Promega), and 500 ng of RNA. The reaction proceeded at room temperature for 10 min, 37°C for 50 min, and 95°C for 5 min and was cooled on ice for 10 min. Twenty units of RNase H (Promega) were added, and the reaction was further incubated for 20 min. PCR primers 5’-ATGTCCTCAGGAGGGGCCACGTCTT-3’ (forward nt position 295–319) and 5’-TCAGGACTCATCCTCCTTGTTGC-3’ (reverse nt position 1354–1377) were designed using OLIGO software and the human p38 MAPK sequence (13). Primers were purchased from Bio-Synthesis (Lewisville, TX).

PCR was performed in a reaction volume of 50 µl in buffer containing 10 µl of 5× PCR buffer (200 mM Tris-HCl, pH 8.4), 2 mM of each dNTP, 0.2 µM of each primer, 5 µl of cDNA (prepared above), and 0.5 units of Taq polymerase. The reaction was performed with an initial denaturation of 3 min, followed by 32 cycles (94°C/1 min, 54°C/1 min, and 72°C/2 min) and a final extension step of 10 min at 72°C in a thermal cycler (Erickomp). Reaction products (10 µl) were electrophoresed through a 1% agarose-Tris-acetate-EDTA gel and visualized with ethidium bromide.

PCR products were subcloned into the pCR2.1 vector (Invitrogen) using direct ligation in a 10-µl reaction containing 2 µl of the amplification products, 1 µl (10 ng) of TA vector DNA, 1 µl of 10× ligation buffer (Invitrogen), and 4 units of T4 ligase (Invitrogen). The ligation reaction was transformed into competent Escherichia coli and spread on ampicillin plates. Colonies were picked by blue-white selection and were sequenced using an ABI Prism 310 DNA automated sequencer. The cDNA sequence was determined using DNA Sequence Analysis software (ABI, Perkin Elmer, Foster City, CA). Further cDNA sequence comparisons, deduced amino acid sequences, and calculation of the isoelectric point of the p38 MAPK were performed using HIBIO DNASIS version 2.0 software (Hitachi Software Engineering, San Bruno, CA). p38 MAPK expression and phosphorylation in intact smooth muscle. To assess expression of p38 MAPK, canine colonic and tracheal smooth muscle strips (~2 × 10 mm) were mounted on stainless steel hooks and incubated for 60 min in oxygenated PSS (37°C). Canine pulmonary artery rings (2–3 mm) were suspended on stainless steel wires. Muscle strips or rings were stimulated three times for 5 min with 70 mM K+ to produce stable, reproducible contractions. Muscles were frozen by immersion in liquid nitrogen, and proteins were extracted in MAPK extraction buffer (20 µl/mg muscle) containing 20 mM Tris (pH 7.5), 5 mM EGTA, 1 mM Na2VO4, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM DTT, 1 µg/µl aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 10,000 g for 10 min (4°C). The protein extract was separated on an SDS-PAGE gel (12% acrylamide) and transferred to nitrocellulose paper in 25 mM Tris, 192 mM glycine, and 20% methanol using a Hoefer TE Transfer electrophoresis unit (0.5 mA, for 14 h, under continuous cooling at 15°C). The blot was probed with anti-p38 MAPK antibody and goat anti-rabbit IgG alkaline phosphatase secondary antibody.

p38 MAPK phosphorylation was measured in colonic smooth muscle strips stimulated with 200 µM sodium arsenite (40 min), 10 µM ACh, or 100 mM NKA for 0, 1, 5, 10, 20, and 30 min. For detection of p38 MAPK phosphorylation, colonic tissue strips were frozen at indicated times and frozen by immersion in liquid nitrogen, and proteins were extracted in MAPK extraction buffer (20 µl/mg muscle). Protein extracts (20 µg/lane) were analyzed by SDS-PAGE (10% acrylamide) and Western blotting as described above. Immunodetection of phosphorylated p38 MAPK was performed using 1:500 dilution of dual anti-phosphotyrosine-threonine-p38 MAPK primary antibody (New England Biolabs). The primary antibody was followed by labeling with goat-anti-rabbit alkaline phosphatase conjugate (1:5,000). Images of immunoblots scanned with a UMAX Powerlook flat-bed scanner were analyzed using the Volume Analyze feature of Molecular Analyst software (Bio-Rad, Hercules, CA). Densitometric data were normalized to the unstimulated muscles.

