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Overexpression of nPKC θ Is Permissive for Myogenic Differentiation

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Abstract Although protein kinase C (PKC) has been shown to participate in skeletal myogenic differentiation, the functions of individual isoforms of PKC in myogenesis have not been completely elucidated. These studies focused on the role of nPKC θ, an isoform of the PKC family whose expression has been shown to be regulated by commitment to the myogenic lineage, myogenic differentiation and innervation. We used the myogenic cell line C_2C_{12} as a tissue culture model system to explore the role of nPKC θ in the formation of multinucleated myotubes. We examined endogenous levels of nPKC θ in C_2C_{12} cells and showed that it is expressed at low levels in myoblasts compared to mouse skeletal muscle and that expression is maintained in myotubes. We overexpressed nPKC θ in C_2C_{12} myoblasts and examined the ability of overexpressing cells to differentiate into myotubes. Using an nPKC θ - green fluorescent protein (GFP) chimera to detect transfected myoblasts, we showed that overexpressed nPKC θ-GFP translocates to the plasma membrane in response to phorbol ester treatment of myoblast cultures in situ. nPKC θ-GFP was found to be completely extracted into the detergent-soluble fraction of cell lysates and was stably expressed throughout the extent of differentiation into myotubes. No difference was seen in the ability of myoblasts either overexpressing nPKC θ - GFP or GFP alone to form myotubes. These studies demonstrate that overexpression of nPKC θ does not interfere with fusion of myoblasts into myotubes suggesting that nPKC θ activity is not inhibitory for myogenesis. These studies also demonstrate a method for transfecting myoblasts and identifying differentiated cells that overexpress nPKC θ-GFP for investigating the function of nPKC θ in living myotubes. J. Cell. Biochem. 79:71–79, 2000.

Key words: nPKC θ, myogenesis; C_2C_{12} cells; GFP; PKC isoforms; overexpression

Activation of signal transduction pathways that involve protein kinase C (PKC) influence skeletal myogenesis. Long-term treatment with phorbol esters, which both activates and downregulates PKC, has been shown to inhibit mouse and chick myoblast fusion into multinucleated myotubes (Cohen et al., 1977; Cossu et al., 1983). In accordance with this observation, total PKC activity declines in chick and mouse cells during myogenesis (Adamo et al., 1989; Vaidya et al., 1991; Capiati et al., 1999). Alternatively, chick myoblasts treated with PKC inhibitors fail to fuse, suggesting that PKC activity is required for muscle cell differentiation (David et al., 1990). These conflicting observations raise the possibility that different isoforms of PKC play different roles during myogenesis.

PKC activity is mediated by a family of enzyme isoforms that are differentially expressed among different tissue types (Newton, 1995). The tissue distribution and subcellular localization of PKC isoforms are thought to confer specificity of function (Jaken, 1996). Some insight has been gained on the role of specific PKC isoforms on myogenesis. Expression of a constitutively active mutant isoform of cPKC α in the myogenic C_2C_{12} cell line leads to phosphorylation of the transcription factor myogenin abolishing its ability to activate muscle specific gene transcription (Li et al., 1992). In support of these findings is a report that cPKC α expression, but not expression of other PKC isoforms, declines in chick myoblasts during differentiation (Capiati et al., 1999).

nPKC θ, a member of the novel subset of PKC isoforms, is the most abundant PKC isoform in skeletal muscle (Osada et al., 1992). Expression of nPKC θ is restricted to skeletal muscle and hematopoietic tissue (Osada et al., 1992; Baier et al., 1993; Chang et al., 1993; Hilgenberg and Miles, 1995). We have previously shown that expression of nPKC θ in skeletal muscle increases dramatically during the first two weeks after birth in rats (Hilgenberg and Miles, 1995) and that high expression of

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this PKC isoform in adult rat skeletal muscle depends upon innervation (Hilgenberg et al., 1996).

These in vivo observations were further confirmed by studies in cultured rat skeletal myoblasts. In primary fetal rat skeletal muscle cells in culture, nPKC θ was detected in myoblasts and not in nonmyogenic fibroblasts (Hilgenberg et al., 1996). Expression of nPKC θ in myoblasts increased during myogenic differentiation. Coculturing primary rat myotubes with neuronal cells also increased nPKC θ levels in myotubes. These data suggest that nPKC θ expression is regulated by commitment to the myogenic lineage, by myogenic differentiation, and by innervation.

nPKC θ expression in cells committed to the myogenic lineage provides the foundation for experiments that address the role of nPKC θ during myogenesis. Overexpression of exogenous nPKC θ in mouse primary skeletal muscle myoblasts did not affect their differentiation as far as myocytes (Zapelli et al., 1996). Recently, nPKC θ has been implicated in inhibiting chick myotube formation by virtue of its ability to phosphorylate the myristoylated alanine-rich C kinase substrate (MARCKS) (Kim et al., 2000). In contrast to rat and mouse, both chick and human skeletal muscle cells displayed decreased nPKC θ expression during differentiation into myotubes (Boczan et al., 2000; Kim et al., 2000). Taken together these observations raise the question as to the role of nPKC θ expression in myotube formation.

