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Beta catenin-independent activation of MyoD in presomitic mesoderm requires PKC and depends on Pax3 transcriptional activity

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Abstract

Early activation of myogenesis in the somite depends on signals from surrounding tissues. Canonical β-catenin dependent Wnt signalling preferentially activates *Myf5*. We now show, in explant experiments with presomitic mesoderm, that the expression of another myogenic determination factor, MyoD, depends on non-canonical Wnt signalling, probably emanating from the dorsal ectoderm. Inhibitors of PKC block MyoD expression, indicating that the intracellular Wnt pathway depends on this kinase. In the absence of Myf5 and Mrf4, this activation is only minorily affected and we identify Pax3 as the transcriptional mediator responsible for MyoD expression. When embryos expressing a constitutively active form of Pax3, PAX3–FKHR, are used for these studies in the presence of PKC inhibitors, MyoD expression is not affected, suggesting that Wnt signalling acts on the transcriptional activity of Pax3.

Keywords: Non-canonical Wnt signalling; PKC; MyoD; Pax3; Onset of myogenesis; Mouse embryo

Introduction

Skeletal myogenesis is initiated in the paraxial mesoderm in response to signalling molecules produced by neighbouring tissues, such as the neural tube, and the notochord or the dorsal ectoderm, leading to the activation of myogenic regulatory factor genes such as *Myf5* and *MyoD* (Tajbakhsh et al., 1998). These signalling molecules include members of the Wnts, Bmp and Shh families. Wnts constitute a large gene family, related to *Drosophila wingless*, and encoding secreted glycoproteins which are implicated in a multitude of developmental processes. Wnts bind to Frizzled (Fz) receptors on target cells to activate different signalling pathways, on the basis of which they can be divided into two main classes. Some Wnts, such as Wnt1, Wnt3a and Wnt8, activate the so-called canonical pathway, leading, through inactivation of glycogen synthase kinase (GSK-β3), to stabilization and subsequent nuclear translocation of β-catenin which then activates TCF/LEF1 (T-cell factor/lymphocyte enhancer factor) transcription factors and induces Wnt-responsive genes (Eastman and Grosschedl, 1999; Filali et al., 2002; Pilon et al., 2006; Reya et al., 2003). In contrast, other Wnts, such as Wnt5a, have been described to activate other signalling pathways in different cell systems, such as the planar cell polarity-like pathway and the Wnt/Ca2+ pathway, through a β-catenin-independent noncanonical intracellular cascade involving protein kinase C (PKC) and c-Jun NH2-terminal kinase (JNK) (Jin et al., 2005; Sheldahl et al., 1999). Wnt7a and Wnt11, on the other hand have been shown to act both through a canonical and a non-canonical pathway (Adamska et al., 2004, 2005; Kengaku et al., 1998; Pandur et al., 2002a; Tao et al., 2005) in different cell systems. It would appear that the same Wnt can signal through different signalling pathways and that

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complex cross-talk between these different pathways may regulate the cellular readout of Wnt signalling (Pandur et al., 2002b).

PKA may be another player in these complex intracellular signalling pathways leading to myogenesis. It has been demonstrated that adenyl cyclase signalling via PKA and its target transcription factor, CREB, are required for Wnt-directed myogenic gene expression (Chen et al., 2005). Wnt proteins can also stimulate CREB-mediated transcription, providing evidence for a Wnt signalling pathway involving PKA and CREB. However it is not clear whether and how this PKA dependent Wnt signalling could affect specifically *MyoD* or *Myf5* or both, and whether there is cross-talk between this pathway and the β-catenin pathway.

We previously showed that Wnt1 and Wnt3a, produced in the dorsal neural tube, adjacent to the site of epaxial myogenesis, preferentially activate *Myf5* whereas Wnt7a and Wnt6, produced in the dorsal ectoderm, preferentially induce activation of *MyoD* (Cossu et al., 1996; Tajbakhsh et al., 1998), which can occur in the absence of Myf5/Mrf4. Recently we showed direct activation of a *Myf5* regulatory element that targets early epaxial myogenesis, by activated β-catenin (Borello et al., 2006). Since Wnt7a may, in some contexts, act through a non-canonical pathway, we decided to investigate whether the *Myf5* independent activation of *MyoD*, first observed during hypaxial myogenesis, was occurring in a β-catenin independent way.

By using a combination of genetic and pharmacological tools, we demonstrate here that in explants of pre-somitic mesoderm (PSM), *MyoD* expression can be activated in the absence of functional β-catenin and of Myf5, indicating that myogenesis can be induced through a PKC dependent, β-catenin independent, pathway. We also demonstrate that Pax3 is required in this pathway and that its transcriptional activity depends on PKC.

Materials and methods

Mouse lines

Generation and genotyping of the $Pax3^{GFP/+}$, $Pax3^{PAX3–FKHR–IRESnlacZ/+}$, P34, $Myf5^{LacZ/LacZ}$ and $Myf5^{LoxP/LoxP}$ have been described elsewhere (Kassar-Duchossoy et al., 2004; Relaix et al., 2003, 2004) $\beta\text{-}catenin^{floxdel/floxed}$ mice were obtained by intercrossing $\beta\text{-}catenin^{floxdel/+}$ with $\beta\text{-}catenin^{floxed/floxed}$ mice (Brault et al., 2001; Lallemand et al., 1998).

Organ and cell culture and infection

For most experiments, embryos ranging in age from 20 to 25 somites (E9.5 days) were isolated in PBS. The tissues were then digested with 0.25% pancreatin–0.1% trypsin for 5 min at 4 °C. After the enzymatic digestion, the various tissues were mechanically separated according to the experimental scheme and then cultured in complete medium (see below). Unless otherwise indicated, presomitic mesoderm (PSM) refers to paraxial mesoderm immediately posterior to the most newly formed somite, alone with no associated structures. In some experiments dorsal ectoderm was left associated with the PSM and this is specified in the text.

All cultures were grown in RPMI medium (SIGMA) supplemented with 15% fetal calf serum (GIBCO), 300 mM β-mercaptoethanol and 50 mg/ml gentamycin. In experiments where co-culture with Wnt expressing cells was required, the tissue fragment were seeded onto a layer of 3T3 cells expressing Wnt1 or Wnt7a as previously described (Borello et al., 1999).

3 h after plating, inhibitors were added to the culture, at the following concentrations: Calphostin C (1 μM), Bisindolylmaleimide I (500 nM), the JNK inhibitor SP600125 w(10 μM), rottlerin (5 μM), Go6976 (5 nM), (CALBIOCHEM), KIC1-1 (Classical PKC Inhibitor), KIE1-1 (Epsilon PKC Inhibitor peptide), KIET1-1 (Eta PKC Inhibitor peptide), KIBI31-1 (BetaI PKC Inhibitor peptide), KIBII31-1 (BetaII PKC Inhibitor peptide), KIG31-1, (Gamma PKC Inhibitor peptide) (1 μM) (KAIPHARMA). Unless otherwise indicated, inhibitors were added every 24 h, the inhibitor peptides every 8 h, and the culture was carried out for a total of 60 h to allow the beginning of myogenic differentiation.

The CRE lentiviral vector (in pRRLsin.PPT.CMV.NTRiresGFPpre) was generated and prepared as described previously (Borello et al., 2006). The final MOI was 5×10^7 PFU/ml. After 6 h culture, the explants were infected with a MOI of 100 in RPMI/15%FCS overnight. The following day the medium was changed. The explants were cultured for a total of 3 days. To test for the correct excision of the floxed *β-catenin*, PCR was performed on genomic DNA from the explant at different times, with the following primers:

RM41: AAG GTA GAG TGA TGA AAG TTG TT;
RM42: CAC CAT GTC CTC TGT CTA TCC;
RM43: TAC ACT ATT GAA TCA CAG GGA CTT.

In addition RT-PCR was performed on RNA extracted from the explants at different times using the following primers:

Cre Fw: GACCAAGTGACAGCAATGCTGTTTCA;
Cre rev: CACCAGCTTGCATGATCTCCGGTATT;
β-catenin Fw: GTGCAATTCCTGAGCTGACA;
β-catenin rev: CTTAAAGATGGCCAGCAAGC-3′;
GAPDH Fw: TGAAGGTCGGAGTCAACGGATTTGGT;
GAPDH Rev: CATGTGGGCCATGAGGTCCACCAC.

At the times indicated, cultures were fixed and immunofluorescence was performed using different antibodies.

Immunofluorescence

Immunofluorescence on cultured explants was carried out as described (Brunelli et al., 2004) using the following antibodies and dilutions: goat polyclonal anti β-galactosidase (Biogenesis) antibody (1:350); MF20 anti-sarcomeric myosin monoclonal antibody (DHSB) (1:4); mouse monoclonal anti-MyoD antibody (DAKO) (1:200); rabbit polyclonal anti Myf5 antibody (Santa Cruz) (1:300); rabbit polyclonal anti-sarcomeric myosin antibody (Cusella-De Angelis et al., 1994) (1:500); anti-GFP rabbit polyclonal antibody (Molecular Probes)(1:300); anti-GFP monoclonal antibody (Molecular Probes) (1:200). Primary antibodies were detected by appropriate secondary antibodies (Molecular Probes), conjugated with either Alexa 594, or Alex 498, for fluorescence detection.