Mono Q chromatography. Colonic smooth muscle cells stimulated with 200 µM sodium arsenite were pulverized and homogenized in (100 µl/106 cells) Tris-EGTA-vanadate extraction buffer (pH 7.5). The buffer contained 20 mM Tris, 5 mM EGTA, 1 mM Na2VO4, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM DTT, 0.1 mM PMSF, 2 µg/ml leupeptin, 0.7 µg/ml pepstatin A, and 1% NP-40. The extracts were clarified by centrifugation at 100,000 g for 10 min at 4°C. MAPKs were separated on a Mono Q HR5/5 column equilibrated with 20 mM Tris (pH 7.5), 2 mM EGTA, 5% glycerol, 0.01% Brij 35, 0.1 mM Na2VO4, 1 mM NaF, 10 mM sodium glyceroxophosphate, 0.1 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A. The column was developed with a 60 mL NaCl gradient (0–4 M) at 0.5 ml/min, and 1.5-ml fractions were collected and analyzed for p38 MAPK, ERK1, and ERK2 using SDS-PAGE and immunoblotting. p38 MAPK activity was assayed by in vitro phosphorylation by fractions that were immunoreactive for p38 MAPK.

In vitro phosphorylation. Purified porcine caldesmon and ATF2 were phosphorylated in vitro with immunoreactive p38 MAPK. Mono Q fractions and Xenopus Mpk2 in a reaction volume of 70 µl containing 0.6 unit of p38 MAPK or 20 µl of Mono Q fraction (0.1 µg/µl total protein) with either 1 µM ATF2 or 1 µM porcine caldesmon. Reactions were performed with and without 25 µM SB-202190, an inhibitor of p38 MAPKs (22). Mono Q fractions with p38 MAPK activity were
pooled and used in phosphorylation reactions as described above. An immunoreactive ERK2 fraction was used as a positive control to phosphorylate caldesmon. All reactions contained 10 µM KN-62 to decrease phosphorylation of caldesmon by potential contaminating calmodulin (CaM) kinase II (1, 25). The reaction was terminated at various times (0, 30, and 180 min) by removing 20-µl aliquots and diluting them 1:4 with concentrated SDS sample buffer [0.24 M Tris (pH 6.8), 8% SDS, 40% glycerol, and 4 mM DTT]. Phosphorylated substrates were isolated by SDS-PAGE (8% acrylamide), and phosphorylation was detected with a Bio-Rad model G5525 Molecular Imager.

Statistical methods. Results are presented as means ± SE. Hypothesis testing was performed by using Student's t-test for unpaired data. The null hypothesis in studies of p38 MAPK phosphorylation was that normalized phosphorylation after treatment was not significantly different from 1. A probability of P < 0.05 was accepted as a significant difference.

RESULTS

Cloning and sequencing of p38 MAPK in mammalian smooth muscle. A full-length clone of the canine p38 MAPK expressed in colonic circular smooth muscle was obtained by RT-PCR using primers designed from the 5'- and 3'-ends of the coding region of human p38 MAPK (13). Amplified PCR products >1,000 nt from five individual subclones were cloned and sequenced. A cDNA of 1,083 bp encoding a predicted 360 amino acids was determined to have a nucleotide sequence identity of 95.8% to human p38 MAPK. The deduced amino acid sequence revealed 99 and 98% identity to the human and rat p38 MAPKs, respectively (Fig. 1; Ref. 13; GenBank accession no. AF003597). The amino acid sequence of canine p38 MAPK differed from the human enzyme in only two residues. There was an aspartic acid residue instead of glutamic acid at amino acid 245 and valine substituted for isoleucine at amino acid 346, both of which are conservative substitutions. The deduced molecular mass of the canine p38 MAPK is 41.2 kDa, with a calculated isoelectric point of 5.41.