C_2C_{12} cells are a mouse skeletal muscle cell line that undergoes a well-described process of myogenic differentiation (Andres and Walsh, 1996). We used these cells as a tissue culture model system to explore the role of nPKC θ in C_2C_{12} myogenesis. In this study, we demonstrate that the enzyme is endogenously expressed at low levels in C_2C_{12} myoblasts and that expression is maintained in myotubes. Using an nPKC θ - green fluorescent protein (GFP) chimeric protein to detect transfected myoblasts overexpressing nPKC θ, we showed that nPKC θ - GFP could translocate to the plasma membrane in response to phorbol esters and be stably expressed throughout the extent of differentiation into myotubes. An equal percentage of myoblasts overexpressing nPKC θ - GFP fused to form myotubes compared to myoblasts overexpressing GFP alone. These studies demonstrate that overexpression of nPKC θ does not interfere with fusion of myoblasts into myotubes, suggesting that nPKC θ activity is not inhibitory for myogenesis.

METHODS

Construct of Plasmid Encoding nPKC θ-GFP Fusion Protein

A cDNA fragment encoding nPKC θ with a *Not* I site in the 5′ terminal and a *Sma* I site at the 3′ terminal was obtained by PCR using pSRD-nPKC θ (Osada et al., 1992) as a template. The sense and antisense primers used were 5′-CGGCGGCCGCGACACCAGGGAACAACC-AT-3′ and 5′-GGATCCCGGGGGAGCAAAT-GAGAGTCTC-3′, respectively. Using *Not* I and *Sma* I restriction sites, the fragment was cloned into pBluescript. A fragment encoding GFP was excised from the pEGFP-N2 plasmid (Clontech) and then cloned into the pBluescript-nPKC θ plasmid using *Sma* I and *Pvu* II restriction sites. A fragment encoding the nPKC θ-GFP fusion protein was then excised and cloned into the pJMF2 expression vector (Lang and Feingold, 1996) using *Not* I restriction sites. Nine amino acids derived from the pEGFP multicloning site were inserted between the C-terminus of nPKC θ and the N-terminus of GFP. Expression of the fusion protein was under the mammalian CMV promoter.

Tissue Culture and Cell Transfection

C_2C_{12} cells were passaged in DMEM growth medium (GM) supplemented with 10% fetal calf serum. Confluent cells were induced to differentiate by switching to DMEM supplemented with 2% horse serum (DM). C_2C_{12} myoblasts were transfected using 6 μls Fugene (Roche Molecular Biochemicals) and 2 μgs DNA (0.2 μgs/μl) per 60 mm dish according to the manufacturer's instructions. To achieve transfection efficiency in the range of $< 10\%$, 1 μg DNA per dish was used instead. For quantitation of green fluorescent cells, areas of equal size and number were randomly marked on culture dishes prior to plating and green fluorescent cells were counted within these areas at 48 h after transfection (~50 myoblasts were counted per dish) and at 24 and 144 h after exposure to DM. Phorbol 12-myristate 13-acetate (PMA) was obtained from Alexis Biochemicals.

Subcellular Fractionation

Mouse hindlimb skeletal muscle was prepared as previously described (Hilgenberg et al., 1996). Cultured C_2C_{12} cells were homogenized using a Dounce homogenizer in 500 μls per 60 mm dish in Buffer A containing 20 mM Tris, pH 7.5, 10 mM EDTA, 10 mM EGTA, 25 μg/ml aprotinin, 50 μg/ml leupeptin, 10 mM benzamidine, 10 mM PMSF, 10 mM β-mercaptoethanol. The homogenates were centrifuged at 6,000 × g for 2 min and supernatants were collected and centrifuged at 95,000 × g for 45 min. The resulting supernatants (cytosolic fractions) were collected and the pellets (membrane fractions) were solubilized in Buffer A containing 1% Triton X-100 (100 μl/pellet). Samples were adjusted such that an equal amount of total protein from each of these two fractions was analyzed. Protein concentrations were determined using the Bio-Rad (Hercules, CA) protein assay based on Coomassie brilliant blue binding.

