Activation of p38α/β MAPK in myogenesis via binding of the scaffold protein JLP to the cell surface protein Cdo

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The p38 mitogen-activated protein kinase (MAPK) pathway plays an important role in cell differentiation, but the signaling mechanisms by which it is activated during this process are largely unknown. Cdo is an immunoglobulin superfamily member that functions as a component of multiprotein cell surface complexes to promote myogenesis. In this study, we report that the Cdo intracellular region interacts with JLP, a scaffold protein for the p38α/β MAPK pathway. Cdo, JLP, and p38α/β form complexes in differentiating myoblasts, and Cdo and JLP cooperate to enhance levels of active p38α/β in transfectants. Primary myoblasts from Cdo−/− mice, which display a defective differentiation program, are deficient in p38α/β activity, and the expression of an activated form of MKK6 (an immediate upstream activator of p38) rescues the ability of Cdo−/− cells to differentiate. These results document a novel mechanism of signaling during cell differentiation: the interaction of a MAPK scaffold protein with a cell surface receptor.

Introduction

MAPKs function as the terminal components of three-tiered cascades of kinases comprised of a MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK and are important signal transducers in development, homeostasis, and disease (Chang and Karin, 2001). For example, the p38 subfamily of MAPKs is involved in a wide variety of biological processes, including inflammation, stress responses, and cell differentiation (Zarubin and Han, 2005). The myriad roles of MAPK cascades indicate that the specificity of MAPK activation and function must be regulated. One mechanism by which this occurs is via MAPK scaffold proteins, which are thought to provide (1) specificity between distinct MAPK subfamilies by assembling individual MAPK modules and (2) precise spatial and temporal regulation to MAPK signaling (Morrison and Davis, 2003). How this latter function is accomplished is unclear, but it suggests that scaffold proteins may interact with cell-type specific factors.

Differentiation of cells in the skeletal muscle lineage is coordinated by the family of myogenic bHLH factors (Myf5, MyoD, myogenin, and MRF4; Arnold and Braun, 2000). During differentiation, these proteins work together with additional transcription factors, notably MEF2, to drive muscle-specific gene expression and promote myoblast fusion into myofibers (Molkentin and Olson, 1996; Penn et al., 2004). Tight control of these transcription factors during myogenesis is required, and their activities are regulated by signal transduction pathways. Much evidence indicates that the p38α/β MAPK pathway plays an important role in myogenesis (Cuenda and Cohen, 1999; Zetser et al., 1999; Wu et al., 2000; de Angelis et al., 2005). p38α/β activity increases and persists in differentiating myoblasts, and differentiation is blocked by the p38α/β inhibitor SB203580. p38α/β phosphorylates several proteins involved in muscle-specific gene expression, including MEF2 isoforms, the myogenic bHLH heterodimeric partner E47, the SWI–SNF complex subunit BAF60, and the RNA decay–promoting factor KH-type splicing regulatory protein (Wu et al., 2000; Simone et al., 2004; Briata et al., 2005; Lluis et al., 2005). MAPKs are generally activated in response to extracellular stimuli, and
many such cues that activate p38 during inflammatory and other responses have been identified (Dong et al., 2002). However, despite the attention p38α/β has received as a modulator of myogenesis, the signaling mechanisms by which it is activated during this process are largely unknown.