Expression of p38 MAPK in canine smooth muscle. p38 MAPK expression was demonstrated by Western blotting with an anti-p38 MAPK polyclonal antibody. Proteins in SDS homogenates of canine trachea, colon, and pulmonary artery smooth muscle were separated by SDS-PAGE and stained with Coomassie blue (data not shown). Canine p38 MAPK was found to be expressed in all three smooth muscles, as evidenced by anti-p38 MAPK immunoreactive bands on Western blots (Fig. 2, A–C). The p38 MAPK bands had an apparent molecular mass of 40.1 kDa, which is similar to a molecular mass of 41.2 kDa calculated from the deduced amino acid sequence in Fig. 1. The polyclonal antibody also cross-reacted with other higher-molecular-mass proteins of unknown relationship to p38 MAPK.

Phosphorylation of p38 MAPK in intact colonic smooth muscle strips. Human p38 MAPK is known to be activated by upstream kinases MKK3 and MKK6 by dual phosphorylation of threonine 180 and tyrosine 182 in the regulatory TGY motif (27). Phosphorylation of threonine-180 and tyrosine-182 has been used as an index of p38 MAPK activation and can be assayed with an anti-p38 phosphotyrosine-threonine-specific antibody recognizing the TGY motif. To test the notion that
stimuli in addition to chemical and physical stressors can activate p38 MAPK in smooth muscle, we first established the linearity of the assay for p38 MAPK phosphorylation. A dilution series of homogenates from a colonic smooth muscle strip stimulated 5 min with 10 µM ACh was separated by SDS-PAGE and analyzed by Western blotting (data not shown). Densitometry of the p38 MAPK phosphotyrosine bands showed the assay was linear over a 10-fold range of protein loading (2.5–20 µg protein).

Activation in response to motor neurotransmitters. ACh and NKA are neurotransmitters in colonic smooth muscle of the dog that are coupled via G protein-linked STM receptors to the contractile apparatus. ACh acts on muscarinic M2 and M3 receptors to increase phosphatidylinositol turnover, elevate intracellular Ca\textsuperscript{2+}, activate some ion channels, and inhibit K\textsuperscript{+} channels (30) leading to phasic contractions (9,32). NKA acts via NK\textsubscript{2} receptors to activate a similar set of intracellular signals, also resulting in phasic contractions (18). Both ACh and NKA also activate the ERK MAPKs in colonic smooth muscle, which were suggested to catalyze phosphorylation of caldesmon in this muscle (10). Because there are many examples of parallel signaling and cross talk between MAPK cascades, we tested the possibility that p38 MAPK is also coupled to G protein-linked STM receptors in colonic smooth muscle. Muscle strips from four animals were incubated for 30 min with 10 µM ACh or 100 nM NKA, which are EC\textsubscript{50} concentrations of these agonists. Muscle strips were also stimulated with 200 µM sodium arsenite to allow comparison to a stress-activated response. Muscle strips were frozen at indicated times (Fig. 3) and homogenized, and total protein extracts were resolved by SDS-PAGE. Phosphorylation of p38 MAPK was detected by Western blotting with anti-phospho-p38 MAPK antibody. Figure 3 (top) shows the relative change in band density after stimulation. Densitometry of the immunoreactive bands showed that there was nearly an eightfold increase in p38 MAPK phosphorylation after a 10-min incubation with ACh (Fig. 3A), a threefold increase with NKA after 10 min (Fig. 3B), and a fourfold increase after a 40-min incubation with sodium arsenite (Fig. 3C). The results show that p38 MAPK is coupled to both muscarinic and neurokinin receptors in colonic smooth muscle.

