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An essential role of phosphatidylinositol 3-kinase in myogenic differentiation

(myogenesis)

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ABSTRACT **The oncogene *p3k*, coding for a constitutively active form of phosphatidylinositol 3-kinase (PI 3-kinase; EC 2.7.1.137), strongly enhances myogenic differentiation in cultures of chicken-embryo myoblasts. It increases the size of the myotubes and induces elevated levels of the muscle-specific proteins MyoD, myosin heavy chain, creatine kinase, and desmin. Inhibition of PI 3-kinase activity with LY294002 or with dominant-negative mutants of PI 3-kinase interferes with myogenic differentiation and with the induction of muscle-specific genes. PI 3-kinase is therefore an upstream mediator for the expression of the muscle-specific genes and is both necessary and rate-limiting for the process of myogenesis.**

During embryogenesis, muscle-cell lineages acquire increased specialization, and their developmental potential becomes progressively restricted. The first gene identified as an important regulator of myogenic differentiation was MyoD (1, 2). MyoD belongs to a family of basic helix–loop–helix muscle regulatory factors (MRFs) that also includes myf5, myogenin, and MRF4. All of these play a role in vertebrate myogenesis; they can induce nonmyogenic cells to express muscle-specific genes and can acquire phenotypic traits of muscle cells (2–6). Myogenic determination and differentiation are reinforced by the myocyte enhancer factor 2 (MEF2), a member of the MADS-box regulators, through interaction with MRFs (7–9). The Pax3 protein can function as a regulator of these muscle-specific transcription factors (10–13). The nature of the upstream signal that initiates myogenic gene regulation remains to be determined.

Expression of oncogenes such as *ras* (14–19), *src* (20–24), *myc* (22, 25, 26), or *jun* (27, 28) commonly inhibits myogenic differentiation. The recently discovered retroviral oncogene v-*p3k*, coding for a homolog of the catalytic subunit p110α of phosphatidylinositol 3-kinase (PI 3-kinase; EC 2.7.1.137), induces oncogenic transformation of chicken embryo fibroblasts in culture and causes hemangiosarcomas in the animal (29). PI 3-kinase is activated by several growth factors (30), functions as a nodal point in cellular signaling, and has been implicated in numerous cellular functions including antiapoptosis (31–40), cell growth (41), and regulation of cytoskeletal structure (42–44). In this study, we show that the oncogene *p3k* can regulate myogenic differentiation and that PI 3-kinase activity is required for this process.

MATERIALS AND METHODS

Myoblast Cell Culture and Virus Production. Chicken embryo myoblasts (CEM) were prepared from thigh muscles of 10-day-old chicken embryos as described (27). CEM were first cultured in myoblast growth (MG) medium, a mixture of nutrient solutions M199 and F10 at a 2:1 ratio, supplemented with 10% fetal bovine serum and 5% chicken embryo extract; they were then infected with the viruses described below. At various times after infection, CEM were switched to myoblast differentiation (MD) medium, which is identical to MG medium except for the substitution of 10% horse serum for fetal bovine serum. The oncogene *p3k* and dominant-negative forms of PI 3-kinase were expressed as inserts in the avian retroviral vector RCAS (29, 43). RCAS constructs were transfected into chicken embryo fibroblasts, which then produced high-titer retroviral progeny with the respective insert (45). These virus preparations were used to infect CEM, allowing expression of the insert in the majority of the cells in a culture.

PI 3-Kinase Assay. The infected and control CEM were washed with ice-cold PBS and scraped from the plates. The cells were incubated for 15 min on ice in lysis buffer (150 mM NaCl/100 mM Tris·HCl, pH 8.0/1% Triton X-100/5 mM EDTA, 10 mM NaF/5 mM DTT/1 mM phenylmethylsulfonyl fluoride/1 mM sodium vanadate/20 μM leupeptin/100 μM aprotinin) and centrifuged at 15,000 $\times$ *g* for 10 min to clarify the supernatants. PI 3-kinase activity was analyzed using 500 μg of protein extracts and anti-p110 antibodies as described (29).