Protein extraction and Western analysis

Embryos or cultured explants were washed in PBS and solubilised in the following lysis buffer: 20 mM MOPS, pH 7. 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, pH 7.2, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl-fluoride, 3 mM benzamidine, 5 μM pepstatin A, 10 μM leupeptin, 0.5% Nonidet P-40. The protein extracts (1 mg/ml) were boiled for 4 min at 100 °C in SDS-PAGE Sample Buffer (31.25 mM Tris–HCl (pH 6.8), 1% SDS (w/v), 12.5% glycerol (v/v), 0.02% bromophenol blue (w/v), and 1.25% β-mercaptoethanol). Alternatively PSM was solubilised in the same lysis buffer minus sodium orthovanadate and with 1 mM EDTA, then incubated for 60 min at 37 °C with 20 units of Calf Intestinal Alkaline Phosphatase (CIP), then boiled in the sample buffer.

Samples were either shipped to KINEXUS (Canada) to be analyzed by KINETWORKS™ PHOSPHO-SITE SCREEN 4.1, or loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels for Western analysis. After electrophoresis, polypeptides were electrophoretically transferred to nitro-cellulose filters (Schleicher and Schuell, Dassel, Germany) and antigens revealed by the following primary monoclonal antibodies: mouse anti-PKCα, PKCβ, PKCδ, PKCε, PKCη, PKCγ, PKCλ (BD-Transduction Laboratories)(Lexington, KY, USA), mouse anti-PAX3 mAb (Hybridoma Bank), anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mAb from Biogenesis (Poole, England). Primary antibodies were detected by chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibodies (Biorad, Hercules, CA, USA).

Image acquisition and manipulation

Images in fluorescence were taken on the Nikon microscope Eclipse E600 (lenses Plan Fuor: 4×/013, 10×/0.33, 20×/0.50, 40×/0.75). All images have been acquired using the NIKON digital camera DXM1200, and the acquisition software NIKON ACT-1. Images were than assembled in panels using Adobe Photoshop 7.0.

Statistical analysis

The results are expressed as means±S.E.M.; *n* represents the number of individual experiments. Statistical analysis was carried out using the ANOVA one way analysis of variance. Asterisks in the figure panels refer to statistical probabilities vs. untreated controls. Statistical probability values (*P*) of less than 0.05 were considered significant.

Results

MyoD activation can occur independently of β-catenin

We investigated the requirement of β-catenin for MyoD or Myf5 expression, by analyzing this in a *β-catenin* null genetic background. We infected presomitic mesoderm explants (PSM), dissected from embryonic day (E) 9.5 *β-catenin*$^{floxdel/floxed}$ embryos (Brault et al., 2001), with a lentiviral vector expressing CRE recombinase (Cre) (Figs. 1E–H, I) or a

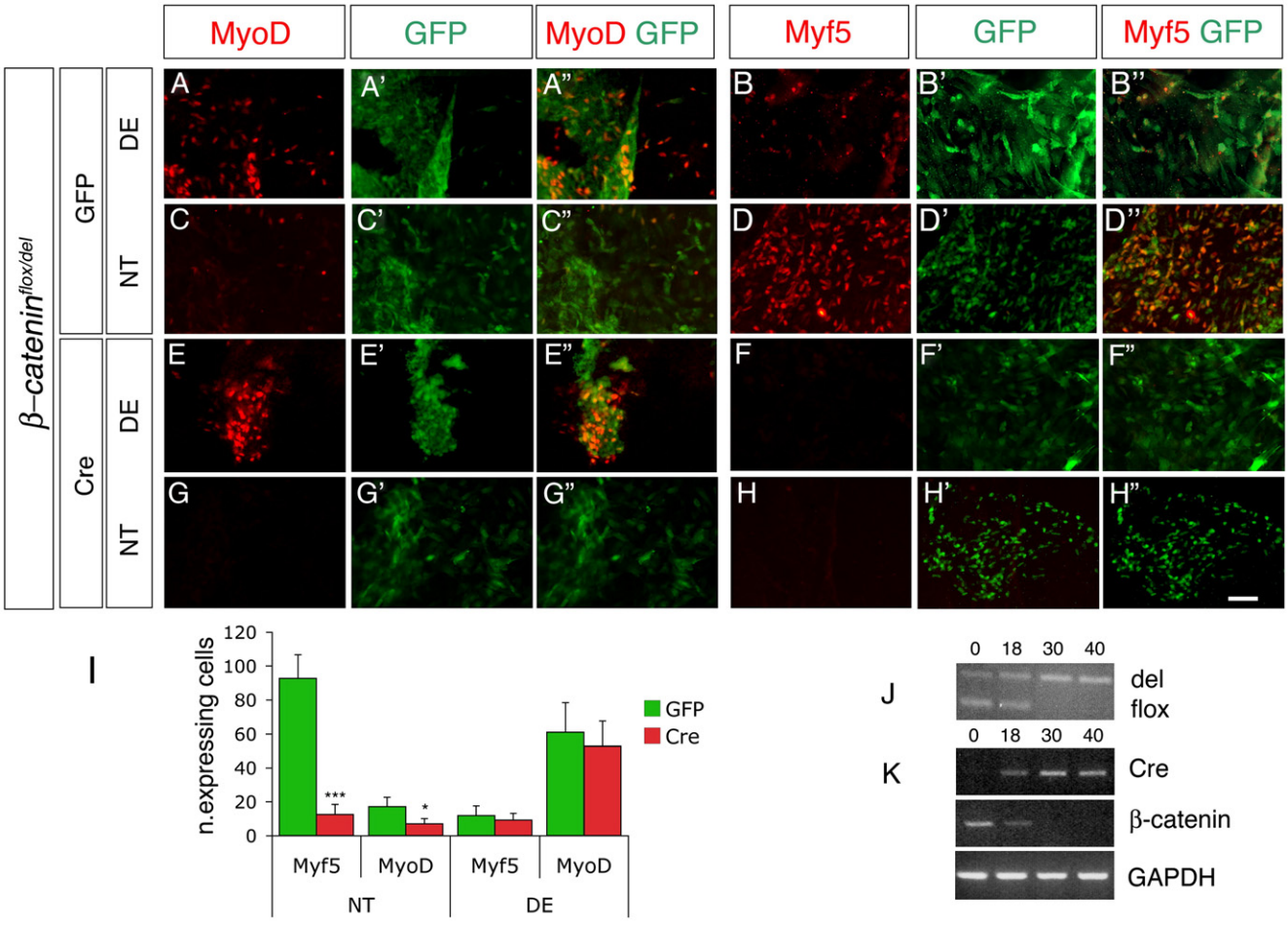

Fig. 1. MyoD activation can occur independently of β-catenin. (A–H) Immunofluorescence on PSM explants dissected from E 9.5 *β-catenin*$^{floxdel/floxed}$ embryos in the presence of dorsal ectoderm (DE) (A–B, E–F) or neural tube (NT) (C–D, G–H), and infected with a lentiviral vector expressing CRE recombinase and GFP (Cre) (A–D) or a control vector expressing GFP only (GFP) (E–H), using antibodies specific for GFP (green) together with MyoD or Myf5 (red). (I) Histogram showing the number (*n*) of Myf5 or MyoD expressing cells in explant cultures under the various conditions. Independent experiments ($n=6$) were performed in triplicate and averaged. Single and triple asterisks represent $P<0.05$ and $P<0.001$ respectively vs. control (C). Bars represent S.E.M. (J) PCR on genomic DNA extracted from the explants, showing the excision of β-catenin in the presence of the lentiviral vector expressing Cre, at different times after infection (18, 30 and 40 h). Flox, floxed *β-catenin* allele; del, deleted *β-catenin* allele. (K) RT-PCR on total RNA extracted from the explant at different times after lentiviral infection, showing expression of Cre recombinase, β-catenin and GAPDH. Scale bar in panel A, 100 μm.

control vector (GFP) (Figs. 1A–D, I), in the presence of dorsal ectoderm (DE) or neural tube (NT) (Figs. 1A–B). Cre-mediated deletion of *β-catenin,* verified by PCR and RT-PCR (Figs. 1J–K), leads to a strong decrease in Myf5 expression in the PSM, induced by signals from the neural tube (Figs. 1H, I), in comparison with a culture infected with the control lentiviral vector (Figs. 1D, I)) as described previously (Borello et al., 2006). Under these conditions MyoD expression is also reduced (Figs. 1C, G, I). On the other hand, MyoD expression, induced in the presence of dorsal ectoderm, was not affected (Figs. 1A, E, I). [Suppl. Fig. 1 provides images of the DAPI staining corresponding to panels A–H in Fig. 1]. These data suggest that β-catenin signalling plays an important role in neural tube mediated expression of these myogenic determination factors in the PSM, but not in that mediated by dorsal ectoderm, indicating that the latter depends on a β-catenin independent pathway.