Substrates for p38 MAPK. Known in vitro substrates for p38 MAPKs include MAPKAP kinases, myelin basic protein, and transcription factors ATF2 and CHOP (27, 31). Because the ERK MAPKs and p38 MAPKs have common in vitro substrates, including myelin basic protein and MAPKAP kinases, we tested the possibility that caldesmon is also a substrate for both types of MAPKs. We previously showed that purified bovine aorta caldesmon is a substrate for canine ERK MAPKs.
We also find that p38 MAPK immunoreactive protein was separated from ERK1 and ERK2 kinases by Mono Q chromatography, but we did not report enzyme activities. In the present study, colonic smooth cells were stimulated with 200 µM sodium arsenite for 40 min and homogenized in MAPK extraction buffer, and p38 MAPK separated from the ERK MAPKs by Mono Q chromatography (10, 20). Proteins were eluted with an NaCl gradient (0.0–0.4 M), and the collected column fractions were probed with polyclonal antibodies to establish the separation of ERK (data not shown) and p38 MAPKs. Figure 4 (top) represents separation of p38 MAPKs assessed by Western blotting. Western blots of the same fractions were probed with a p38 MAPK antibody and with the ERK1-CT antibody (UBI), which recognizes both ERK1 and ERK2 (data not shown). p38 MAPK eluted at 0.30–0.36 M NaCl in fractions 45–54. p38 MAPK activity was assayed by reacting fractions with 1 µM ATF2 in presence (+) and absence (−) of p38 MAPK inhibitor SB-202190. Kinase reaction was stopped by addition of SDS-PAGE sample buffer after 30 min. Phosphoproteins were resolved by SDS-PAGE, and radioactive phosphorus incorporation was measured by imaging gels with a Bio-Rad Molecular Imager.

Fig. 4. p38 MAPK separation by Mono Q chromatography. First-passage muscle cells were stimulated with 200 µM sodium arsenite and homogenized, and extracts were clarified by centrifugation at 100,000 g for 10 min at 4°C. MAPKs were separated on a Mono Q HR5/5 column. Column was developed with a 60-ml NaCl gradient (0–0.4 M) at 0.5 ml/min, and 1.5-ml fractions were collected and analyzed for extracellular signal-regulated kinase (ERK; data not shown) and p38 MAPKs using SDS-PAGE and immunoblotting (top). Fraction numbers indicate milliliters collected from 60-ml gradient. Proteins eluting during salt gradient were detected by measuring absorbance at 280 nm. p38 MAPK eluted at 0.30–0.35 M NaCl in fractions 45–54. p38 MAPK activity was assayed by reacting fractions with 1 µM ATF2 in presence (+) and absence (−) of p38 MAPK inhibitor SB-202190. Kinase reaction was stopped by addition of SDS-PAGE sample buffer after 30 min. Phosphoproteins were resolved by SDS-PAGE, and radioactive phosphorus incorporation was measured by imaging gels with a Bio-Rad Molecular Imager.

As an alternative approach to establishing caldesmon as a substrate, Mpk2 was reacted with ATF2 and porcine caldesmon in the absence and presence of 25 µM SB-202190, a p38 MAPK inhibitor. All caldesmon reactions also contained 10 µM KN-62 to inhibit phosphorylation by contaminating CaM kinase II (1, 26). The kinase reaction was stopped by addition of concentrated SDS-PAGE sample buffer, and the phosphoproteins were resolved by SDS-PAGE. Radioactive phosphorus incorporation was measured by imaging dried gels with a Bio-Rad Molecular Imager. ATF2 was phosphorylated by p38 MAPK in vitro (Fig. 5A), consistent with previous reports (16, 17). Porcine caldesmon was also phosphorylated in vitro by p38 MAPK (Fig. 5B). The p38 MAPK inhibitor SB-202190 blocked phosphorylation of both substrates (Fig. 5), verifying the specificity

