p27\textsuperscript{Kip1} Is Essential for the Antiproliferative Action of 1,25-Dihydroxyvitamin D\textsubscript{3} in Primary, but Not Immortalized, Mouse Embryonic Fibroblasts*

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Received for publication, April 29, 2002, and in revised form, July 16, 2002
Published, JBC Papers in Press, August 5, 2002, DOI 10.1074/jbc.M204162200

1a,25-Dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) inhibits the growth of numerous cancer cell types. The intracellular proteins that mediate 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced growth inhibition are poorly defined, although it is speculated that p21 and p27 are involved. We tested the requirement of p21 and p27 by treating primary wild-type, p21\textsuperscript{-/-}, and p27\textsuperscript{-/-} mouse embryonic fibroblasts (MEFs) with 100 nm 1,25(OH)\textsubscript{2}D\textsubscript{3}. In response to treatment, the wild-type and p21\textsuperscript{-/-} MEFs exhibited 54 and 60% growth inhibition (p < 0.05), respectively, whereas the growth of p27\textsuperscript{-/-} MEFs was unaffected. Western analyses indicated that p27 expression is induced by 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment in wild-type and p21\textsuperscript{-/-} MEFs, although the effect is less robust than for p27. Next, we spontaneously immortalized each MEF strain, which resulted in a gain of responsiveness to 1,25(OH)\textsubscript{2}D\textsubscript{3} by the p27\textsuperscript{-/-} MEFs, as exhibited by 87% growth inhibition (p < 0.05). Both wild-type and p21\textsuperscript{-/-} MEFs retained responsiveness (43 and 72% growth inhibition (p < 0.05), respectively). These data from primary and immortalized MEFs demonstrate that there are both p27-dependent and -independent pathways that mediate the antiproliferative action of 1,25(OH)\textsubscript{2}D\textsubscript{3}.

The observations of increased p21 and p27 mRNA and protein expression after 1,25(OH)\textsubscript{2}D\textsubscript{3} treatment are based on correlative Northern or Western data. There are no genetic data to support a requirement for either protein. In addition, data indicating that 1,25(OH)\textsubscript{2}D\textsubscript{3} induces alterations in p21 and p27 expression are inconsistent among various reports; some researchers demonstrate increases in both CDK inhibitors (2, 13, 14), whereas others demonstrate an increase in one but not the other (3, 12). Different groups working with the same cell type have reported these contradictions, weakening the possibility that cell-type specificity is responsible for these differences (12, 15). Presently, no definitive data exist that support the idea that 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced growth inhibition is caused by p21 and/or p27 increase.

Here, we tested the hypotheses that p21 and p27 are essential mediators of the action of 1,25(OH)\textsubscript{2}D\textsubscript{3}. To test these hypotheses, we used mouse embryonic fibroblasts (MEFs) from wild-type mice or from mice with targeted disruption of either p21 or p27. Our results show that p27, not p21, is essential for the antiproliferative action of 1,25(OH)\textsubscript{2}D\textsubscript{3} in primary, but not immortalized, MEFs. These results indicate that there are p27-dependent and -independent mechanisms involved in the action of 1,25(OH)\textsubscript{2}D\textsubscript{3}.

EXPERIMENTAL PROCEDURES

Mouse Embryonic Fibroblasts—MEFs from inbred 129/sv wild-type and p27 knock-out mice (16), as well as MEFs from Swiss wild-type and p21 knock-out mice (17), were generous gifts of Dr. James Roberts (Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center). We spontaneously immortalized these MEFs following the NIH 3T3 protocol (18). Briefly, cells were split every 3 days and plated...
at $3 \times 10^5$ cells per 60-mm dish. Early passage MEFs were defined as passage 8 or below. Late passage MEFs were defined as passage 20 or greater.