PKC inhibitors abrogate dorsal ectoderm or Wnt7a dependent myogenesis in the PSM

Two non-canonical Wnt pathways have been described, namely the Wnt/Ca(2+) pathway, which goes through protein kinase C (PKC), and the Wnt/PCP pathway involving Rho/Rac small GTPases and Jun N-terminal kinase (JNK) classes of transduction molecules. We examined whether the expression of MyoD, which depends on dorsal ectoderm and can be induced by Wnt7a (Tajbakhsh et al., 1998), requires the presence of functional PKC or JNK activities.

PSM was dissected free of dorsal ectoderm or neural tube, from E9.5 embryos and cocultured on a layer of 3T3 cells expressing either Wnt7a (Figs. 2A–F and Suppl. Figs. 2A–F for DAPI staining) or Wnt1 (Figs. 2G–L and Suppl. Figs. 2G–L for DAPI staining), in the presence or absence of PKC inhibitors, Calphostin C or Bisindolylmaleimide or the JNK inhibitor, SP600125. The explants were left to differentiate for 3 days (drugs were added 3 h after plating and then every 24 h), before examination of Myf5 or MyoD expression. As previously described (Tajbakhsh et al., 1998), Wnt1 preferentially activates *Myf5* (Figs. 2G, J, M) and Wnt7a, *MyoD* (Figs. 2A, D, M), leading to myogenesis, as seen by the appearance of Myosin Heavy Chain expressing cells (Figs. 2A, G, M). In the presence of PKC inhibitors, expression of MyoD induced by Wnt7a was reduced (Figs. 2B, E) while Myf5 expression was not, since we still can see the few Myf5 expressing cells, some of which are also MyoD positive (Figs. 2D–E and Suppl. Figs. 2M, P, S, N, Q, T). In explants where myogenesis is induced by Wnt1, neither Myf5 nor MyoD appear to be reduced (Figs. 2G–H, J–K, M). We obtained similar results on co-culturing the PSM explant with neural tube or dorsal ectoderm, instead of Wnt1 or Wn7a expressing cells respectively (Fig. 2M). JNK inhibitors, on the other hand, did not have any effect (Figs. 2C, F, I, L, M and Suppl. Figs. 2O, R, U).

These results indicate that dorsal ectoderm (and Wnt7a) signals mainly through activation of the PKC pathway. Myf5 is not strongly activated by this signal but its expression may account for the residual MyoD expression in the presence of

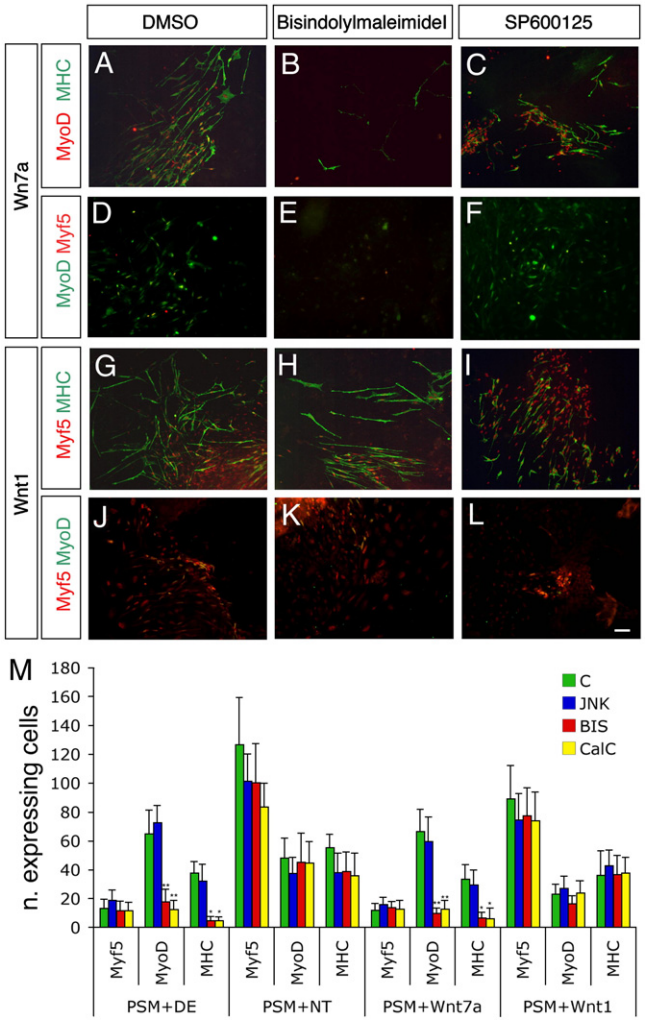

Fig. 2. PKC inhibitors abrogate dorsal ectoderm dependent myogenesis in PSM explants. (A–L) Immunofluorescence on PSM explants from E9.5 embryos left to differentiate on a feeder layer of Wnt7a (A–F) or Wnt1 (G–L) expressing cells, in the presence of the PKC inhibitor Bisindolylmaleimide I (0.5 μM) (B, E, H, K) or the JNK inhibitor SP600125 (10 μm) (C, F, I, L) or DMSO as a control (A, D, G, J) using antibodies specific for Myosin Heavy Chain, MyoD or Myf5, colour coded as indicated. (M) Summary of the experiments shown as examples in panels A–L, together with experiments in which explants were cultured with the neural tube or dorsal ectoderm. Histograms representing the number of cells expressing Myosin Heavy Chain, MyoD or Myf5, in the different culture conditions are shown. Independent experiments ($n=6$) were performed in triplicate and averaged. Single and double asterisks represent $P<0.01$ and $P<0.001$ respectively vs. control (C). Bars represent S.E.M. DE, dorsal ectoderm; NT, neural tube; C, control (with DMSO alone); Bis, Bisindolylmaleimide I (0.5 μM); CalC, Calphostin C (1 μM); JNK, SP600125 (10 μM). Scale bar in panel A, 100 μm.

PKC inhibitors, since *MyoD* can be directly activated by Myf5 (Tajbakhsh et al., 1997).

We therefore decided to perform similar experiments in *Myf5* mutant mice in which early myogenesis is driven only by MyoD. We dissected presomitic mesoderm (PSM), including dorsal ectoderm or neural tube from $Myf5^{nlacZ/nlacZ}$ embryos, which are null mutant for both *Myf5* and *Mrf4*, or the sibling $Myf5^{nlacZ/+}$, or $Myf5^{LoxP/LoxP}$ embryos, in which Mrf4 is still present (Kassar-Duchossoy et al., 2004). The explant cultures were left to

differentiate in the presence or absence of a PKC inhibitor. MyoD expressing cells in explants of PSM from *Myf5*$^{nlacZ/nlacZ}$ embryos in the presence of dorsal ectoderm and treated with PKC inhibitors are reduced with respect to *Myf5*$^{nlacZ/+}$ embryos ($P<0.02$, $n=8$) (Figs. 3C, K, Q; Suppl. Figs. 3Q–V). In addition in these experiment we were able to test the expression of β-Galactosidase (β-Gal) as a readout of *Myf5* activation (Figs. 3B, D, F, H, L, N, P, Q). This confirms that while the neural tube strongly activated β-Gal expression in PSM explants (Figs. 3F, N, Q), fewer βGal expressing cells were present in dorsal ectoderm induced explants (Figs. 3B, J, Q). This expression was not affected by the presence of PKC inhibitors (Figs. 3D, H, L, P Q), suggesting that dorsal ectoderm signalling leading to *Myf5* activation is not PKC dependent. We observed a similar reduction in MyoD expressing cells in explants from *Myf5*$^{LoxP/LoxP}$ embryos when PKC, but not JNK signalling, was inhibited, in all sets of experiments (Suppl. Fig. 3W and not shown). This shows that Mrf4, is not implicated in *MyoD* regulation by the dorsal ectoderm signal. These results confirm that MyoD expression, induced by the dorsal ectoderm, is compromised in the absence of activated PKC and that this effect is even more pronounced in the absence of Myf5.