Alternatively, cells were harvested in 50 μls per 60 mm dish Buffer B containing 20 mM Tris pH 7.6, 0.5% NP40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM $NaVO_3$, 50 μg/ml leupeptin, 10 mM benzamidine, 10 mM PMSF. The mixture was vigorously vortexed, incubated on ice for 5 min and then centrifuged at 6,000 × g for 5 min. SDS-sample buffer was added to the entire supernatant (detergent-soluble fraction) or pellet (detergent-insoluble fraction) from each dish and samples were boiled for 5 min before analysis by SDS-PAGE and immunoblotting.

Immunoblot Analysis

Proteins were subjected to SDS-PAGE and then transferred electrophoretically to nitrocellulose membranes. The membranes were incubated overnight at 4°C in Blotto buffer containing 200 mM NaCl, 50 mM Tris, 0.1% Triton, 0.2% Tween-20 and 5% nonfat dry milk (w/v), pH 7.4 to block nonspecific binding. nPKC θ was detected using S22 antiserum raised against the C-terminus 17 amino acids of mouse nPKC θ (Hilgenberg and Miles, 1995). cPKC α was detected using a previously characterized monoclonal antibody, M6 (UBI, Lake Placid, NY), and myosin heavy chain (MHC) was detected using the MF20 antibody generously provided by Dr. Donald Fishman (Cornell Medical College, NY). Incubation with monoclonal antibodies was followed by a rabbit anti-mouse secondary antibody (Dako, Carpinteria, CA). Membranes were then incubated with ^{125}I-Protein A (NEN, Boston MA), washed and exposed to a PhosphorImager screen. Radioactivity was detected using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

PKC Isoform Expression During C_2C_{12} Myogenic Differentiation

Two PKC isoforms, cPKC α and nPKC θ, have been shown to be highly expressed in skeletal muscle (Hilgenberg and Miles, 1995; Osada et al., 1992). nPKC θ expression is restricted to cells committed to the myogenic lineage and lymphoid cells whereas cPKC α is ubiquitously expressed. Since the expression pattern of these PKC isoforms in C_2C_{12} cells is not known, we initially sought to determine the endogenous levels of nPKC θ and cPKC α in C_2C_{12} myoblasts and myotubes. nPKC θ was found at very low but detectable levels in C_2C_{12} myoblasts and was concentrated in the cytosolic fraction rather than the membrane fraction (Fig. 1). During C_2C_{12} myogenic differentiation total protein synthesis per culture increased; however, the ratio of nPKC θ to total cytosolic protein did not change (Fig. 1).

In C_2C_{12} cells cPKC α was found to be concentrated predominantly in the cytosolic fraction at levels comparable to mouse skeletal muscle. The ratio of cPKC α expression with respect to total protein in the cytosolic fraction did not change after differentiation into myotubes (Fig. 1). A slight decrease in expression of cPKC α with respect to total protein was detected in the membrane fraction of C_2C_{12} myotubes compared to that of myoblasts. These data show that expression of both nPKC θ and cPKC α is maintained during C_2C_{12} myogenesis.

To demonstrate that the C_2C_{12} cells used in these studies can differentiate to express molecular markers of myogenesis as well as form myotubes, we measured the expression of myosin heavy chain (MHC) (Fig. 1). MHC expression was dramatically increased during myogenesis verifying that these cells undergo a well-characterized process of myogenic differentiation (Andres and Walsh, 1996).

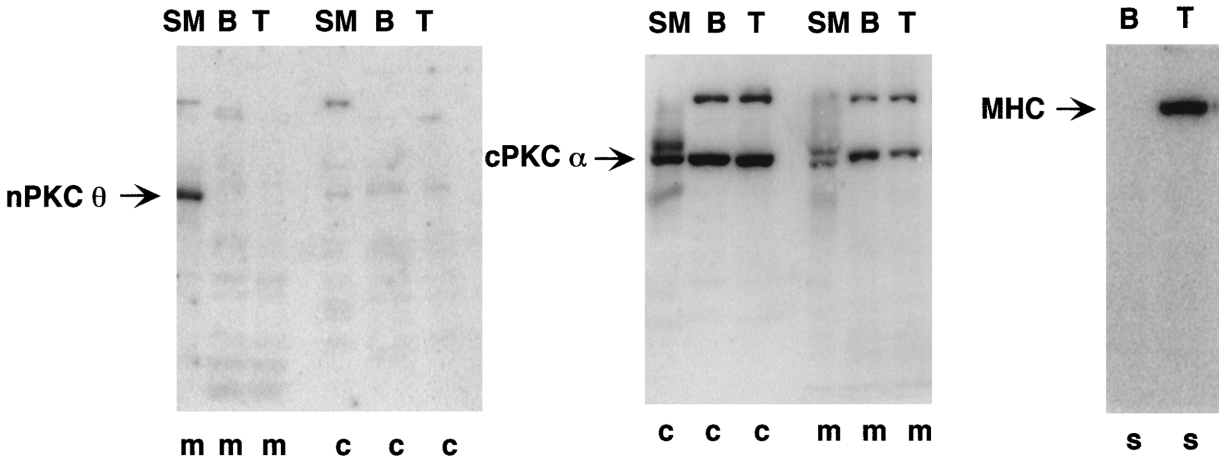
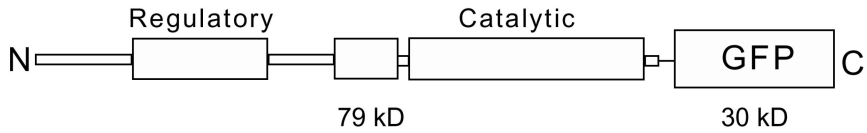


