Prostacyclin has many effects in the vasculature; one of the less well understood is the ability to block cell cycle progression through G1 phase. We previously reported that the prostacyclin mimic, cicaprost, selectively inhibits cyclin E-cyclin-dependent kinase-2 (Cdk2), and now we show that it acts by regulating the expression of Skp2, the F-box protein that targets cyclin D1 in mesenchymal cells. In contrast, cyclin E is expressed throughout the G1 phase, and the activation of Cdk2 is typically regulated by the rate at which Cdk2 inhibitors, p21\(^{Cip1}\) and p27\(^{Kip1}\), are down-regulated (9). Once activated, cyclin D-Cdk4/6 and cyclin E-Cdk2 phosphorylate the pocket protein family of transcriptional regulators (pRb, p107, p130), which in turn modulate the activation of E2F-dependent genes such as cyclin A (10). cyclin A is induced at the G1/S interface, and the consequent formation of cyclin A-Cdk2 complexes marks entry into the S phase of the cell cycle.

We recently reported that the prostacyclin mimic, cicaprost, inhibits G1 to S phase entry in aortic smooth muscle cells by inhibiting CREB- and pocket protein-dependent cyclin A gene expression (11). The inhibitory effect of cicaprost on pocket protein phosphorylation was associated with a selective inhibition of cyclin E-Cdk2 activity; the induction of cyclin D1 and the activation of cyclin D-Cdk4 were unaffected by cicaprost. Cicaprost did not affect the levels of cyclin E or Cdk2, but we were unable to define the molecular mechanism underlying the inhibitory effect of cicaprost on cyclin E-Cdk2 activity, probably because the smooth muscle cells used in that study (primary murine smooth muscle cells and the A10 smooth muscle cell line) do not cycle efficiently. In this study, we used established mouse embryonic fibroblasts (MEFs) to show that cicaprost exerts its inhibitory effect on cyclin E-Cdk2 activity by specifically regulating the expression of p27\(^{Kip1}\). Furthermore, we link this effect on p27\(^{Kip1}\) to a cicaprost-mediated block in expression of the Skp2 gene. The Skp2 gene encodes the substrate-targeting component of the E3 ubiquitin ligase complex that degrades p27\(^{Kip1}\) (12, 13). These data identify a new mechanism for antimitogenesis with potential implications for cardiovascular disease.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Spontaneously immortalized embryonic fibroblasts from wild-type and p27\(^{Kip1}\)−/− mice were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). In cell cycle experiments, near confluent monolayers of MEFs were G0/G1 phase arrested by serum starvation for 36 h. Serum-starved transfectants were trypsinized, reseeded (~10\(^6\) cells per 10 ml of 10% FBS-Dulbecco’s modified Eagle’s medium) into 100-mm tissue culture dishes, and incubated in the absence or presence of 200 nM cicaprost. For HA-Skp2 overexpression studies, MEFs were transfected as described (14) using LipofectAMINE Plus and 5 μg of either pcDNA3.1 (vector control) or a pcDNA3.1-based HA-tagged mouse Skp2 expression vector (HA-Skp2). After an overnight recovery, the cells were serum-starved for 36 h. Serum-starved transfecants were trypsinized, reseeded (~10\(^6\) cells per 10 ml of 10% FBS-Dulbecco’s modified Eagle’s medium) into 100-mm culture dishes, and incubated in the absence or presence of 200 nM cicaprost. Cicaprost was the generous gift of Garret FitzGerald.

To monitor entry into S phase, quiescent MEFs were added to 100-mm (~10\(^6\)) dishes containing autoclaved glass coverslips and incubated in 2 ml of Dulbecco’s modified Eagle’s medium, 10% FBS, 3 μg/ml BrdUrd (Amer sham Biosciences) in the absence or presence of...
200 nM cicaprost. Formaldehyde-fixed cells were incubated with anti-BrdUrd as described (11). Approximately 200 cells in 3–4 fields of view were counted per sample to determine the percent of BrdUrd-labeled nuclei. Results shown are typically representative of at least three independent experiments.

Cyclin A promoter-luciferase assays were performed as described (11) using the p434/cyclin A promoter-luciferase expression vector (bases −270 to +164 of the human cyclin A promoter cloned upstream of a firefly luciferase reporter (15)), the Renilla luciferase vector (pRL-CMV), and an E7 expression vector. Results shown are representative of three independent experiments.