Cdo is a cell surface Ig superfamily member with a long intracellular region (Krauss et al., 2005). Cdo promotes myogenesis in vivo and in vitro; mice lacking Cdo display delayed skeletal muscle development, and primary myoblasts (satellite cells) obtained from such animals differentiate defectively (Cole et al., 2004). Cdo functions in myoblasts as a component of multiprotein complexes that also include the closely related factor Boc, the Ig superfamily receptor neogenin and its ligand netrin-3, and the adhesion molecules N- and M-cadherin (Krauss et al., 2005). Experiments with myoblast cell lines and reporter assays in fibroblasts indicate that one way Cdo promotes myogenesis is to signal to posttranslationally activate myogenic bHLH factors in a fashion that requires its intracellular region (Cole et al., 2004; Krauss et al., 2005). We report that the Cdo intracellular region binds JLP, a scaffold protein for the p38α/β MAPK pathway (Lee et al., 2002; Kelkar et al., 2005). Cdo and JLP cooperate to activate p38α/β in transfectants, and endogenous Cdo, JLP, and p38α/β form complexes during myoblast differentiation. Cdo−/− satellite cells are deficient in their ability to activate p38α/β, and their defective differentiation phenotype is rescued by the expression of an activated form of a p38 MAP2K, MKK6. Thus, one way p38α/β is activated during myogenesis is through the interaction of a pathway-specific scaffolding module with a Cdo-containing cell surface complex.

Results and discussion

Cdo interacts with JLP

To identify proteins that interact with the Cdo intracellular region, a yeast two-hybrid screen was performed with a construct containing the Cdo transmembrane plus intracellular region as bait (Fig. 1 A). Several positive clones corresponded to a portion of JLP. The transmembrane plus intracellular region of a different Ig protein, Necl-2, was used as a control bait, and JLP did not interact with Necl-2. Conversely, a Necl-2–binding protein, Pals2 (Shingai et al., 2003), bound the Necl-2 bait but not the Cdo bait. JLP, JIP4, and SPAG9 are alternatively spliced products of a single gene (Kelkar et al., 2005), and RT-PCR analysis suggested that JLP is the major product in C2C12 myoblasts (unpublished data). To assess whether Cdo and JLP interact in mammalian cells, lysates from Cdo−/+ and Cdo−/− satellite cells were immunoprecipitated with antibodies to Cdo and blotted for the presence of JLP (Fig. 1 B). JLP was detected in immunoprecipitates from Cdo+/+ but not Cdo−/− lysates, indicating that Cdo was required to bring down JLP and that the antibody was specific. In a reciprocal experiment, C2C12 myoblasts were transiently transfected with an expression vector encoding S epitope-tagged JLP or a
control vector, and lysates were precipitated with anti-S agarose (Fig. 1 C). Full-length endogenous Cdo was coprecipitated from the JLP transfected but not the control transfectants. We concluded that Cdo and JLP interact in myoblasts.

Coimmunoprecipitation experiments in COS cells were then used to identify regions of JLP and Cdo involved in binding. A series of S-tagged fragments of JLP were coexpressed with Cdo and lysates pulled down with anti-S agarose (Fig. 1 D). Only a fragment encoding amino acids 465–1,008 coprecipitated Cdo; because a positive yeast two-hybrid clone contained JLP amino acids 317–647 (Fig. 1 A), the major Cdo-binding region of JLP resides between amino acids 465–647. p38α binds to two sites within JLP (amino acids 1–110 and 160–209; Lee et al., 2002), neither of which overlaps the Cdo-binding region (Fig. 1 E), suggesting that Cdo, JLP, and p38 could form a ternary complex. An analogous experiment was performed with Cdo deletion mutants that lack portions of the intracellular region. Loss of JLP binding was seen in each case (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200608031/DC1). It is possible that multiple regions of the Cdo cytoplasmic domain are required to provide structural integrity sufficient for JLP binding or that the Cdo deletion mutants may not be targeted to an appropriate subcellular compartment for interaction.