Fig. 1. Expression of PKC θ and α isoforms in C_2C_{12} myoblasts and myotubes. 50 μgs protein from membrane (m) and cytosolic (c) fractions of adult mouse hindlimb skeletal muscle (SM), C_2C_{12} myoblasts (B) or myotubes (T) or the entire detergent-soluble (s) extract from one parallel culture dish were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were probed with antiserum to nPKC θ, antibodies to cPKC α or antibodies to MHC as indicated followed by ^{125}I-labeled protein A. Blots were exposed for PhosphorImager analysis. Immunoblot analysis was repeated, using different culture preparations, four times for anti nPKC θ and twice for anti-cPKC α with similar results in each case.

Expression of nPKC θ-GFP Fusion Protein in C_2C_{12} Myoblasts

The above studies show that low levels of nPKC θ are present in myoblasts and maintained in myotubes, suggesting that elevated levels are not required for nPKC θ function during myogenesis or that nPKC θ activity might be inhibitory for myogenesis. To begin to address these possibilities, we sought to express high levels of nPKC θ in C_2C_{12} cells and examine its effect on myogenesis. To identify those cells overexpressing nPKC θ in culture we created an expression construct encoding mouse nPKC θ fused to the N-terminus of the eukaryotic GFP (Fig. 2A). We transiently transfected the chimeric protein into C_2C_{12} cells and detected its presence in cell extracts using an antiserum raised against a



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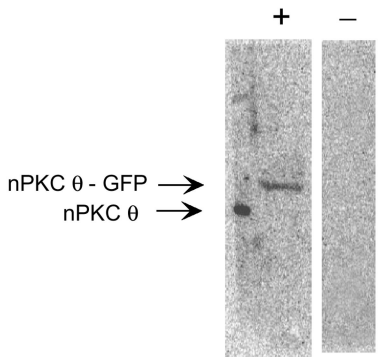

B

Fig. 2. Diagram of nPKC θ-GFP fusion protein (**A**). Expression of nPKC θ-GFP in C_2C_{12} myoblasts (**B**). C_2C_{12} myoblasts were transfected with (+) or without (−) pJMF2-nPKC θ-GFP. After 48 h, cells were harvested and total soluble protein per 60 mm dish was treated as described in the legend to Fig. 1. 50 μgs protein from adult mouse skeletal muscle membrane fraction was loaded in lane 1. The immunoblot was probed with antiserum to nPKC θ.