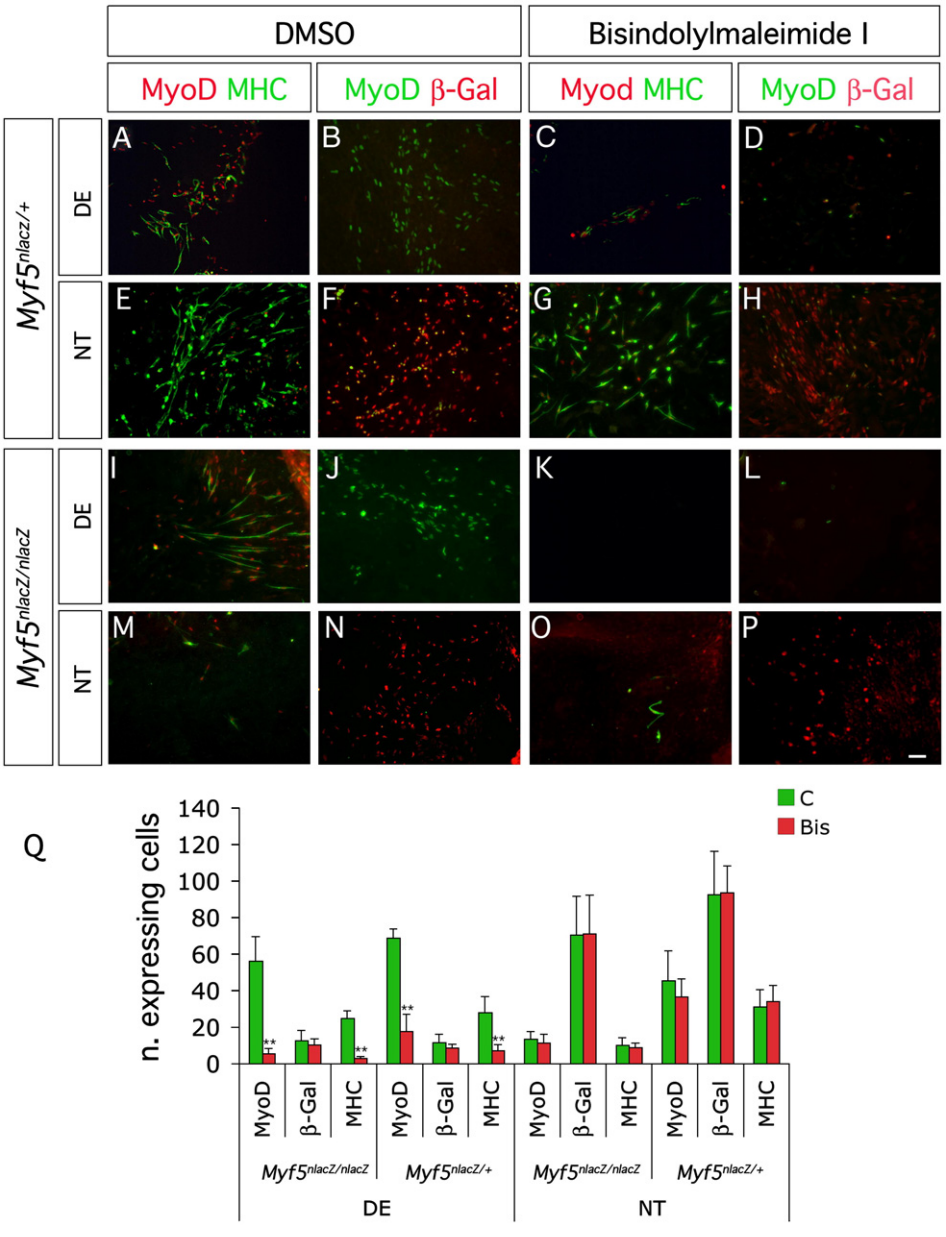

Fig. 3. Myf5 independent myogenesis depends on PKC activity. (A–P) Immunofluorescence on PSM explants from E9.5 *Myf5*$^{nlacZ/+}$ (A–H) or *Myf5*$^{nlacZ/nlacZ}$ (I–P) embryos in the presence of dorsal ectoderm (DE) (A–D, I–L) or neural tube (NT) (E–H, M–P), left to differentiate in the presence (C–D, G–H, K–L, O–P) or absence of Bisindolylmaleimide I (0.5 μM) (A–B, E–F, I–J, M–N), using antibodies specific for Myosin Heavy Chain (MHC), MyoD or β-Galactosidase (Myf5). DMSO is added to control cultures. (Q) Histogram representing the number of Myosin Heavy Chain, MyoD or β-Galactosidase positive cells in the single explant cultures under the different culture conditions. Independent experiments ($n=8$) were performed in triplicate and averaged. Double asterisks represent $P<0.001$ vs. control (C). Bars represent S.E.M. Bis, Bisindolylmaleimide I (0.5 μM). Scale bar in panels A–P, 100 μm.

Several PKC isoforms are expressed in presomitic mesoderm and are involved in MyoD expression

The tissue distribution of different PKC isoforms has only been partially documented during development (Bogatkevich et al., 2005; Hamplova et al., 2005; Oehrlein et al., 1998; Pauken and Capco, 2000; Redling et al., 2004; Wilda et al., 2001) and we therefore explored whether any particular isoform was specifically expressed in the PSM, where it could play a role in the non-canonical pathway leading to MyoD expression.

We initially assessed the expression of various PKC isoforms in extracts of the PSM, compared to the total embryo, at E9.5 by Western blot analysis. As shown in Fig. 4A, PSM expresses PKCα, β, η, γ, δ, ε, λ at high to detectable levels, the majority at similar levels to those in the total embryo, except for α and δ, which appear to be enriched in the PSM. We took advantage of several newly identified peptides that inhibit specific PKC isoforms (Disatnik et al., 2002) to investigate which of the PSM isoforms were mainly responsible for MyoD expression. We tested inhibitors of PKCβI, βII, γ, ε, and η on the differentiation of *Myf5*$^{LoxP/LoxP}$ PSM explants. We also tested two drugs that show a certain degree of selectivity for PKCα (Go6976) and PKCδ (rottlerin) (Renault-Mihara et al., 2006). While inhibition of PKCβII, γ or ε did not have significant effects on MyoD expression, we observed that this is reduced in the presence of selective inhibitors of PKCβI and η and to some extent by inhibitors that preferentially effect PKCδ and PKCδ (Fig. 4B). This suggests that, of the isoforms tested, βI, η, and also possibly α and δ, may be involved in the transduction of non-canonical Wnt signalling, leading to MyoD expression.

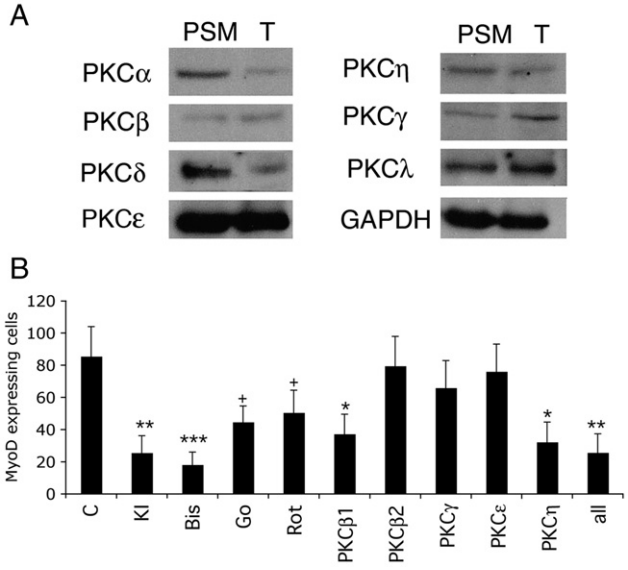

Fig. 4. Several PKC isoforms are expressed in presomitic mesoderm and are involved in MyoD expression. (A) Western blot analysis on PSM or total extracts from E9.5 embryos, using PKC antibodies specific for the different PKC isoforms, PKCα, β, η, γ, δ, ε, λ. (B) Histogram representing the number of MyoD expressing cells in the PSM explant culture from E9.5 *Myf5*$^{LoxP/LoxP}$ embryos differentiated in the presence of the different isoform-specific inhibitors. Independent experiment ($n=8$) were performed in triplicate and averaged. Single cross, single, double and triple asterisks represent $P<0.05$, $P<0.03$, $P<0.01$ and $P<0.001$ vs. control (C). Bars represent the S.E.M. C, not treated control; KI, Kaipharma, classical general PKC inhibitor (1 μM); Bis, Bisindolylmaleimide I (0.5 μM); Rot, rottlerin PKCδ inhibitor (5 μm), Go, Go6796, PKCα inhibitor (5 nM), PKCβI, peptide inhibitor of PKCβI; PKCβII, peptide inhibitor of PKCβII; PKCγ, peptide inhibitor of PKCγ; PKCε, peptide inhibitor of PKCε; PKC η, peptide inhibitor of PKC η, (all at 1 μM); all, combination of available PKC inhibitors.

Inhibition of PKC activity in the PSM alters the phosphorylation state of many kinases playing a role in different signal transduction pathways

While the involvement of PKC and, in some instances, the final transcriptional target of the non-canonical Wnt pathway has been described in many systems (Jin et al., 2005; Koyanagi et al., 2005; Pandur et al., 2002a), in the case of somitic myogenesis it is not known which are the substrates of PKC phosphorylation and how this signal is transduced.