Immunoblots. CEM were washed with ice-cold PBS and scraped from plates. The cells were lysed by incubation for 15 min on ice in RIPA buffer (150 mM NaCl/100 mM Tris·HCl, pH 8.0/1% Triton X-100/1% deoxycholic acid/0.1% SDS/5 mM EDTA/10 mM NaF) supplemented with 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 20 μM leupeptin, and 100 μM aprotinin. The lysates were clarified by centrifugation at 15,000 $\times$ *g* for 10 min. Aliquots of the protein extracts were resolved in SDS/polyacrylamide gels and transferred to nitrocellulose membranes in 20 mM Tris·HCl (pH 8.0) containing 150 mM glycine and 20% (vol/vol) methanol. Membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated with antibodies specific for MyoD (Santa Cruz Biotechnology), myosin heavy chain (MHC), desmin (ICN), and actin (Sigma). Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Amersham) and a chemiluminescence reagent (DuPont/NEN).

RESULTS AND DISCUSSION

To investigate the potential role of the v-P3k protein in myogenic differentiation, we used CEM, the precursors of chicken skeletal-muscle cells. CEM undergo myogenic differentiation *in vitro*; they exit the cell cycle, fuse into multinucleated myotubes, and turn on muscle-specific gene programs.

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Abbreviations: CEM, chicken embryo myoblasts; PI 3-kinase, phosphatidylinositol 3-kinase; MRF, muscle regulatory factor; CK, creatine kinase; MD, myoblast differentiation medium; MG, myoblast growth medium.

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Fig. 1*A* documents the expression of the v-P3k protein in CEM by demonstrating increased PI 3-kinase activity. The presence of v-P3k in these cells was confirmed by immunoblots using polyclonal antibodies directed against the retroviral Gag portion of the v-P3k protein (ref. 29; data not shown). v-P3k had a pronounced effect on the morphology of CEM cultures. It enhanced myogenic differentiation, inducing the formation of multinucleated myotubes that were significantly larger than those found in control cultures. As negative controls, CEM expressing the *src* oncogene failed to differentiate, as expected (Fig. 1*B*). v-P3k also elevated the expression of muscle-specific genes over the levels seen in differentiating CEM cultures infected with the RCAS vector alone. Creatine kinase (CK) activity began to increase on day 4 postinfection with the RCAS-v-P3k virus, and on day 9 climbed to more than double the levels of control cultures infected with the RCAS virus alone. Expression of the activated Src kinase kept CK at the predifferentiation level (Fig. 2*A*). Other muscle-specific proteins were likewise up-regulated in v-P3k-expressing as compared with RCAS-infected myoblasts. These include MyoD, myosin heavy chain, and desmin (Fig. 2*B*). To determine whether PI 3-kinase activity is required for myogenic differentiation, we inhibited the endogenous enzyme activity in CEM cultures with the PI 3-kinase-specific inhibitor, LY294002. Exposure of CEM cultures to LY294002 for 3 days at a concentration of 12.5 μM strongly interfered with the formation of myotubes (Fig. 3 *A* and *B*). At 50 μM LY294002, myotube formation was completely inhibited. The PI 3-kinase inhibitor also interfered in a dose-dependent manner with the induction of muscle-specific proteins. Levels of CK (Fig. 3*C*), MyoD, myosin heavy chain, and desmin (Fig. 3*D*) were significantly reduced in LY294002-treated CEM compared with untreated controls. Levels of actin (Fig. 3*D*) and of the extracellular signal-regulated kinase Erk1 (data not shown) were not affected by the inhibitor. These data suggest that PI 3-kinase not only enhances myogenesis, functioning as a rate-limiting component, but is essential for this differentiation process. This conclusion is supported by experiments with two dominant-negative mutants of the regulatory subunit of PI 3-kinase, p85ΔiSH2-N and p85ΔiSH2-C (43), deletion mutants that lack critical domains required for the interaction of the regulatory subunit p85 with the catalytic subunit p110 of PI