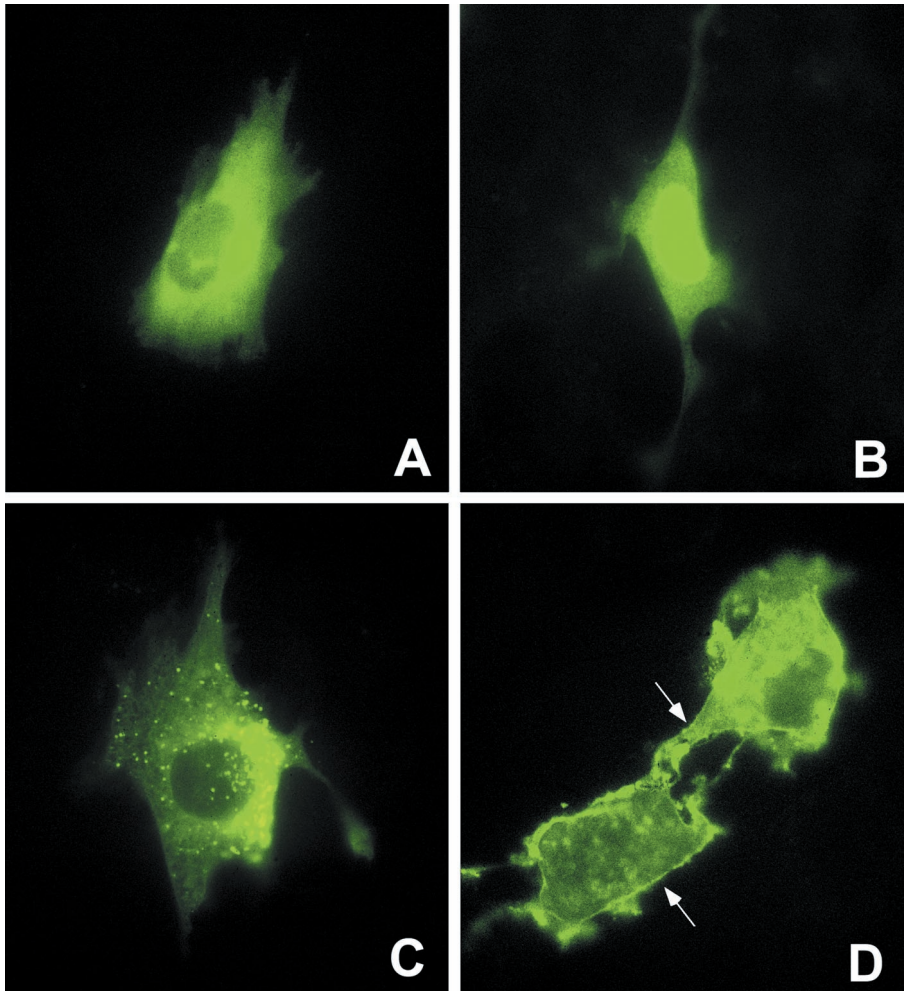

Fig. 3. Expression and phorbol ester-induced translocation of nPKC θ-GFP in C_2C_{12} myoblasts. C_2C_{12} myoblasts were transfected with pEGFP (**A, B**) or pJMF2-nPKC θ-GFP (**C, D**). 48 h later, cells were treated with 100 nM PMA for 60 mins (**B, D**). Cells were then washed in PBS, fixed in 2% paraformaldehyde and visualized by epifluorescence microscopy.

C-terminus peptide of mouse nPKC θ (Fig. 2B) (Hilgenberg and Miles, 1995). A protein of approximately 110 kDs corresponding to the predicted molecular weight of nPKC θ-GFP was present in transfected but not untransfected cells (Fig. 2B).

Phorbol Ester-Induced Translocation of nPKC θ-GFP Fusion Protein in C_2C_{12} Myoblasts

Since transfected C_2C_{12} myoblasts overexpress an intact fusion protein we can conclude that in our studies green fluorescent cells are overexpressing exogenous nPKC θ-GFP. We next examined whether nPKC θ-GFP exhibited enzymatic activity by determining whether it can translocate to the membrane in response to phorbol esters. GFP linked to several different PKC isoforms have previously been used to monitor translocation of the enzyme as a measure of activation in living cells (Sakai et al., 1997; Feng et al., 1998; Oancea et al., 1998; Almholt et al., 1999; Ng et al., 1999; Wang et al., 1999). C_2C_{12} myoblasts were transfected with either the nPKC θ-GFP expression construct or a construct encoding GFP alone. nPKC θ-GFP appeared in a punctate distribution in the cytoplasm of C_2C_{12} myoblasts (Fig. 3C) whereas GFP appeared diffusely distributed throughout the cytoplasm (Fig. 3A). Phorbol ester treatment of these cultures for one hour resulted in increased fluorescence intensity of the plasma membrane in myoblasts expressing nPKC θ-GFP (Fig. 3D, see arrows) suggesting that nPKC θ-GFP translocated from the cytosol to the plasma membrane. In contrast, GFP did not translocate to the plasma membrane after phorbol ester treatment (Fig. 3B)(Sakai et al., 1997). These in situ observations, taken together with the immunoblot data (Fig. 2B), provide evidence that nPKC

θ-GFP is stably expressed in C_2C_{12} myoblasts and that it is catalytically active.