We thus performed a differential phosphorylation profile analysis on protein extracts from explants of PSM and dorsal ectoderm, grown in the presence or absence of PKC inhibitor for 24 h. The analysis was carried out by the company KINEXUS using a panel of antibodies specific for phosphosites (Kinetworks™KPSS-4.1—Phospho-Site) (Fig. 5A, Table 1).

The inhibition of PKC activity in the PSM leads to the modification of the phosphorylation status of many different transduction molecules (Figs. 5A–B). Of particular interest is the decreased phosphorylation of GSK3β, suggesting the existence of a cross-talk with the β-catenin dependent pathway, and of p38a MAPK and MKK6, known to be involved in the regulation of myotome development (De Angelis et al., 2005). Increased phosphorylation was seen for ERK1, ERK2, PKCδ and Rb, but the significance of this remains to be investigated.

Pax3 transcriptional activity, but not Pax3 expression, is abrogated by PKC inhibitors, and is necessary to initiate MyoD dependent myogenesis

Pax3 is another key regulator of myogenesis. In the absence of both Myf5 and Mrf4, *MyoD* can still be activated. However the combined mutation in mouse embryos of *Myf5*, *Mrf4* and *Pax3* results in the absence of *MyoD* activation and the loss of all skeletal muscles in the trunk and limbs (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997). It is, however, still unknown how Pax3 influences *MyoD* activation. We therefore decided to investigate whether Pax3 was involved in the Wnt7A/PKC pathway of MyoD expression. PSM explants from *Pax3*$^{GFP/+}$ embryos were left to differentiate with dorsal ectoderm, in the presence or absence of PKC inhibitors (Figs. 6A–B and Suppl. Figs. 3A–B). As shown previously, we observed a reduction of MyoD expression and myogenesis in the presence of PKC inhibitors (Figs. 6B–C and Suppl. Fig. 6B), while GFP, which reflects expression of *Pax3* (Relaix et al., 2005), was unaffected. The presence of Pax3 protein was confirmed by Western blot analysis (Fig. 6J): Pax3 is still

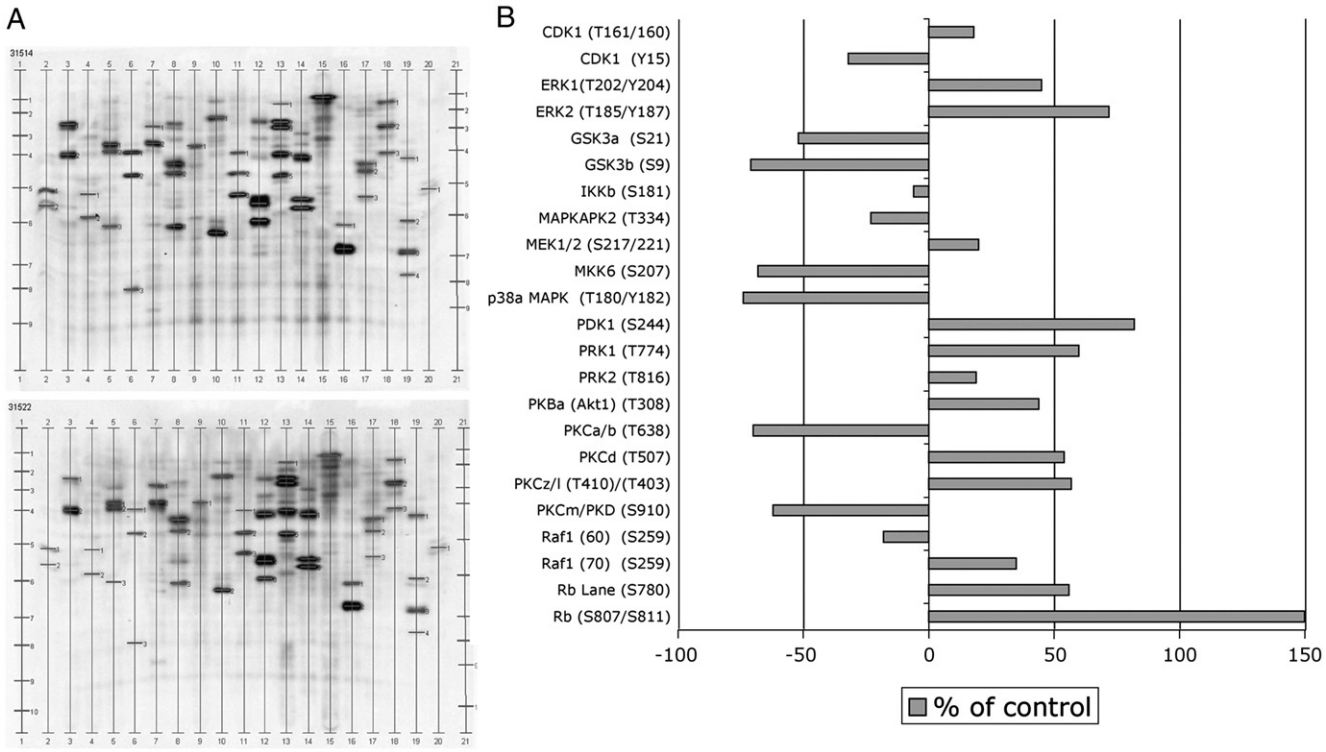

Fig. 5. Phosphosite profiling of PSM. (A) Western blot analysis of the phosphorylation status of 40 different phosphoproteins (see Table 1 for the bands position) in PSM explants from E9.5 embryos co-cultured with dorsal ectoderm, treated (lower gel) or untreated (upper gel) with Bisindolylmaleimide I (0.5 μN) for 24 h, using antibodies for specific phosphosites. (B) Histogram showing the relative increase or decrease in phosphorylation of the phosphoproteins in Bisindolylmaleimide I (Bis) treated explants as a percentage of control explants. Only phosphoproteins that gave a positive signal, showing a difference higher than 5% between treated and control explants are reported here.

detectable in treated explants, although the level appears slightly lower. This suggested that in the absence of activated PKC, the Pax3 protein is unable to activate *MyoD*.

In order to test the transcriptional activity of Pax3 in this situation, we performed the same experiment on explants derived from *P34* transgenic embryos, that express the reporter gene *nlacZ* driven by a *Pax3* responsive element (Relaix et al., 2004). In these mice the activity of the reporter depends on Pax3 transcriptional activity. We observed a drastic reduction in *nlacZ* expression, measured as expression levels of β-galactosidase, in explants grown in the presence of PKC inhibitors (Figs. 6D–F and Suppl. Figs. 5C–D). Since Pax3 expression is not affected by these inhibitors, we conclude that the transcriptional activity of Pax3 requires PKC activity.

A constitutively active form of Pax3, PAX3–FKHR, overcomes the PKC requirement for MyoD expression

PAX3–FKHR arises in humans as a result of a chromosomal translocation, t(2;13), which leads to the fusion of the potent C-terminal transcriptional activation domain of FKHR (FOX01A) to the N-terminal region of PAX3, which includes the intact paired-box and homeobox DNA-binding elements. PAX3–FKHR is a potent gain-of-function mutation and is able to rescue the *Pax3* mutant phenotype (Relaix et al., 2003). We decided to test whether this activated form of Pax3 was still dependent on PKC activity, by examining MyoD expression in explants of PSM and dorsal ectoderm from $Pax3^{PAX3–FKHR–IRESnlacZ/+}$ embryos, in the presence or absence of PKC inhibitors.

Explants from embryos expressing PAX3–FKHR are able to express MyoD and differentiate in the presence of PKC inhibitors (Figs. 6G–I and Suppl. Figs. 6E–F). This therefore demonstrates that expression of MyoD at this stage depends on Pax3 transcriptional activity, since PKC inhibition is rescued by PAX3–FKHR. Pax3 contains several putative consensus sites for Ser/Thr phosphorylation, amongst which there are 8 sites for PKC, which are conserved in different species (mouse, human, chicken, xenopus). Pax3 mobility shift on SDS-PAGE gels is nevertheless not affected when PKC is inhibited by different inhibitors, but it is when the protein extract is treated with alkaline phosphatase, indicating that if phosphorylation of Pax3 is at stake, only a few phosphosites are affected by PKC (Fig. 6J).