**Western Blotting and Cyclin E-Cdk2 Kinase Assays**—Western blotting was performed as described (14) using 75–100 µg of total cellular protein and the following antibodies: Cdk4 (sc-280, Santa Cruz Biotechnology or DCS-31, BioSource), cyclin D1 (sc-8396, Santa Cruz Biotechnology), p27Kip1 (sc-7252, Transduction Laboratories), p21Cip1 (sc-6246, Santa Cruz Biotechnology), cyclin E (sc-481, Santa Cruz Biotechnology), Cdk2 (06-505, Upstate Biotechnology), pRb (28-007, Zymed Laboratories Inc.), pAKT (9271S, Cell Signaling), pERK (9101S, Cell Signaling) and Skp2 (32-3400, Zymed Laboratories Inc.). Rabbit polyclonal cyclin A antibody was prepared in our laboratory using recombinant cyclin A as an immunogen. Cyclin E-Cdk2 kinase assays were performed by incubating identically prepared lysates (150 µg) with anti-cyclin E (sc-481, Santa Cruz Biotechnology) as described previously (18); reaction products were analyzed by SDS gel electrophoresis, autoradiography, and immunoblotting with anti-Cdk2 (06-505, Upstate Biotechnology). Results shown are typically representative of at least three independent experiments.

**Quantitative Real Time Reverse Transcrip-tase-PCR**—Quiescent MEFs were trypsinized, replated at subconfluence (∼5 × 10⁵ cells in 5 ml of medium per 60-mm culture dish) and incubated in 10% FBS in the absence or presence of 200 nM cicaprost for selected times. Cells were washed with phosphate-buffered saline and lysed in 1 ml of TRIzol (Invitrogen) per 60-mm dish to extract total RNA. Total RNA (∼400 ng) from each sample was used for cDNA synthesis using reverse transcription reagents from Applied Biosystems Inc. (5.5 mM MgCl₂, 2 mM dNTP, 2.5 µg oligo(dT) for 2.5 µg random hexamers, 8 units of RNase A, and 25 units of reverse transcriptase per 20 µl of reaction.) The reverse transcription was performed separately from the PCR reaction. An aliquot (10%) of cDNA was then subjected to 40 amplification cycles of PCR (Applied Biosystems Prism 7000 sequence detection system) using Taq-man universal PCR master mix. For mouse Skp2, the real-time PCR reaction contained 200 nM forward primer 5′-ATC ACT GAG TTC GAC AGG TCC AT, 900 nM reverse primer 5′-GCC GTC TGA ACA TCC CAA T, 900 nM reverse primer 5′-GTC GTT CGG TGA CCA A, and 250 nM MGB probe VIC-ATC CAT CAG TCG TAC A-MGB-NFQ. Each sample was analyzed in duplicate, and mRNA expression was quantified using ABI Prism 7000 sequence detection system software. Mean quantities were calculated from duplicate samples. Skp2 mRNA expression was normalized to Cdk4 mRNA expression, which did not vary reproducibly during serum stimulation or cicaprost treatment. Results shown are representative of three independent experiments.

**Results**

Cicaprost Inhibits S Phase Entry in MEFs by Selectively Regulating p27Kip1—We reported previously that cicaprost selectively inhibited the activation of cyclin E-Cdk2 in vascular smooth muscle cells. Yet levels of cyclin D1, the activation of Cdk4, and the levels of cyclin E and Cdk2 were unaffected (11). However, inefficient cycling of those cells prevented us from characterizing the responsible mechanism in further detail. Therefore we tested the effect of cicaprost in established MEFs, which efficiently re-enter G1 phase from quiescence (Fig. 1A, control). Cicaprost strongly inhibited S phase entry in MEFs (Fig. 1A) with a dose response that was similar to that of vascular smooth muscle cells (IC₅₀ ∼30 nM; data not shown). Moreover, cicaprost blocked the phosphorylation of pRb and the induction of cyclin A in MEFs (Fig. 1B) in a manner very similar to that seen in vascular smooth muscle cells (11). However, unlike its effects in vascular smooth muscle cells (11), cicaprost did not regulate CREB-dependent cyclin A gene expression in MEFs and did not inhibit the phosphorylation of CREB or the binding of CREB and phospho-CREB to the cyclin A CRE (supplemental Fig. 1, A and B, respectively). Moreover, the inhibitory effect of cicaprost on wild-type cyclin A promoter activity in MEFs was overcome by ectopic expression of human papillomavirus E7, a protein that sequesters pocket proteins and mimics inactivation by the G1 phase Cdks (Fig. 1C). E7 expression fails to overcome the inhibitory effect of cicaprost on cyclin A promoter activity in vascular smooth muscle cells unless the CRE-inhibitory effect of cicaprost is first eliminated by the use of a CRE-deleted or mutated promoter (11). Thus, the antimitogenic effect of cicaprost on pocket protein phosphorylation is conserved in these mesenchymal cells whereas the antimitogenic effect on CREB is not.