Time Course of nPKC θ-GFP Expression During Myogenesis

To monitor the expression and stability of nPKC θ-GFP during differentiation of C_2C_{12} myoblasts into myotubes, myoblast cultures were transfected and 24 h later shifted to differentiation medium. Each day starting 24 h after transfection, individual cultures were harvested and proteins contained in detergent-soluble and insoluble fractions were analyzed by immunoblotting. nPKC θ-GFP was found to accumulate intact within the detergent-soluble fraction of cells throughout differentiation suggesting that the protein is stable in differentiating C_2C_{12} cells for as long as 6 days (Fig. 4A). nPKC θ-GFP was not detected in the detergent-insoluble fraction, indicating that the enzyme is completely processed in cells throughout differentiation (Fig. 4B) (Keranen, 1995).

Expression of nPKC θ-GFP Fusion Protein in C_2C_{12} Myotubes

We next investigated whether overexpression of nPKC θ-GFP in myoblasts affects their ability to differentiate into myotubes. Parallel cultures overexpressing either PKC θ-GFP or GFP (Fig. 5B and F, respectively), were shifted to differentiation medium and examined for the formation of myotubes after 3 days. Green fluorescent multinucleated myotubes were present in both cases (Fig. 5D and H). No differences were observed between the average size or length, or the number of nuclei present in myotubes overexpressing nPKC θ-GFP compared to myotubes overexpressing GFP (data not shown). nPKC θ-GFP fluorescence was distributed throughout myotubes and often appeared randomly in punctate patches. The distribution of nPKC θ-GFP throughout myotubes is comparable to that observed for endogenous nPKC θ in primary fetal myotubes in culture (Zapelli et al., 1996). In contrast, GFP fluorescence was always seen as diffusely distributed throughout the entire C_2C_{12} myotube.

To quantitate the number of myotubes that form from overexpressing myoblasts, cultures were transfected such that the frequency of overexpressing cells was low (< 10%) thus limiting the likelihood that two transfected cells would fuse with each other. Cultures overexpressing either nPKC θ-GFP or GFP were examined 48 h after transfection and the number of green fluorescent myoblasts in randomly located, equally sized areas was quantitated. The cultures were then reexamined after 2–3 days in DM, and green fluorescent multinucleated myotubes were counted within the same areas that were previously designated. The ratio of the number of green fluorescent myotubes to the number of green fluorescent myoblasts in each case was calculated. Approximately 10% of the number of green fluorescent myoblasts subsequently appeared as multinucleated green fluorescent myotubes in each case (Fig. 5I). These results suggest that overexpression of nPKC θ does not perturb myogenic differentiation as assayed by myotube formation.

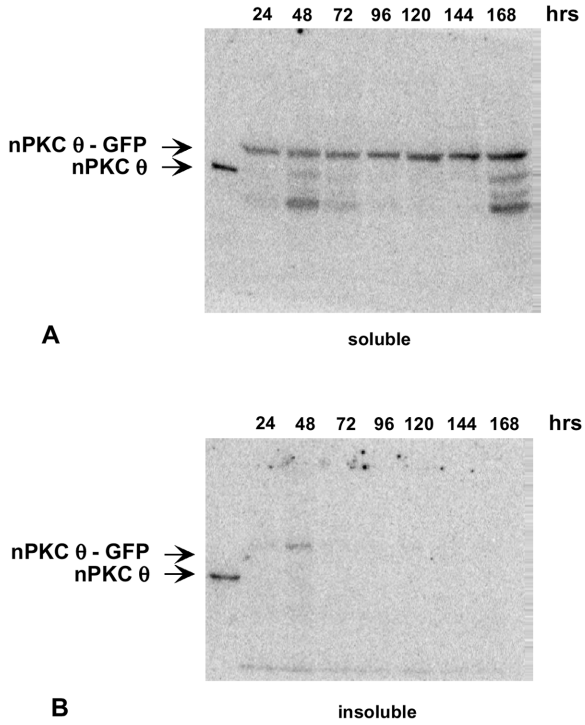

Fig. 4. Time course of nPKC θ-GFP expression during myogenesis. C_2C_{12} myoblasts were transfected with pJMF2-nPKC θ-GFP. Cultures were transferred to DM at 24 h post transfection. At times indicated after transfection, cells were harvested and separated into total soluble (**A**) and insoluble (**B**) fractions and analyzed as described in the legend to Fig. 2. 50 μgs protein from adult mouse skeletal muscle membrane fraction was loaded in lane 1 of each gel.