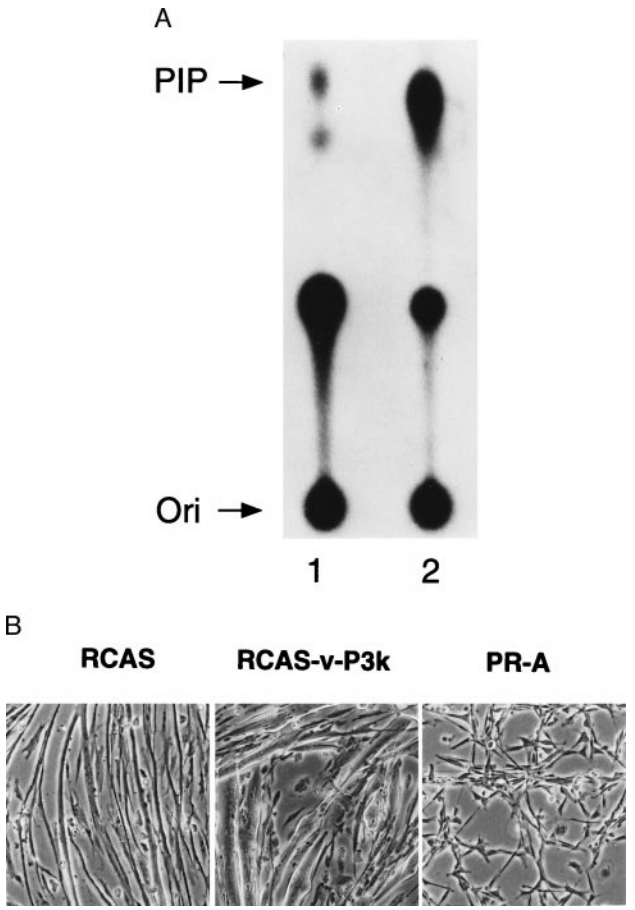

FIG. 1. (*A*) Increase of PI 3-kinase activity in CEM expressing v-P3k. CEM were prepared as described (27) and were infected with the retroviral vector RCAS (lane 1) or with RCAS-v-P3k (lane 2). The cells were cultured for 2 days in MG medium, followed by three days in MD medium as described in *Materials and Methods*. PI 3-kinase activity was assayed *in vitro* as described (29) PIP, phosphatidylinositol 3-phosphate; Ori, origin of the chromatogram. (*B*) Enhancement of myogenic differentiation by v-P3k. CEM were infected with the RCAS vector, RCAS-v-P3k, or Prague strain Rous sarcoma virus subgroup A (PR-A) and cultured as above. Representative fields were photographed 5 days postinfection (×6.5 objective, phase-contrast).