Cdo-p38 signaling
To begin to determine whether Cdo–JLP interaction has a positive effect on p38α/β activity, transfections in heterologous systems were used. 293T cells were transfected with a vector encoding T7-tagged p38α and various combinations of other expression vectors, and lysates were blotted with antibodies to the dually phosphorylated (active) form of p38α/β (pp38α/β; Fig. 2 A). The expression of p38α alone resulted in very low levels of pp38α, but the coexpression of ASK1 (a p38 MAP3K) produced abundant pp38α. The expression of Cdo increased the levels of pp38α above that seen with p38α alone, and this was further increased in a dose-dependent manner by the coexpression of JLP. The expression of JLP without Cdo was less effective. Cdo–JLP interaction was also tested with a MyoD-dependent reporter gene assay in fibroblasts, in which an activated form of the p38 MAP2K, M KK6 (M KK6EE), enhances MyoD activity (Wu et al., 2000). Although the cotransfection of Cdo or JLP separately each increased MyoD-dependent reporter activity above what p38α alone produced, cotransfection of the two together produced ~80% above what would be expected from a purely additive response (Fig. 2 B). Although the effects of Cdo and JLP coexpression on p38α activity in these heterologous systems are relatively modest, they are clearly stimulatory.

We next asked whether endogenous Cdo, JLP, and p38α/β could be found in complexes during myogenesis. C2C12 cells were harvested while proliferating in growth medium (GM), at the time of transfer to differentiation medium (DM), and 48 h after transfer, when they were differentiating. Lysates were immunoprecipitated with Cdo antibodies and blotted for JLP, p38α/β, and Cdo; whole lysates were also probed for the...
expression of total p38, JLP, pp38α/β, and the differentiation markers myogenin and myosin heavy chain (MHC; Fig. 2 C). JLP and p38α/β coprecipitated with Cdo only when the cells were actively differentiating despite the fact that total levels of all three proteins were unchanged in the various conditions examined. Furthermore, the formation of this complex correlated with pp38α/β production. It is likely that p38α/β coprecipitates with Cdo via binding to JLP, which interacts with Cdo, as p38α and Cdo did not interact in the yeast two-hybrid system (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200608031/DC1). To address this notion more directly, an RNAi approach was used. The pSilencer vector containing a sequence corresponding to mouse Jlp or an irrelevant sequence (as a control) was cotransfected into C2C12 cells with a GFP expression vector, and the cultures were sorted for the presence of GFP. Sorted cultures were replated and subsequently transferred into DM for 48 h, at which point whole lysates and lysates immunoprecipitated with Cdo antibodies were blotted for JLP, p38α/β, which point whole lysates and lysates immunoprecipitated with MKK6EE in Cdo−/− cells that were similarly infected Cdo−/− cells were ~50/50. The expression of MKK6EE in Cdo−/− cells restored the 80/20 percent ratio of MHC+ versus MHC− cells seen in Cdo−/− cells but had little effect on Cdo+/+ cells (Fig. 3 D). Likewise, GFP vector–infected Cdo+/+ cells formed elongated myotubes and had a fusion index more than fourfold higher than Cdo−/− cells, which failed to elongate (Fig. 3, C and E). Infection of Cdo−/− cells with the MKK6EE virus led to the production of elongated myotubes by these cells and raised their fusion index to a level similar to that seen with Cdo+/+ cells, which were much more modestly affected by the expression of MKK6EE (Fig. 3, C and E).

Similar results were obtained in C2C12 myoblasts. Differentiation of these cells was inhibited by the expression of RNAi to Cdo, but production of MHC and multinucleated cells
was restored by the coexpression of MKK6EE (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200608031/DC1). It is concluded that the expression of MKK6EE specifically rescues the defects in myogenic differentiation caused by Cdo deficiency, presumably via restoration of p38\(^{\alpha/\beta}\) activity.

**Activation of p38\(^{\alpha/\beta}\) during myogenesis**

p38\(^{\alpha/\beta}\) MAPK is established as a promyogenic kinase, but the mechanisms by which it is activated during differentiation are not well understood. Certain soluble signaling factors, including ATP and amphoterin, stimulate p38\(^{\alpha/\beta}\) activity and enhance myogenesis when added exogenously to cultured myoblasts; likewise, the expression of a dominant-negative amphoterin receptor blocks production of pp38\(^{\alpha/\beta}\) and differentiation (Ryten et al., 2002; Sorci et al., 2004). Additionally, MyoD activity stimulates a feed-forward pathway that involves activation of p38 via induction of target genes (Penn et al., 2004). However, in general, the signaling mechanisms underlying p38\(^{\alpha/\beta}\) activation by these factors are not clear nor are they confirmed by genetic loss of function data.