DISCUSSION

Our strategy to investigate the role of distinct PKC isoforms in myogenesis is focused on

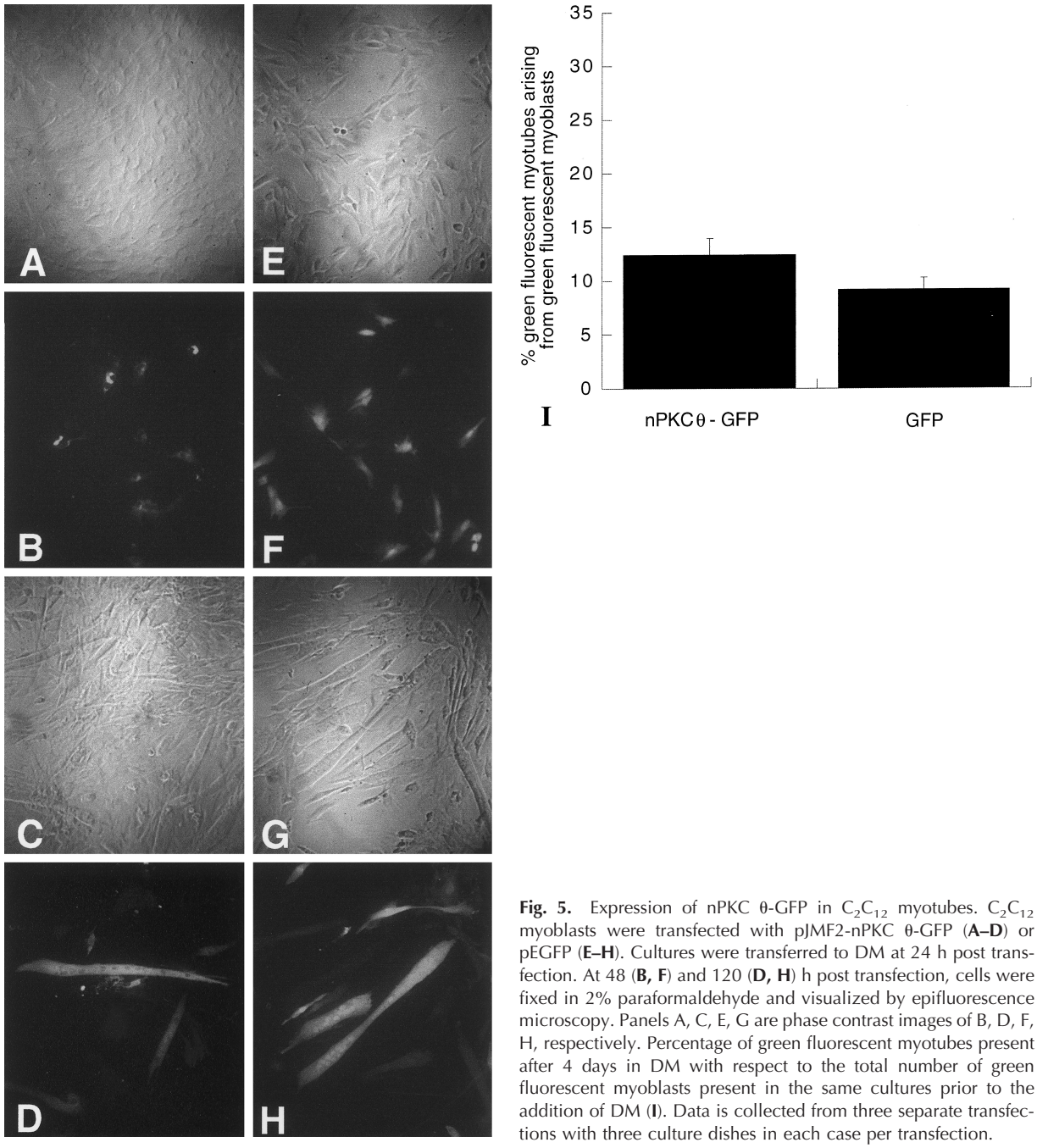

Fig. 5. Expression of nPKC θ-GFP in C_2C_{12} myotubes. C_2C_{12} myoblasts were transfected with pJMF2-nPKC θ-GFP (**A–D**) or pEGFP (**E–H**). Cultures were transferred to DM at 24 h post transfection. At 48 (**B, F**) and 120 (**D, H**) h post transfection, cells were fixed in 2% paraformaldehyde and visualized by epifluorescence microscopy. Panels A, C, E, G are phase contrast images of B, D, F, H, respectively. Percentage of green fluorescent myotubes present after 4 days in DM with respect to the total number of green fluorescent myoblasts present in the same cultures prior to the addition of DM (**I**). Data is collected from three separate transfections with three culture dishes in each case per transfection.

nPKC θ, a PKC isoform that is restricted to cells of the myogenic lineage and certain lymphoid cells. We first examined and compared the endogenous expression in C_2C_{12} cells of nPKC θ and cPKC α, another PKC isoform shown to be abundant in skeletal muscle (Osada et al., 1992). Similar to our findings in rat primary muscle cell cultures (Hilgenberg et al., 1996), expression of nPKC θ was low whereas expression of cPKC α was high in both C_2C_{12} myoblasts and myotubes compared to adult skeletal muscle. During myogenesis both nPKC θ and cPKC α levels increased in proportion to total protein synthesis in differentiated C_2C_{12} cells. We previously observed that cPKC α levels increased dramatically during differentiation of primary rat myoblasts whereas the opposite has been observed in chick myoblasts undergoing differentiation (Capiati et al., 1999). Perhaps these differences reflect func-

tional differences between mammalian and avian PKC isoforms.