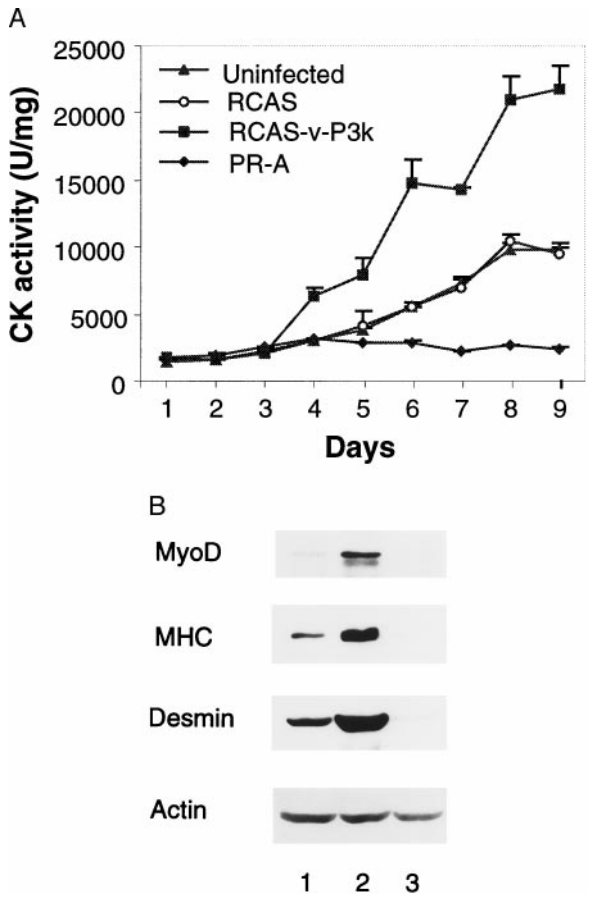

FIG. 2. (*A*) Induction of creatine kinase (CK) activity by v-P3k. Noninfected CEM or CEM infected with RCAS viruses or PR-A as described in Fig. 1 were kept in MG medium for 2 days and thereafter were maintained in MD medium. Cells were harvested at daily intervals, and CK activity was assayed by using a commercial kit (Sigma). Mean ± SE values were obtained from two (uninfected and PR-A-infected) or three (RCAS and RCAS-v-P3k-infected) independent experiments (two replicate plates per experiment). The CK activity is expressed in μmol of creatine formed per min per mg of total protein extract (U/mg). (*B*) Induction of muscle-specific proteins by v-P3k. Total proteins were prepared from CEM at day 8 postinfection with RCAS vector (lane 1), RCAS-v-P3k (lane 2), or PR-A (lane 3). Muscle-specific protein levels were analyzed by immunoblot assay by using antibodies specific for MyoD (Santa Cruz Biotechnology), myosin heavy chain (MHC), and desmin (ICN). Actin served as ubiquitously expressed control was detected with an antibody from Sigma. Similar results were obtained in three independent experiments.