**Single-point Growth Assays**—Cells (1 x $10^4$) were placed in 35-mm dishes with experimental media (Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (both from Invitrogen) and supplemented with either 0.1% ethanol (vehicle control) or 100 nM 1,25(OH)$_2$D$_3$ (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) ($n = 4$). Treatment media were changed every 2 days. When cells on control plates reached 80–90% confluence (7–8 days), plates were washed twice with Hepes-buffered saline, and cells were trypsinized and collected by centrifugation at 3600 x $g$ for 4 min. Pellets were resuspended in trypan blue dye, and cells were counted on a hemocytometer. Live and dead cell numbers were recorded. Total cell number was calculated based on volume in tubes.

**Dose-Response Growth Assays**—Cells (1 x $10^4$) were plated onto twenty 35-mm dishes containing treatment media (four each for 0.1% ethanol, and 0.1, 1, 10, and 100 nM 1,25(OH)$_2$D$_3$). Media were changed every 2 days. When cells on control plates reached 80–90% confluence, cells were harvested and counted as described under “Single-point Growth Assays.”

**Time Course**—Cells were plated at 1 x $10^5$ cells per dish onto 35 35-mm dishes in Dulbecco's modified Eagle's medium/5% fetal bovine serum. The first day after plating, media, washes, and cells from four dishes were collected and counted as described. Twenty-eight dishes were treated with 0.1% ethanol, and 28 dishes with 100 nM 1,25(OH)$_2$D$_3$. Each day thereafter, four dishes of vehicle control and four dishes of 1,25(OH)$_2$D$_3$-treated cells were collected and counted. In some cases in which the cells did not grow as well, the experiment was extended beyond 8 days.

**Western Analysis**—Cells were plated at 1 x $10^5$ cells per 10-cm dish. Once cells reached ~50% confluence, medium containing 0.1% ethanol or 100 nM 1,25(OH)$_2$D$_3$ was added. At the specified time point, plates were washed twice with cold phosphate-buffered saline, then cells were scraped in 1 ml of scraping buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 150 mM sodium chloride with a protease inhibitor mixture (final concentrations: chymostatin, 2 µg/ml; leupeptin, 0.5 µg/ml; soybean trypsin inhibitor I, 50 µg/ml; pepstatin, 1.4 µg/ml; benzamidine, 333 µg/ml)). Cells were collected by centrifugation at 3600 x $g$ for 4 min at 4°C, and pellets were then snap-frozen on dry ice and stored at −80°C until all time points were harvested.

Once time-point harvest was complete, cells were resuspended in lysis buffer (scraping buffer with 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture) and kept on ice for 15 min with occasional vortexing. Lysates were cleared by centrifugation at 12,300 x $g$ at 4°C and then stored at −80°C.

Protein concentration was determined by DC protein assay (Bio-Rad), and 40 µg of protein from each of the samples was resolved on a 15% SDS-PAGE gel. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The membrane was incubated with blocking buffer (5% dry milk in Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature, then incubated with either mouse anti-p21 (PharMingen) (1:1000) or mouse anti-p27 (BD Transduction Laboratories, Lexington, KY) (1:3000) antibodies in blocking buffer for 1 h at room temperature, then incubated with either mouse anti-p21 or p27 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) (1:2000) in blocking buffer for 1 h at room temperature. After washing in wash buffer, the horseradish peroxidase signal was detected using the ECL Plus kit (Amersham Biosciences). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences). Films were scanned with a UMAX Astra 2400 X scanner, transmissive setting at 150 dpi. Band densities were analyzed using NIH Image analysis software version 1.62. Time point expression of p21 or p27 relative to actin expression from wild-type and p27−/− MEFs was calculated. The values were then standardized to the control expression of the indicated protein for each time point.

**Statistics**—Comparisons between groups in single-point growth assays were performed by Student’s $t$ test, and $p \leq 0.05$ was considered significant. Dose response and growth curve results were analyzed by analysis of variance with post-hoc analysis by Scheffe’s F-test, and $p \leq 0.05$ was considered significant.