![Fig. 5. Substrates for p38 MAPK. Activated recombinant p38 MAPK (Mpk2) was reacted with 1 µM ATF2 (A) and 1 µM porcine caldesmon (B) in presence (● and ○, respectively) and absence (■ and ▲, respectively) of p38 MAPK inhibitor SB-202190. Kinase reaction was stopped by addition of SDS-PAGE sample buffer after indicated time points. Phosphoproteins were resolved by SDS-PAGE, and radioactive phosphorus incorporation was measured by imaging gels with a Bio-Rad Molecular Imager.](http://ajpcell.physiology.org/)
of the phosphorylation by the purified recombinant enzyme. Caldesmon phosphorylation by smooth muscle p38 MAPK. The partially purified p38 MAPK from the pooled Mono Q fractions and an ERK2-enriched fraction (fraction 21; data not shown) were used to phosphorylate caldesmon in vitro as described above (Fig. 6). The activated p38 MAPK from colonic smooth muscle phosphorylated porcine caldesmon to a stoichiometry of 1.5 mol phosphate/mol caldesmon, similar to ERK phosphorylation stoichiometry, which was 1.6 mol phosphate/mol caldesmon. The p38 MAPK activity was inhibited by 25 µM SB-202190, verifying the specificity of phosphorylation by p38 MAPK and not a contaminating enzyme. These data suggest that p38 MAPK can phosphorylate caldesmon in vitro and has a similar activity to ERK.

DISCUSSION

In nonmuscle cells, the p38 MAPKs are known to participate in the response to stressful stimuli, including osmotic shock, ultraviolet radiation, and proinflammatory cytokines. The p38 MAPK is a homologue of the HOG1 kinase in Saccharomyces, which is activated by osmotic stress (12). The mammalian homologue is thought to participate in inflammatory responses by being activated by interleukin-1 and tumor necrosis factor-α (8, 27). The kinase cascade upstream of p38 MAPKs is thought to include small G proteins rac and cdc42 and activation of MKK3 and/or MKK6 and PAK1.
lated by phosphorylated HSP27 in vivo. Bitar et al. (6) suggested a functional role for HSP27 phosphorylation in smooth muscle by showing that bombesin-induced contraction of isolated, permeabilized rectosigmoid smooth muscle cells was blocked by preincubation with a monoclonal antibody against HSP27. These results and our recent study of MAPKAP kinase activation and HSP27 phosphorylation in smooth muscle (20) suggest p38 MAPK activation may be linked to several functions, including activation of transcription factors, cell motility, and smooth muscle contraction.

We also considered the possibility that p38 MAPKs are coupled to phosphorylation of caldesmon in smooth muscles because the amino acid sequences of the p38 MAPK and ERK MAPKs are >50% homologous and both can phosphorylate myelin basic protein in vitro (16). Adam and Hathaway (2) showed that h-caldesmon is a substrate for the ERK MAPKs and that caldesmon is phosphorylated in vivo at proline-directed sites similar to MAPK consensus sequences. We showed that ERK1 and ERK2 MAPKs are expressed in intact colonic smooth muscle and that bovine aorta caldesmon is a substrate in vitro for the agonist-activated ERK MAPKs (11). The in-gel kinase assay used in our previous study would not distinguish p38 MAPK (41.2 kDa) from ERK2 MAPK because both enzymes migrate at ~39–40 kDa on SDS-polyacrylamide gels that we use in the assay. We directly tested the notion that caldesmon might be a substrate for p38 MAPKs by using partially purified p38 MAPK from stimulated colon smooth muscle cells and purified Mpk2, the p38 MAPK (32). We found that p38 MAPK can phosphorylate porcine smooth muscle h-caldesmon, suggesting that both ERK and p38 MAPKs in smooth muscles might phosphorylate h-caldesmon. Further studies are required to establish the necessity and sufficiency of p38 MAPK activation in catalyzing caldesmon phosphorylation in vivo, the site(s) phosphorylated, and the functional significance of this reaction, if any.

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