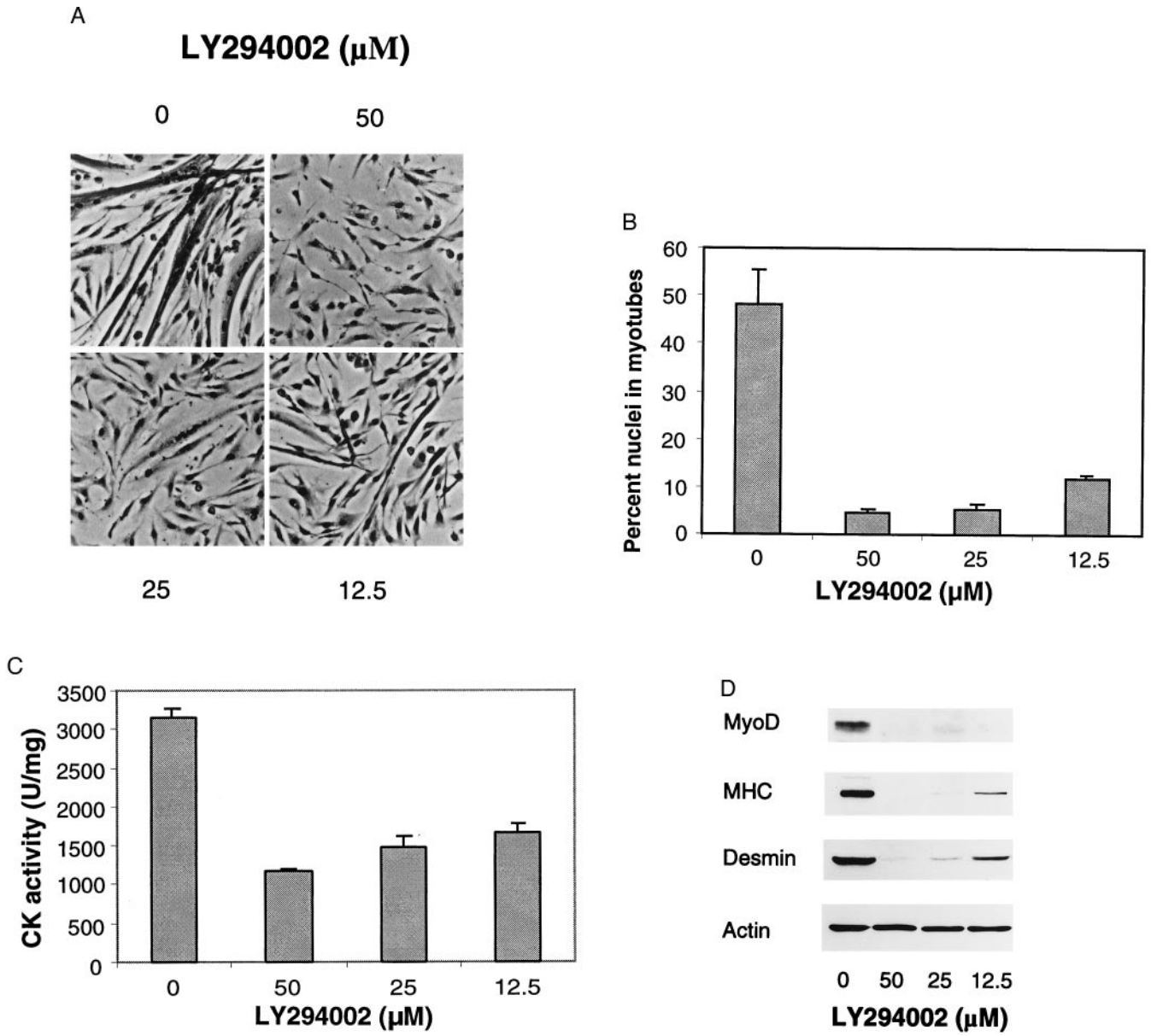

FIG. 3. (*A*) Inhibition of myoblast differentiation by the PI 3-kinase-specific inhibitor LY294002. CEM were cultured in MG medium for 1 day after seeding, and continued to grow for 3 days in MD medium containing the indicated concentrations of LY294002 or dimethyl sulfoxide solvent. Cells were stained on day 4 with the LeukoStat kit (Fisher) to distinguish the cytoplasm and the nucleus, and representative fields were photographed (×16 objective, brightfield illumination). (*B*) Inhibition of multinucleated myotube formation by LY294002. Cells on day 4 were stained as above, and counted under ×16 objective lens to determine the percentage of nuclei in myotubes (containing at least three nuclei per cell). Three independent plates with three random fields per plate were used. (*C*) Inhibition of CK activity by LY294002. CK activity on day 4 was determined in two experiments with three replicate plates in the presence of LY294002 as indicated. (*D*) Inhibition of muscle-specific gene expression by LY294002. Total protein was extracted from CEM treated for 3 days with LY294002 as indicated and processed for immunoblot assay as described in Fig. 2*B*.

3-kinase. The mutants prevent activation of the catalytic subunit by interfering with upstream signals that require docking to p85. Expression of these dominant-negative mutants from the avian replication competent (RCAS) vector inhibited myotube formation in CEM cultures (Fig. 4*A*). It also reduced the levels of CK activity compared with CEM infected with the empty RCAS vector alone (significance was verified by Student's *t* test: $P = 0.008$ for p85ΔiSH2-N and $P = 0.006$ for p85ΔiSH2-C; Fig. 4*B*). In control experiments, RCAS-v-P3k significantly increased CK activity and expression of v-*src* inhibited CK activity (Fig. 4*B*). Levels of other muscle-specific proteins, MyoD, myosin heavy chain, and desmin were also down-regulated by the dominant-negative mutants (Fig. 4*C*). These results suggest that PI 3-kinase is an essential upstream component controlling the expression of MyoD; through the induction of MyoD, it may mediate myogenic differentiation. A recent study on the mechanism by which insulin-like growth factors induce myogenesis in cultures of the rat skeletal-muscle myoblast cell line L6E9 also identified PI 3-kinase as an essential component in this process (46). These results demonstrate a general role of PI 3-kinase in myogenic differentiation in cells of mammalian and avian origin. Constitutively active forms of PI 3-kinase such as v-P3k transform chicken embryo fibroblasts in culture and are oncogenic in the animal (29), yet unlike other oncogenes, they not only fail to interfere with myogenic differentiation, they strongly stimulate it. Although this induction of a differentiation program by an oncogene is uncommon, it is not unprecedented. Expression of oncogenic *ras* in PC12 cells leads to neurite outgrowth, presumably by the tyrosine phosphorylation of mitogenactivated