**FIG. 1.** Early passage wild-type MEFs are growth inhibited by 100 nM 1,25-dihydroxyvitamin D$_3$. A, early passage Swiss wild-type MEFs (passage 5) were inoculated onto 35-mm dishes, treated, and counted 8 days later as described under “Experimental Procedures.” Black bars, 0.1% ethanol; white bars, 100 nM 1,25(OH)$_2$D$_3$. Asterisk denotes statistical significance as shown by Student’s $t$ test ($p \leq 0.05$). B, Western blot analysis of p27 and p21 expression in early passage Swiss wild-type MEF (passage 6). Times in hours are given at the top of the blot. Treatment media: E, ethanol; D, 100 nM 1,25(OH)$_2$D$_3$. C, densitometric analysis of p27 expression relative to actin, normalized to control, from blot in B. D, densitometric analysis of p21 expression from blot in B. Black bars, 0.1% ethanol; white bars, 100 nM 1,25(OH)$_2$D$_3$.
p27Kip1 Is Essential for 1,25(OH)2D3 Action in Primary MEFs

RESULTS

To determine the responsiveness of early passage wild-type MEFs to the active metabolite of vitamin D, 1,25(OH)2D3, we treated Swiss wild-type MEFs with vehicle or 100 nM 1,25(OH)2D3. We observed that these MEFs were 70% growth-inhibited, as shown in Fig. 1A (4.0 + 1.35 x 10^4 cells for 0.1% ethanol and 1.19 + 0.19 x 10^4 cells for 1,25(OH)2D3). We next performed Western analysis to examine the effects of 1,25(OH)2D3 treatment on the expression of the CDK inhibitors p21 and p27. This analysis showed that p27 protein expression is rapidly and transiently induced after 1,25(OH)2D3 treatment, with a greater than 3-fold induction at 1 h (see Fig. 1B and C). The expression of p21 is also transiently induced after treatment, although this induction is modest, with only a 25% increase (see Fig. 1D). These results are consistent with results from three repeated experiments.

Because both p21 and p27 were induced after treatment with 1,25(OH)2D3, we tested the response of MEFs derived from p21−/− or p27−/− mice. After confirming the knock-out status of these MEFs by Western analysis (data not shown), we plated and treated early passage Swiss p21−/− and 129/sv wild-type and p27−/− MEFs with vehicle or 100 nM 1,25(OH)2D3. Fig. 2A demonstrates that 1,25(OH)2D3-treated early passage p21−/− MEFs were 70% growth-inhibited (8.06 + 0.5 x 10^4 cells for 0.1% ethanol, 2.39 + 0.11 x 10^4 cells for 100 nM 1,25(OH)2D3). Fig. 2A also demonstrates that both wild-type MEF strains (Swiss and 129/sv) are growth-inhibited by 1,25(OH)2D3. In contrast, p27−/− MEFs are not.

FIG. 2. Early passage wild-type and p21−/− MEFs are growth-inhibited by 1,25(OH)2D3, whereas p27−/− MEFs are not. A, Swiss wild-type (passage 0), p21−/− (passage 5), 129/sv wild-type (passage 3), and p27−/− (passage 4) MEFs were plated, treated, and counted as described in Fig. 1. Black bars, cell numbers treated with 0.1% ethanol; white bars, cell numbers treated with 100 nM 1,25(OH)2DH11002. Asterisk denotes statistical significance as determined by Student’s t test (p ≤ 0.05). B, dose response of early passage wild-type (square) and p21−/− MEFs (triangle) (same passage numbers as in A). Asterisk denotes dose at which growth of MEFs became statistically significant from 0.1% ethanol as analyzed by Scheffe’s F test.

Time-course experiments demonstrate that growth rates of control and 1,25(OH)2D3-treated early passage wild-type (Swiss) and p21−/− MEFs become significant at days 6 and 4, respectively (data not shown). At later time points, live cell number in control groups increases rapidly, whereas that of 1,25(OH)2D3-treated groups increases but at a much slower rate. Although significant growth occurred during the duration of the experiment, the growth rates of control versus 1,25(OH)2D3-treated p21−/− MEFs were not different at any time point measured (data not shown). In all of the above trypan blue assays, the number of dead cells was consistently between 5 and 10% of the number of live cells for both control and 1,25(OH)2D3-treated cells for all MEF types (data not shown). Also, all MEFs expressed nuclear vitamin D receptors
as was shown by immunofluorescence (data not shown).