Discussion

PKC, but not β-catenin, is required for MyoD expression at the onset of myogenesis

Early myogenesis can be triggered by two principal cascades, initiated by either the axial structures (neural tube and notochord) or the dorsal ectoderm via signalling molecule such as the wnts (Wnt1, Wnt3a and Wnt4 or Wnt6 and Wnt7A respectively) leading to the expression of Myf5 or MyoD in the

Table 1
Legend of the Western blots in Fig. 5A

Lane	Band	Protein name	Abbreviation	Epitope	~MW
2	1	Glycogen synthase kinase-3 alpha (S21)	GSK3a	S21	47
2	2	Glycogen synthase kinase-3 beta (S9)	GSK3b	S9	42
3	1	Protein kinase D (Protein kinase mu) (S910)	PKCm/PKD	S910	134
3	2	Protein kinase C zeta (T410)/lambda (T403)	PKCz/l	T410/403	79
4	1	Unclassified	–	–	46
4	2	p38 MAPK (T180/Y182)	p38a MAPK	T180/Y182	39
5	1	90 kDa Ribosomal S6 Kinases (T573/577)	RSK1/2	T573/577	86
5	2	Unclassified	–	–	81
5	3	Unclassified	–	–	37
6	1	Protein kinase C alpha/ beta (T638)	PKCa/b	T638	79
6	2	Unclassified	–	–	55
6	3	elF4E binding protein (S65) (18)	4E-BP1	S65	18
7	1	Retinoblastoma protein (S807/S811)	Rb	S807/S811	115
7	2	90 kDa Ribosomal S6 Kinases (S380/386)	RSK1/2	S380/386	87
8	1	Raf (S259) (70)	Raf1	S259	67
8	2	Raf (S259) (60)	Raf1	S259	57
8	3	Type1 protein phosphatase alpha (T320)	PP1a	T320	36
9	1	I-kappa-B kinase beta (S181)	IKKb	S181	86
10	1	Unclassified	–	–	135
10	2	MKK6(2) (S207)	MKK6	S207	33
11	1	Unclassified	–	–	76
11	2	Protein kinase B (T308)	PKBa (Akt1)	T308	55
11	3	Unclassified	–	–	45
12	1	Unclassified	–	–	72
12	2	MAPK/Erk kinase 1/2 (S217/221)	MEK1/2	S217/221	42
12	3	Unclassified	–	–	37
13	1	The mammalian target of Rapamycin (S2448)	mTOR	S2448	169
13	2	PKC-related kinase 2 (T816)	PRK2	T816	128
13	3	PKC-related kinase 1 (T774)	PRK1	T774	117
13	4	Unclassified	–	–	75
13	5	Phosphoinositide-dependent protein kinase 1 (S244)	PDK1	S244	54
14	1	Protein kinase C delta (T507)	PKCd	T507	71
14	2	Extracellular signal-regulated kinase 1 (T202/Y204)	ERK1	T202/Y204	43
14	3	Extracellular signal-regulated kinase 2 (T185/Y187)	ERK2	T185/Y187	41
15	1	Unclassified	–	–	187
16	1	Unclassified	–	–	35
16	2	Cyclin-dependent kinase 1 (Y15)	CDK1	Y15	26
17	1	p85 S6 kinase 2 (T444/S447)	S6K2 p85	T444/S447	66
17	2	p70 S6 kinase (T421/T424)	S6Ka p70	T421/T424	55
17	3	MAP kinase activated protein kinase 2 (T334)	MAPKAPK2	T334	43
18	1	Unclassified	–	–	172
18	2	Retinoblastoma P rotein (S780)	Rb	S780	116
18	3	Unclassified	–	–	76
19	1	p70 S6 kinase (T389)	S6Ka p70	T389	68
19	2	Unclassified	–	–	37
19	3	Cyclin-dependent kinase 1 (T161/160)	CDK1	T161/160	25
19	4	Unclassified	–	–	20
20	1	Lyn (Y507) (46)	Lyn	Y507	46

mouse embryo (see Cossu et al., 1996; Tajbakhsh et al., 1998). In the case of *Myf5*, we recently showed that its epaxial activation is due to a cooperative interaction between the Wnt/β-catenin and the Shh/Gli pathways (Borello et al., 2006). However it remains unknown whether Wnts activate *MyoD* through a canonical or a non-canonical pathway. We show here that Wnt-dependent expression of MyoD can occur independently of β-catenin but not of PKC signalling, therefore demonstrating the implication of a non-canonical Wnt pathway.

The non-canonical Wnt pathway has been described to regulate many developmental and differentiation processes (Kohn and Moon, 2005; Medina et al., 2000). In particular, a PKC dependent non-canonical Wnt pathway has been associated with cardiomyogenesis both during Xenopus development and more recently in the differentiation of human circulating progenitor cells (Koyanagi et al., 2005; Pandur et al., 2002a). Similarly to our findings, inhibition of PKC activity blocked cardiomyocyte differentiation. An involvement of PKC in skeletal myogenesis has been demonstrated, at least in cultured myoblasts and in the cell line C2C12. In contrast to our results, down-regulation of PKC appeared to be necessary to allow differentiation and myoblast fusion (Goel and Dey, 2002a,b). However in these experiments PKC appeared not to mediate Wnt signalling, but probably an insulin or integrin signalling pathway.

PKC and PKA in the activation of myogenesis

Another kind of non-canonical pathway has been recently described in muscle progenitor cells in the PSM (Chen et al., 2005). Wnt1 and Wnt7a potently induce transcription from a cyclic AMP-responsive element (CRE) promoter. By testing a barrage of inhibitors and dominant-negative mutants, evidence is presented that noncanonical Wnt signalling – specifically the adenylate cyclase (AC) cascade –activates CREB-mediated transcription and this process is physiologically relevant for Wnt-induced myogenesis. Increasing AC activity (or elevating cAMP levels) specifically enhanced expression of MyoD and Myf5 induced by Wnt1, whereas blocking AC or PKA activity inhibited both Wnt1- and Wnt7a-mediated myogenesis.

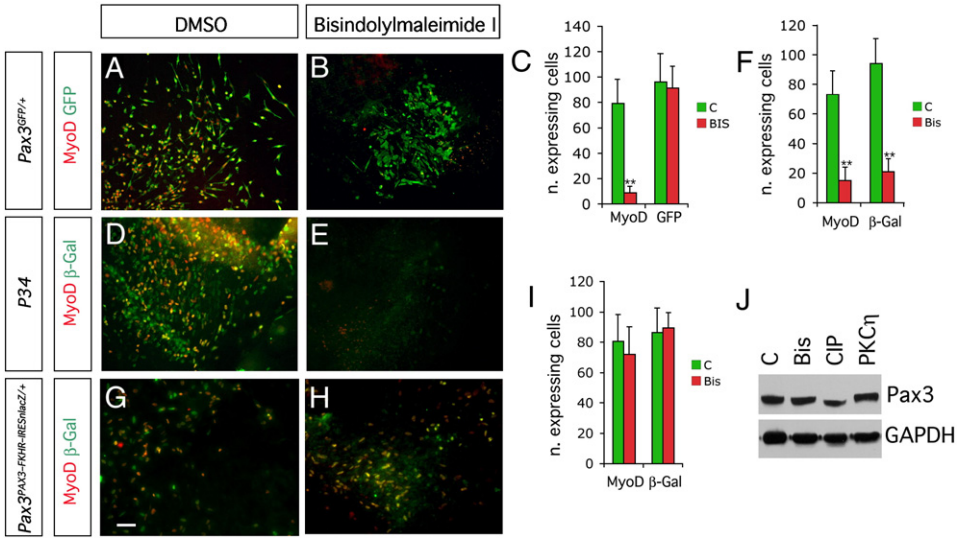

Fig. 6. Pax3 and PKC dependent expression of MyoD. (A–B) Immunofluorescence on PSM explants with dorsal ectoderm from E9.5 $Pax3^{GFP/+}$ embryos left to differentiate in the presence of DMSO (A) or of Bisindolylmaleimide I (0.5 μM) (B), using antibodies specific for GFP (green) and MyoD (red). (C) Histogram representing the number of MyoD or GFP expressing cells in the PSM explant culture from E9.5 $Pax3^{GFP/+}$ embryos differentiated in the presence or absence of Bisindolylmaleimide I (0.5 μM). Independent experiment ($n=6$) were performed in triplicate and averaged. Double asterisks represent $P<0.005$ vs. control (C). Bars represent S.E.M. (D–E) Immunofluorescence on PSM explants with dorsal ectoderm, from E9.5 *P34* embryos, left to differentiate in the presence or DMSO or Bisindolylmaleimide I (0.5 μM) (E) of using antibodies specific for β-Galactosidase (β-Gal) (green) and MyoD (red). (F) Histogram representing the number of MyoD or β-Galactosidase expressing cells in the PSM explant culture from E9.5 *P34* embryos differentiated in the presence or absence of Bisindolylmaleimide I (0.5 μM). Independent experiment ($n=5$) were performed in triplicate and averaged. Double asterisks represent $P<0.005$ vs. control (C). Bars represent S.E.M. (G–H) Immunofluorescence on PSM explants with dorsal ectoderm, from E9.5 $Pax3^{PAX3–FKHR–IRESnlacZ/+}$ embryos, left to differentiate in the presence of DMSO (G) or of Bisindolylmaleimide I (0.5 μM) (H), using antibodies specific for β-Gal (green) and MyoD (red). (I) Histogram representing the number of MyoD expressing cells in the PSM explant culture from E9.5 $Pax3^{PAX3–FKHR–IRESnlacZ/+}$ embryos differentiated in the presence or absence of Bisindolylmaleimide I (0.5 μM). Independent experiment ($n=5$) were performed in triplicate and averaged. Bars represent S.E.M. (J) Western blot analysis of PSM extracts from E9.5 embryos co-cultured with dorsal ectoderm, untreated (C) or treated with Bisindolylmaleimide I (0.5 μM) (Bis), PKCη inhibitor peptide (1 μm) or in a PSM protein extract dephosphorylated in vitro with Alkaline Phosphatase (CIP), using antibodies specific for Pax3 and GAPDH. Scale bar in panels A–B, D–E and panels G–H: 100 μm.