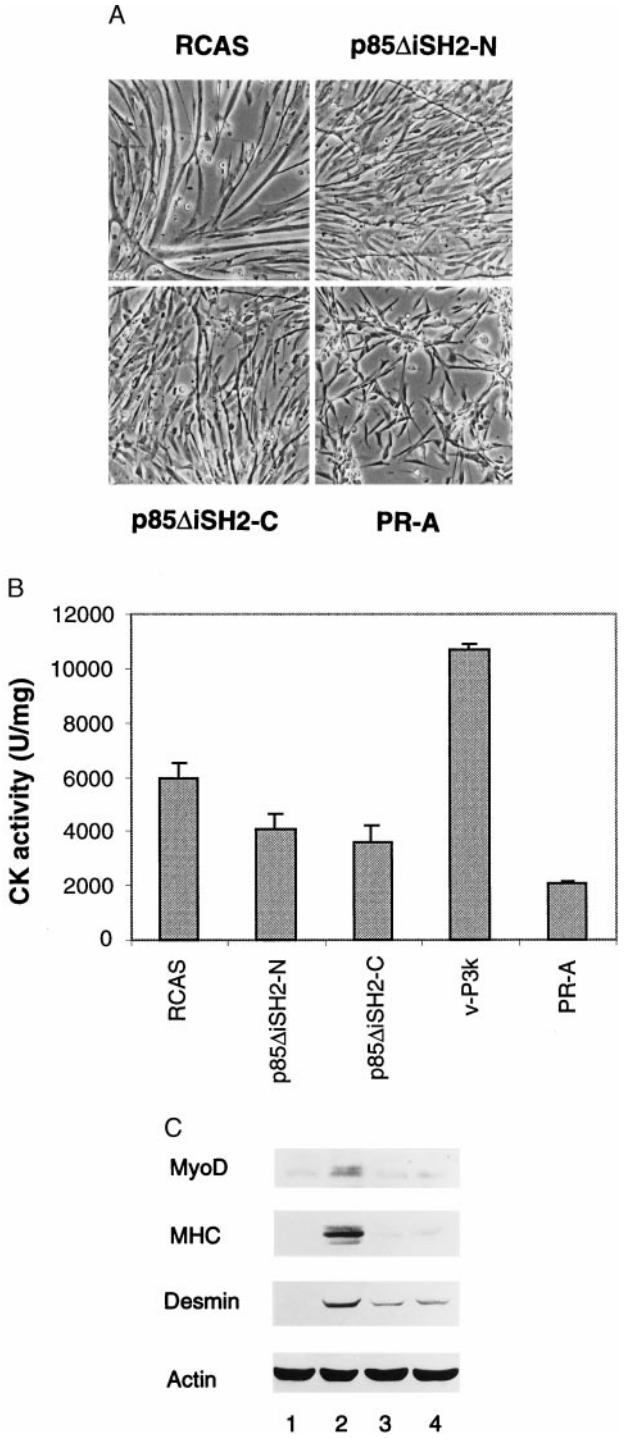

FIG. 4. (*A*) Dominant-negative PI 3-kinase constructs inhibit myogenesis. CEM were infected with RCAS, PR-A, or RCAS viruses expressing two dominant-negative mutants of the regulatory subunit of PI 3-kinase, p85ΔiSH2-N and p85ΔiSH2-C (43). The cells were cultured in MG medium for 3 days postinfection, followed by 2 days in MD medium to induce myoblast differentiation. Representative fields were photographed on day 5 (×6.5 objective, phase-contrast). (*B*) Dominant-negative PI 3-kinase mutants interfere with the induction of CK. CK activity was determined on day 6 in CEM cultures infected as described above or infected with RCAS-v-P3k as a positive control. Mean ± SE values were from two experiments with three replicate assays. (*C*) Dominant-negative PI 3-kinase mutants inhibit the expression of muscle-specific proteins. Muscle-specific protein levels were studied in the cells on day 6 postinfection with PR-A (lane 1), RCAS (lane 2), RCAS-p85ΔiSH2-N (lane 3), or RCAS-p85ΔiSH2-C (lane 4) viruses and assayed by immunoblot as in Fig. 2*B*. Similar results were obtained in three replicate experiments.

protein kinases (47–49). v-*rel* Expression induces the differentiation of P19 embryonal carcinoma cells (50). The retroviral oncogene v-*ski* also activates myogenic differentiation (51–53). Further work must now define all upstream signals that elicit the differentiation-inducing activity of PI 3-kinase and the downstream targets that mediate this activity. The explanation of tissue specificity for this PI 3-kinase signal will be a major challenge.

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