We performed Western analysis of p27 expression in the growth-inhibited p21\textsuperscript{KO}/H11002/MEFs. Like the wild-type (Swiss) MEFs, we observed a rapid and transient increase in p27 expression in 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated p21\textsuperscript{KO}/H11002/MEFs. There was a 2-fold induction at 2 h of treatment (data not shown). Upon investigation of p21 expression in the non-growth-inhibited p27\textsuperscript{KO}/H11002/MEFs, we observed a 2-fold induction at 10 h of treatment as shown in Fig. 3.

With the intention of expanding our supply of MEFs for future experiments, we spontaneously immortalized these Swiss wild-type, p21\textsuperscript{KO}/H11002/ and p27\textsuperscript{KO}/H11002/ MEFs using the NIH 3T3 protocol. Knock-out status of late passage MEFs was confirmed by Western analysis (data not shown). Upon analysis of the immortalized MEF response to 100 nM 1,25(OH)\textsubscript{2}D\textsubscript{3}, we observed that wild-type and p21\textsuperscript{KO}/H11002/MEFs retained their responsiveness to 1,25(OH)\textsubscript{2}D\textsubscript{3} as shown by 43 and 72% growth inhibition, respectively (see Fig. 4A). Surprisingly, immortalized p27\textsuperscript{KO}/H11002/MEFs gained responsiveness to 1,25(OH)\textsubscript{2}D\textsubscript{3}, as shown by an 87% growth inhibition. Dose-response assays demonstrate that growth inhibition of late passage wild-type MEFs was significant at 10 nM 1,25(OH)\textsubscript{2}D\textsubscript{3}, whereas the growth inhibition of late passage p21\textsuperscript{KO}/H11002/ and p27\textsuperscript{KO}/H11002/ MEFs was significant at 1 and 100 nM, respectively (see Fig. 4B). Time course

as was shown by immunofluorescence (data not shown).

We performed Western analysis of p27 expression in the growth-inhibited p21\textsuperscript{KO}/H11002/MEFs. Like the wild-type (Swiss) MEFs, we observed a rapid and transient increase in p27 expression in 1,25(OH)\textsubscript{2}D\textsubscript{3}-treated p21\textsuperscript{KO}/H11002/MEFs. There was a 2-fold induction at 2 h of treatment (data not shown). Upon investigation of p21 expression in the non-growth-inhibited p27\textsuperscript{KO}/H11002/MEFs, we observed a 2-fold induction at 10 h of treatment as shown in Fig. 3.

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assays show that differential growth between control and treated curves is apparent in all three cell lines at day 4 (see Fig. 4C). Video time-lapse microscopy analysis of 1,25(OH)2D3-treated late passage p27/Kip1−/− MEFs showed no indication of apoptosis (data not shown).

We then performed Western analysis of p21 expression in control and treated samples of late passage, spontaneously immortalized p27/Kip1−/− MEFs (see Fig. 5, A and B). We observed that there was a 1.5–2-fold increase in p21 expression after treatment. This induction was observed for at least 12 h of treatment.

**DISCUSSION**

Although the inhibitory effects of 1,25(OH)2D3 treatment on cell growth have been widely published, the mechanisms that mediate those effects remain elusive. Using mouse knock-out models, we definitively showed here that p27 was one essential mediator of 1,25(OH)2D3 growth-inhibitory action. Data from single-point and dose-response growth assays definitively demonstrated that p27 expression was essential for the antiproliferative action of 1,25(OH)2D3 in early passage MEFs. Loss of p21 expression did not affect the ability of early passage MEFs to be growth-inhibited by 1,25(OH)2D3.

Wild-type (Swiss), p21−/−, and p27−/− early passage MEFs were also treated with increasing doses of the soy isoflavone genistein, another known antiproliferative agent (19). We observed that all cell types responded to genistein in a dose-dependent manner (data not shown). These data strengthen the argument that the p27−/− MEFs were unable to respond to 1,25(OH)2D3-induced growth inhibition because they lacked p27 not because of some intrinsic inability to respond to antiproliferative agents.