In contrast to primary rat myoblasts or myotubes and mouse and rat skeletal muscle, nPKC θ appeared in the cytosolic fraction rather than concentrated in the membrane fraction in both C_2C_{12} myoblasts and myotubes. This difference in subcellular localization may reflect variation in levels of nPKC θ activation, differences in PKC anchoring proteins (Jaken, 1996) or different functional roles for nPKC θ among these cell types. In general, however, our observations on nPKC θ expression in C_2C_{12} cells concur with our previous results in primary fetal rat muscle cells, suggesting that nPKC θ plays a similar role in C_2C_{12} cells (Hilgenberg et al., 1996).

To address the question as to whether nPKC θ activity in C_2C_{12} cells affects differentiation into myotubes we sought to overexpress this enzyme in myoblasts and subject them to conditions that induce differentiation. We designed an nPKC θ-GFP fusion protein as a means of detecting transfected cells and following their fate during differentiation. Since GFP fluorescence can be visualized in living cells, using nPKC θ-GFP enabled us to monitor differentiation within a culture of nPKC θ-GFP overexpressing cells. Comparisons of myotube formation between nPKC θ-GFP and GFP overexpressing cultures obviated the need for high transfection efficiency in these experiments.

nPKC θ-GFP was stably expressed in C_2C_{12} myoblasts and throughout myogenesis. The fusion protein could be completely extracted into the detergent-soluble fraction of cell lysates and application of phorbol esters caused nPKC θ-GFP to translocate to the plasma membrane. These data provide evidence that overexpressed nPKC θ-GFP is completely processed and functionally active in C_2C_{12} cells.

Overexpression of PKC isoforms has been used in other systems to examine the role of specific PKC isoforms in physiological processes. In several instances, overexpression of specific isoforms of PKC, in the absence of activation by exogenously added phorbol esters or ligands, led to effects that were attributed to the specific activity of that isoform (Mischak et al., 1993; Sun et al., 1999). An overexpressed kinase negative mutant of nPKC θ, which contains a point mutation in the ATP-binding domain and is therefore unable to phosphorylate, was found to translocate to the surface membrane in endothelial cells under unstimulated conditions. Translocation of this kinase negative mutant was thought to underlie the dominant negative inhibition of endogenous nPKC θ activity that was observed (Tang et al., 1997). Taken together these results suggest that overexpression of a given PKC isoform raises intracellular activity compared to endogenous levels and can be used to reveal a functional role for PKC isoforms.

When exposed to DM, not all C_2C_{12} cells myoblasts fuse to form multinucleated myotubes (Wang and Walsh, 1996). The mechanisms that determine the differentiation pathway of a myoblast have not been completely explicated. Since C_2C_{12} myoblasts express low levels of nPKC θ we examined whether overexpression of nPKC θ would inhibit myotube formation. An equal percentage of myotubes arose from overexpressing nPKC θ-GFP compared to GFP myoblasts indicating that the enzyme permits myotube formation in C_2C_{12} cells. Future studies will be directed at determining whether endogenous ligands that activate nPKC θ affect myogenesis in nPKC θ-GFP overexpressing cells. For example, nPKC θ has previously been shown to mediate transforming growth factor β (TGF β) induced inhibition of myogenesis in mouse embryonic myoblasts (Zapelli et al., 1996). The presence or absence of nPKC θ was thought to confer the differential sensitivity of fetal and embryonic myoblasts to endogenous TGF β during limb muscle development.

The demonstration of stable expression of nPKC θ-GFP throughout myogenesis and its ability to permit formation of differentiated myotubes provides a means of investigating other functions of this skeletal muscle-enriched PKC isoform by overexpression in differentiated muscle cells in culture. Given that introduction and overexpression of exogenous genes into myoblasts could potentially affect myogenesis and that differentiated myotubes are refractory to transfection, these studies demonstrate a convenient method to transfect nPKC θ-GFP into C_2C_{12} myoblasts and later identify transfected cells to study effects of nPKC θ overexpression on certain properties of differentiated myotubes.

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