Although these data show that AC activity affects both *Myf5* and *MyoD*, they do not clarify whether either gene may be a direct target of CREB activity since the experiments were performed in wild type embryos with both MyoD and Myf5 present at the same time. Likewise, it is not obvious whether PKA dependent myogenesis occurs independently of the canonical pathway. Even if it appears that dominant negative CREB does not affect targets of the canonical pathway (Buttitta et al., 2003), other data suggest that PKA, by directly phosphorylating β-catenin, inhibits its ubiquitination, therefore allowing its accumulation and activating the canonical Wnt signalling cascade (Hino et al., 2005).

PKC intracellular pathways in myogenesis

How does PKC affect early myogenesis? The data presented here suggest that several molecules may mediate PKC activity. Some indications come from the analysis of the phosphorylation profile of a panel of phosphoproteins in the presence or absence of PKC activity. We observed that inhibition of PKC activity leads to a strong modification of the phosphorylation state of many proteins involved in important signal transduction pathways. In particular when PKC is inactive, phosphorylation of MKK6 and p38MAPK are strongly reduced. p38 MAPK signalling has been implicated in myogenesis with varying reports of its effects on Myf5 or MyoD expression (De Angelis et al., 2005; Keren et al., 2005; Penn et al., 2004; Wu et al., 2000).

Inhibition of PKC led to a reduction of GSK3β phosphorylation. Cross-talk between the canonical and non-canonical Wnt pathways has been described, probably because they share common components such as Dsh (Kuhl, 2002; Kuhl et al., 2001) or GSK3β, already identified as a PKC substrate (De Servi et al., 2005; Oriente et al., 2001). It is possible that during myogenesis cross-talk exists between the Wnt/PKC pathway and the Wnt/β-catenin pathway as suggested by the effect of PKC inhibitors on GSK3β phosphorylation, although in our PKC inhibition experiments we do not observe detectable effects on the low level of Myf5 expression in dorsal ectoderm induced myogenesis.

Pax3 transcriptional activation requires PKC

Another important player in early myogenic induction is Pax3. Analysis of mutant mice have shown that in the absence of the myogenic regulatory factors *Myf5* and *Mrf4,* Pax3 is required for *MyoD* activation and the entry of cells into the skeletal muscle programme (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997). Wnt signalling from the neural tube, acting through the β-catenin dependent pathway, leads to the early activation of myogenic regulatory genes, notably *Myf5*, which is a direct target of TCF/β-catenin (Borello et al., 2006),

in the epaxial dermomyotome of the somite. In the epaxial domain, the early expression of *MyoD* depends on *Myf5/Mrf4* (Kassar-Duchossoy et al., 2004; Tajbakhsh et al., 1997). *Myf5* and *Mrf4* are also transcribed in the hypaxial dermomyotome, where Pax3 is also strongly expressed. In this domain, MyoD expression initially depends on Myf5/Mrf4 but this is also transitory and indeed in the explant experiments reported here only a small additional effect was observed using *Myf5/Mrf4* mutant embryos.

Wnt signalling from the dorsal ectoderm is probably most important for myogenesis in this hypaxial context, and also potentially for the Pax3/Pax7 positive progenitor cells derived from the central region of the dermomyotome of the mouse embryo (Relaix et al., 2005). In the *Myf5/Mrf4* double mutant, there is a minor delay in early *MyoD* activation in the hypaxial domain (Tajbakhsh et al., 1997). The results reported here demonstrate that non-canonical Wnt signalling regulates MyoD expression in a Pax3-dependent manner, since in the presence of this transcription factor, MyoD expression is downregulated by PKC inhibitors, whereas in the presence of PAX3–FKHR it is not. Furthermore this observation provides insight into the way PKC affects Pax3 activity. Pax3 itself is a poor transcriptional activator and probably requires a co-activator or modifications in order to act efficiently (Relaix et al., 2003). PAX3–FKHR, which has the strong activation domain of FKHR circumvents this requirement. We identify several potential phosphorylation sites for PKC in the Pax3 protein, which are conserved in other species. No detectable change in size is seen for Pax3, on the basis of electrophoretic mobility, in the presence of PKC inhibitors, however this may reflect the large number of phosphorylated residues, since a size change is detected on electrophoresis only after global dephosphorylation. Alternatively, or in addition, co-activator phosphorylation may be involved; we are currently looking for Pax3 interacting proteins in the myogenic context. We favour this explanation since Pax3 can also activate Myf5, probably through a different PKC-independent co-activator (Bajard et al., 2006). Wnt signaling has been implicated in the maintenance of Pax3 expression in pre-somitic mesoderm (Fan et al., 1997). Canonical Wnts such as Wnt1 derived from the neural tube have more effect than dorsal ectoderm derived Wnts such as Wnt6 although both signalling pathways act on Pax3. This represents a further level of regulation, in addition to that exerted by PKC dependent Wnt signalling from the dorsal ectoderm on Pax3 activity. Indeed the latter may affect Pax3 transcription through an autoregulatory feedback loop. In $Pax3^{nlacZ/nlacZ}$ mice we continue to see β-galactosidase positive cells (Relaix et al., 2004), indicating that Pax3 is not essential for at least some level of *Pax3* transcription. Indeed in the presence of PKC inhibitors, Pax3 is still present, although slightly reduced.

In conclusion, we have provided new insights into the complex regulatory network that drives the early phases of embryonic myogenesis. We demonstrate that MyoD expression can occur in the absence of an active Wnt/β-catenin pathway, through a Wnt/PKC non-canonical pathway. We show that expression of MyoD is mainly Pax3 dependent and that Pax3 transcriptional activity is modulated by PKC, directly or indirectly by phosphorylation of the factor itself or putative co-factors. In addition we provide some insight into how activation of PKC by Wnt may act on the signal transduction of other signalling pathways, including the Wnt/β-catenin canonical pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.01.006.

References

Adamska, M., MacDonald, B.T., Sarmast, Z.H., Oliver, E.R., Meisler, M.H., 2004. En1 and Wnt7a interact with Dkk1 during limb development in the mouse. Dev. Biol. 272, 134–144.

Adamska, M., Billi, A.C., Cheek, S., Meisler, M.H., 2005. Genetic interaction between Wnt7a and Lrp6 during patterning of dorsal and posterior structures of the mouse limb. Dev. Dyn. 233, 368–372.

Bajard, L., Relaix, F., Lagha, M., Rocancourt, D., Daubas, P., Buckingham, M.E., 2006. A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. Genes Dev. 20, 2450–2464.

Bogatkevich, G.S., Gustilo, E., Oates, J.C., Feghali-Bostwick, C., Harley, R.A., Silver, R.M., Ludwicka-Bradley, A., 2005. Distinct PKC isoforms mediate cell survival and DNA synthesis in thrombin-induced myofibroblasts. Am. J. Physiol.: Lung Cell. Mol. Physiol. 288, L190–L201.

Borello, U., Buffa, V., Sonnino, C., Melchionna, R., Vivarelli, E., Cossu, G., 1999. Differential expression of the Wnt putative receptors Frizzled during mouse somitogenesis. Mech. Dev. 89, 173–177.

Borello, U., Berarducci, B., Murphy, P., Bajard, L., Buffa, V., Piccolo, S., Buckingham, M., Cossu, G., 2006. The Wnt/{beta}-catenin pathway regulates Gli-mediated Myf5 expression during somitogenesis. Development 133, 3723–3732.

Brault, V., Moore, R., Kutsch, S., Ishibashi, M., Rowitch, D.H., McMahon, A.P., Sommer, L., Boussadia, O., Kemler, R., 2001. Inactivation of the beta-catenin gene by Wnt1–Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 128, 1253–1264.

Brunelli, S., Tagliafico, E., De Angelis, F.G., Tonlorenzi, R., Baesso, S., Ferrari, S., Niinobe, M., Yoshikawa, K., Schwartz, R.J., Bozzoni, I., Cossu, G., 2004. Msx2 and necdin combined activities are required for smooth muscle differentiation in mesoangioblast stem cells. Circ. Res. 94, 1571–1578.