Western analysis data showed rapid and transient induction of p27 expression in wild-type (Swiss) and p21−/− MEFs. The greatest induction of p27 occurred at 1 h in the wild-type and 2 h in the p21−/− MEFs, suggesting that a rapid event led to an increase in the intracellular level of p27. Given the short time period, it is unlikely that this increase was due solely to an increase in transcription and/or translation of p27. We presume that other events, such as decreased degradation of p27 or nongenomic vitamin D-dependent effects, were responsible for the increase in p27 protein expression. These nongenomic effects have been reported to include rapid transport of calcium through membrane channels and activation of mitogen-activated protein kinase and protein kinase C (20). These rapid actions may be mediated by a membrane-bound vitamin D receptor that differs from the nuclear vitamin D receptor. These actions may be involved in cross-talk with other signaling pathways that affect cellular proliferation and differentiation (21).

Based on these Western and growth assay data, we predict that the antiproliferative action of 1,25(OH)2D3 might be mediated by the early increase in p27 expression. Because the differential growth of treated and untreated cells did not become apparent until days 3–5 of treatment in wild-type and p21−/− MEFs, it is likely that induction of p27 might be one of the initial steps of mechanistic action stimulated by treatment with 1,25(OH)2D3. The proteins and steps involved after p27 induction remain to be defined in the MEF model. We speculate that increased availability of p27 leads to increased inhibition of Cdk2 activity, which would lead to decreased phosphorylation of proteins such as pRb. These events would culminate in decreased cell proliferation.

We also demonstrated a modest, transient increase in p21 expression in growth-inhibited wild-type MEFs as well as an increase in the non-growth-inhibited p27−/− MEFs. The increase in p21 expression in p27−/− MEFs whose growth was not affected by 1,25(OH)2D3 demonstrated that correlative data may not always distinguish between cause and effect. Our approach highlights the value of using genetic models, as opposed to correlative data, to determine the essential roles of potential mediators of 1,25(OH)2D3 action.

Growth assays using the late passage (spontaneously immortalized) MEFs demonstrated that neither p27 nor p21 alone was essential for the antiproliferative action of 1,25(OH)2D3 in these MEFs. The gain of responsiveness of the spontaneously immortalized p27−/− MEFs demonstrated that there were also p27-independent mechanisms that mediated the antiproliferative action of 1,25(OH)2D3. Over the course of immortalization, other undefined mechanisms emerged that had the ability to mediate 1,25(OH)2D3 action. In light of the induction of p21 expression in these p27−/− MEFs, it is possible that a p21-dependent mechanism for 1,25(OH)2D3-induced growth inhibition emerged in these cells to compensate for the loss of p27. This phenomenon of compensation has been observed in other models. In serum-starved p21/p27 double knock-out MEFs, p130, a pRB-related protein, is able to compensate for the loss of these proteins to elicit the antiproliferative response (22).

Further investigation is necessary to determine the cause of this gain-of-responsiveness to 1,25(OH)2D3 in the late passage p27−/− MEFs.

The disparity between the resulting effects on the growth of early and late passage p27−/− MEFs raises concerns about key issues in the area of cancer therapeutic research. Foremost is that most research is performed in established cell lines. Based on the data presented here, it is possible that the response of primary cells is different from that of established cell lines from the same tissue type. Second, the observation that both primary and immortalized MEFs respond to 1,25(OH)2D3 suggests that vitamin D growth inhibition can be mediated via multiple, perhaps redundant, mechanisms in untransformed and transformed tissues. This conclusion further supports the use of noncalcemic vitamin D analogues as drugs for both the prevention and the treatment of various cancers.

In conclusion, we showed definitively that p27 was essential for the antiproliferative action of 1,25(OH)2D3 in early passage MEFs. We also showed that both p27 and p21 were transiently induced by 1,25(OH)2D3 treatment. Spontaneous immortalization of these MEFs resulted in a gain of responsiveness of p27−/− MEFs to 1,25(OH)2D3. These data demonstrate the existence of both p27-dependent and -independent mechanisms, which may include the induction of p27 protein, that mediate the antiproliferative action of 1,25(OH)2D3 in early passage and spontaneously immortalized MEFs.

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