The results described here reveal a novel mechanism of p38\(^{\alpha/\beta}\) activation during myogenesis: the interaction of JLP, a p38\(^{\alpha/\beta}\) MAPK scaffold protein, with Cdo, which is a component of multiprotein cell surface complexes comprised of promyogenic signaling receptors and adhesion molecules (Fig. 4). The binding of a MAPK scaffold protein to the intracellular region of a transmembrane receptor protein is unusual; furthermore, this mechanism is distinct from other known receptor-mediated signaling mechanisms, such as intrinsic enzyme activity (e.g., receptor tyrosine kinases) or direct coupling to nonreceptor tyrosine kinases (e.g., cytokine receptors). JIP3, which is structurally related to JLP, binds the cytoplasmic tail of Toll-like receptor 4 (Matsuguchi et al., 2003), suggesting that direct interaction with transmembrane receptors may be a feature of this class of scaffold protein. It is anticipated that in its role as a scaffold (Lee et al., 2002; Kelkar et al., 2005), JLP brings additional components of the pathway, such as MAP3Ks and MAP2Ks, to these complexes. Furthermore, the interaction of a pathway-specific scaffolding module with Cdo-containing cell surface complexes may allow the coordination of additional signals required for p38\(^{\alpha/\beta}\) activity via the actions of other membrane components of such complexes (e.g., regulation of small GTPases by cadherins; Charrasse et al., 2002). Cdo is also expressed in and promotes the differentiation of neuronal precursors (Zhang et al., 2006b), and similar signaling mechanisms may be involved in myogenesis and neurogenesis. Assembly at sites of cell–cell contact of higher order structures comprised of multiprotein cell surface complexes and intracellular signaling modules is an appealing mechanism for coordinating changes in gene expression and cell morphology during cell differentiation in general.

**Materials and methods**

**Yeast two-hybrid screen**

A cDNA encoding the transmembrane and intracellular regions of mouse Cdo (amino acids 948–1,250) was fused in frame to the Gal4 DNA-binding domain in the pGADUC1 vector. The yeast strain PJ69-4A was sequentially transformed with this vector and a library containing mouse embryonic cell DNA fused to the Gal4 activation domain via lithium acetate. Approximately 4.2 \(\times\) 10\(^5\) transformants were obtained and screened as described previously (James et al., 1996). Four identical clones encoding amino acids 317–647 of JLP were isolated.

**Cell culture**

C2C12, 10T1/2, 293T, COS-7, and satellite cells isolated from Cdo\(^{−/−}\) and Cdo\(^{+/+}\) mice were cultured as described previously (Cole et al., 2004; Kang et al., 2004). To induce differentiation, C2C12 and satellite cells were transferred into medium containing 2% horse serum or 5% FBS, respectively. Quantification of myotube formation was performed as described previously (Kang et al., 2004).

For RNAi studies shown in Fig. 2, the Jlp sequence 5′-AGATGGCTTATGAAGC1G-3′ was inserted into the psilencer 2.0-U6 vector (Ambion). pSilencer 2.0-U6 expressing an irrelevant sequence (Ambion) was used as a control. These vectors were transfected along with a GFP expression vector. Cells were sorted for the presence of GFP and analyzed by coimmunoprecipitation and Western blotting as described in the next paragraph and for the ability to form myotubes. RNAi-mediated knockdown of Cdo was as described previously (Zhang et al., 2006a), and transient assay of its effects on C2C12 differentiation was performed as described previously (Kang et al., 2004). Replication-deficient adenoviruses encoding GFP or HA-tagged MKK6EE were provided by M. Meseck (Mount Sinai School of Medicine, New York, NY) and L. Puri (Burnham Institute, La Jolla, CA; Simone et al., 2004), respectively, and amplified in 293T cells. Satellite cell cultures were infected at an MOI of 50 and assessed for the expression of MKK6EE, pp38\(^{\alpha/\beta}\), and MHC and for myotube formation.