Buttitta, L., Tanaka, T.S., Chen, A.E., Ko, M.S., Fan, C.M., 2003. Microarray analysis of somitogenesis reveals novel targets of different WNT signaling pathways in the somitic mesoderm. Dev. Biol. 258, 91–104.

Chen, A.E., Ginty, D.D., Fan, C.M., 2005. Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. Nature 433, 317–322.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., Buckingham, M., 1996. Activation of different myogenic pathways: Myf5 is induced by

the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. Development 122, 429–437.
Cusella-De Angelis, M.G., Molinari, S., Le Donne, A., Coletta, M., Vivarelli, E., Bouche, M., Molinaro, M., Ferrari, S., Cossu, G., 1994. Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis. Development 120, 925–933.
de Angelis, L., Zhao, J., Andreucci, J.J., Olson, E.N., Cossu, G., McDermott, J.C., 2005. Regulation of vertebrate myotome development by the p38 MAP kinase–MEF2 signaling pathway. Dev. Biol. 283, 171–179.
De Servi, B., Hermani, A., Medunjanin, S., Mayer, D., 2005. Impact of PKCdelta on estrogen receptor localization and activity in breast cancer cells. Oncogene 24, 4946–4955.
Disatnik, M.H., Boutet, S.C., Lee, C.H., Mochly-Rosen, D., Rando, T.A., 2002. Sequential activation of individual PKC isozymes in integrin-mediated muscle cell spreading: a role for MARCKS in an integrin signaling pathway. J. Cell Sci. 115, 2151–2163.
Eastman, Q., Grosschedl, R., 1999. Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr. Opin. Cell Biol. 11, 233–240.
Fan, C.M., Lee, C.S., Tessier-Lavigne, M., 1997. A role for WNT proteins in induction of dermomyotome. Dev. Biol. 191, 160–165.
Filali, M., Cheng, N., Abbott, D., Leontiev, V., Engelhardt, J.F., 2002. Wnt-3A/beta-catenin signaling induces transcription from the LEF-1 promoter. J. Biol. Chem. 277, 33398–33410.
Goel, H.L., Dey, C.S., 2002a. PKC-regulated myogenesis is associated with increased tyrosine phosphorylation of FAK, Cas, and paxillin, formation of Cas–CRK complex, and JNK activation. Differentiation 70, 257–271.
Goel, H.L., Dey, C.S., 2002b. Role of protein kinase C during insulin mediated skeletal muscle cell spreading. J. Muscle Res. Cell Motil. 23, 269–277.
Hamplova, B., Novakova, O., Tvrzicka, E., Kolar, F., Novak, F., 2005. Protein kinase C activity and isoform expression during early postnatal development of rat myocardium. Cell Biochem. Biophys. 43, 105–117.
Hino, S., Tanji, C., Nakayama, K.I., Kikuchi, A., 2005. Phosphorylation of beta-catenin by cyclic AMP-dependent protein kinase stabilizes beta-catenin through inhibition of its ubiquitination. Mol. Cell Biol. 25, 9063–9072.
Jin, E.J., Park, J.H., Lee, S.Y., Chun, J.S., Bang, O.S., Kang, S.S., 2005. Wnt-5a is involved in TGF-beta3-stimulated chondrogenic differentiation of chick wing bud mesenchymal cells. Int. J. Biochem. Cell Biol.
Kassar-Duchossoy, L., Gayraud-Morel, B., Gomes, D., Rocancourt, D., Buckingham, M., Shinin, V., Tajbakhsh, S., 2004. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature 431, 466–471.
Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La Pena, J., Johnson, R.L., Belmonte, J.C., Tabin, C.J., 1998. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. Science 280, 1274–1277.
Keren, A., Bengal, E., Frank, D., 2005. p38 MAP kinase regulates the expression of XMyf5 and affects distinct myogenic programs during Xenopus development. Dev. Biol. 288, 73–86.
Kohn, A.D., Moon, R.T., 2005. Wnt and calcium signaling: beta-catenin-independent pathways. Cell Calcium 38, 439–446.
Koyanagi, M., Haendeler, J., Badorff, C., Brandes, R.P., Hoffmann, J., Pandur, P., Zeiher, A.M., Kuhl, M., Dimmeler, S., 2005. Non-canonical Wnt signaling enhances differentiation of human circulating progenitor cells to cardiomyogenic cells. J. Biol. Chem. 280, 16838–16842.
Kuhl, M., 2002. Non-canonical Wnt signaling in Xenopus: regulation of axis formation and gastrulation. Semin. Cell Dev. Biol. 13, 243–249.
Kuhl, M., Geis, K., Sheldahl, L.C., Pukrop, T., Moon, R.T., Wedlich, D., 2001. Antagonistic regulation of convergent extension movements in Xenopus by Wnt/beta-catenin and Wnt/Ca2+ signaling. Mech. Dev. 106, 61–76.
Lallemand, Y., Luria, V., Haffner-Krausz, R., Lonai, P., 1998. Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre site-specific recombinase. Transgenic Res. 7, 105–112.
Medina, A., Reintsch, W., Steinbeisser, H., 2000. Xenopus frizzled 7 can act in canonical and non-canonical Wnt signaling pathways: implications on early patterning and morphogenesis. Mech. Dev. 92, 227–237.
Oehrlein, S.A., Maelicke, A., Herget, T., 1998. Expression of protein kinase C gene family members is temporally and spatially regulated during neural development in vitro. Eur. J. Cell Biol. 77, 323–337.
Oriente, F., Formisano, P., Miele, C., Fiory, F., Maitan, M.A., Vigliotta, G., Trencia, A., Santopietro, S., Caruso, M., Van Obberghen, E., Beguinot, F., 2001. Insulin receptor substrate-2 phosphorylation is necessary for protein kinase C zeta activation by insulin in L6hIR cells. J. Biol. Chem. 276, 37109–37119.
Pandur, P., Lasche, M., Eisenberg, L.M., Kuhl, M., 2002a. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature 418, 636–641.
Pandur, P., Maurus, D., Kuhl, M., 2002b. Increasingly complex: new players enter the Wnt signaling network. Bioessays 24, 881–884.
Pauken, C.M., Capco, D.G., 2000. The expression and stage-specific localization of protein kinase C isotypes during mouse preimplantation development. Dev. Biol. 223, 411–421.
Penn, B.H., Bergstrom, D.A., Dilworth, F.J., Bengal, E., Tapscott, S.J., 2004. A MyoD-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. Genes Dev. 18, 2348–2353.
Pilon, N., Oh, K., Sylvestre, J.R., Bouchard, N., Savory, J., Lohnes, D., 2006. Cdx4 is a direct target of the canonical Wnt pathway. Dev. Biol. 289, 55–63.
Redling, S., Pfaff, I.L., Leitges, M., Vallon, V., 2004. Immunolocalization of protein kinase C isoenzymes alpha, beta I, beta II, delta, and epsilon in mouse kidney. Am. J. Physiol.: Renal Physiol. 287, F289–F298.
Relaix, F., Polimeni, M., Rocancourt, D., Ponzetto, C., Schafer, B.W., Buckingham, M., 2003. The transcriptional activator PAX3–FKHR rescues the defects of Pax3 mutant mice but induces a myogenic gain-of-function phenotype with ligand-independent activation of Met signaling in vivo. Genes Dev. 17, 2950–2965.
Relaix, F., Rocancourt, D., Mansouri, A., Buckingham, M., 2004. Divergent functions of murine Pax3 and Pax7 in limb muscle development. Genes Dev. 18, 1088–1105.
Relaix, F., Rocancourt, D., Mansouri, A., Buckingham, M., 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. Nature 435, 948–953.
Renault-Mihara, F., Beuvon, F., Iturrioz, X., Canton, B., De Bouard, S., Leonard, N., Mouhamad, S., Sharif, A., Ramos, J.W., Junier, M.P., Chneiweiss, H., 2006. PEA-15 expression inhibits astrocyte migration by a PKC delta-dependent mechanism. Mol. Biol. Cell.
Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., Weissman, I.L., 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 423, 409–414.
Sheldahl, L.C., Park, M., Malbon, C.C., Moon, R.T., 1999. Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Curr. Biol. 9, 695–698.
Tajbakhsh, S., Rocancourt, D., Cossu, G., Buckingham, M., 1997. Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell 89, 127–138.
Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M., Cossu, G., 1998. Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. Development 125, 4155–4162.
Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C.C., Lin, X., Heasman, J., 2005. Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. Cell 120, 857–871.
Wilda, M., Ghaffari-Tabrizi, N., Reisert, I., Utermann, G., Baier, G., Hameister, H., 2001. Protein kinase C isoenzyme: selective expression pattern of protein kinase C- and theta; during mouse development. Mech. Dev. 103, 197–200.
Wu, Z., Woodring, P.J., Bhakta, K.S., Tamura, K., Wen, F., Feramisco, J.R., Karin, M., Wang, J.Y., Puri, P.L., 2000. p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. Mol. Cell Biol. 20, 3951–3964.