**Protein and reporter assays**

Western blot analyses were performed as described previously by Kang et al. (2004). For immunoprecipitations, cells were lysed in extraction buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl\(_2\), 10 mM NaF,
2 mM DTT, 1 mM Na2VO4, and 0.5% Triton X-100 supplemented with 1 to 2 ml of Complete protease inhibitor cocktail (Roche). 2 ng of whole cell extract from each sample was prefiltered with protein G-Sepharose (GE Healthcare) conjugated with 2 μg of normal rabbit IgG (Santa Cruz Biotechnology, Inc.) for 1 h at 4°C followed by immunoprecipitation with 2 μg anti-Cdo antibody for 2 h at 4°C. Immunocomplexes were washed three times with and suspended in extraction buffer, and samples were analyzed by Western blotting. For pull-down experiments, 4 × 10^5 cells were seeded onto 100-mm plates 1 d before transfection with plasmids encoding a series of Stagged JLP proteins (Lee et al., 2002). 2 d after transfection, cells were harvested and lysed in extraction buffer. Whole cell extracts were incubated with 20 μl of 50% slurry Strep-tag protein beads (Novagen) for 3.5 h at 4°C. Beads were washed three times with and suspended in extraction buffer, and samples were analyzed by Western blotting.

Antibodies used in this study are as follows: anti-Cdo (Zymed Laboratories), anti-JLP (Abcam), anti-p38ά/β (Sigma-Aldrich), anti-p38β (Cell Signaling Technology), anti-T7 (Novagen), anti-S probe (Santa Cruz Biotechnology, Inc.), anti-MHC (MF-20; Developmental Studies Hybridoma Bank), and antimyogenin (Santa Cruz Biotechnology, Inc.).

For reporter assays, 3 × 10^5 10T1/2 cells were seeded onto individual wells of a six-well plate 1 d before transfection with FuGene6 (Roche). For each well, 200 ng 4RTK luc and, as an internal control, 100 ng CMV-lucZ plasmids were transfected along with expression vectors for MyOD (50 ng), E47 (10 ng), p38α (100 ng), Cdo (200 ng), and JLP (400 ng) as indicated in Fig. 2 B. Cells were incubated for 48 h after transfection and harvested to determine luciferase and β-galactosidase activity as described previously (Cole et al., 2004; Zhang et al., 2006a).

Microscopy

Cultures were processed as described previously (Kang et al., 2004) and examined on a phase-contrast microscope (Eclipse TS100; Nikon) with plan Fluor 10× NA 0.3 and 20× NA 0.45 objectives (Nikon) at room temperature. Images were captured with a camera (model 2.2.1 Spot RT Color; Diagnostic Instruments) using Spot software (version 3.5.9; Diagnostic Instruments) and Photoshop 7.0 (Adobe).

Online supplemental material

Fig. S1 shows that Cdo deletion mutants do not bind to JLP, and Fig. S2 shows that p38α does not bind to Cdo. Fig. S3 shows that MKK6EE rescues the block to C2C12 cell differentiation imposed by RNAi to p38α. Fig. S3 shows that MKK6EE rescues the block to C2C12 cell differentiation imposed by RNAi to p38α. MKK6EE was transfected to C2C12 cells together with 200 ng p38α siRNA and Cdo siRNA. Western blotting shows that p38α does not bind to Cdo. This work was supported by grants from National Institutes of Health (AR46207) and the T.J. Martell Foundation to R.S. Krauss.

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