As with vascular smooth muscle cells, cicaprost did not affect the levels of cyclin E and Cdk2 in MEFs (Fig. 2A) but strongly inhibited the late G1 phase activation of cyclin E-Cdk2 as determined by both the gel shift of Cdk2 (a hallmark of cdk-activating kinase mediated Cdk2 activation; Fig. 2A) and in vitro kinase assays (Fig. 2B). Cicaprost also blocked the down-regulation of p27Kip1 but did not affect the G1 phase levels of p21Cip1 (Fig. 2A). The absence of an effect on p21Cip1 suggested...
that the antimitogenic effect of cicaprost was not caused by mere inhibition of mitogenic signaling, and indeed we found that cicaprost did not inhibit activation of several well established mitogenic targets, including ERK1/2, AKT, and cyclin D1 (Fig. 2C). Finally, the effects of cicaprost on p27Kip1 levels were causally related to its antimitogenic effect; cicaprost strongly blocked cyclin E-Cdk2 activity, cyclin A induction, and S phase entry in wild-type MEFs but not in MEFs lacking p27Kip1 (Fig. 3, A and B). Overall, these results show that a selective effect of cicaprost on the levels of p27Kip1 is causally related to its antimitogenic effect on S phase entry.

**Cicaprost Inhibits Expression of the Skp2 Gene**—One of the most common mechanisms for regulating p27Kip1 involves its phosphorylation at Thr-187 by cyclin E-Cdk2, an event that targets p27Kip1 for degradation by the SCF ubiquitin-ligase complex (17, 18). The substrate-targeting component of the SCF complex that is required for the ubiquitin-mediated degradation of p27Kip1 is the F-box protein, Skp2 (12, 13). Skp2 is poorly expressed in quiescent cells, and its expression is stimulated by mitogens during late G1 and S phase (12, 19) (Fig. 4, A and B; control). Cicaprost blocked the serum-stimulated expression of Skp2 (Fig. 4A), and again this effect was not caused by an overall inhibition of mitogenic signaling (Fig. 2C).

Recent reports have identified the anaphase promoting complex (Cdh1) as a major regulator of Skp2 proteolysis as cells progress from mitosis into G1, as well as when cells are serum-starved to enter quiescence (20, 21). Although these results raised the possibility that cicaprost could regulate Skp2 levels post-transcriptionally by regulating anaphase promoting complex (Cdh1), quantitative real-time PCR showed that cicaprost strongly inhibited the induction of Skp2 mRNA (Fig. 4C). Moreover, we could rescue the expression of Skp2, the down-regulation of p27Kip1, the induction of cyclin A, and entry into S phase in cicaprost-treated cells by transfecting cells with a Skp2 cDNA and enforcing the expression of ectopic Skp2 at near normal levels (Fig. 5, A and B). The rescue of ectopic Skp2 protein expression in cicaprost-treated cells would be highly unlikely to occur if cicaprost strongly stimulated Skp2 proteolysis. Thus, our data indicate that prostacyclin is antimitogenic because it inhibits gene expression of the F-box protein that controls the degradation of p27Kip1. Note that MEFs expressing HA-Skp2 have reduced levels of endogenous Skp2 (Fig. 5A) consistent with a study indicating that Skp2 itself is a target of a Skp2-containing SCF complex (22).

**8-bromo-cAMP Induces G1 Arrest without Affecting Skp2**—Cicaprost binds to the IP receptor, a heterotrimeric G-protein-coupled receptor that canonically couples to Gs and increases cAMP (23). Because cAMP can increase p27Kip1 levels and inhibit S phase entry (24), we asked whether the effect of cicaprost on Skp2 could be recapitulated merely by increasing
cAMP. Consistent with previous studies (24), the cAMP analog, 8-bromo-cAMP, increased p27Kip1 levels, inhibited the induction of cyclin A, and blocked S phase entry in a dose-dependent manner (Fig. 6, A and B). However, this effect on p27Kip1 was not accompanied by a decreased expression of Skp2 (Fig. 6A). Similar results were seen with forskolin (data not shown). These results show that the antimitogenic effect of cicaprost on Skp2 expression is not a general response to increased cAMP levels and indicate that the effects of prostacyclin and cAMP on p27Kip1 are mechanistically distinct. They also emphasize that the inhibitory effect of cicaprost on the expression of Skp2 is not merely a secondary consequence of G1 phase arrest; MEFs treated with 1 mM 8-bromo-cAMP are arrested in G1 phase yet still express Skp2 (Fig. 6, A and B).

DISCUSSION

Our results show that prostacyclin exerts its antimitogenic effects by selectively regulating p27Kip1. We first showed that cicaprost acts through a pocket protein-dependent pathway to inhibit cyclin A promoter activity. Moreover, cicaprost inhibited the activation of cyclin E-Cdk2 and the down-regulation of p27Kip1 that accompanies cell cycle progression through G1 phase, and these antimitogenic effects of cicaprost on cyclin E-Cdk2 activity, cyclin A induction, and S phase entry are overcome by deleting p27Kip1. Interestingly, p27Kip1-null MEFs retain the ability to undergo G1 arrest in response to contact inhibition, serum starvation, and /H9253-irradiation (25). Thus, although the inhibitory effect of p27Kip1 can be compensated for in these settings, p27Kip1 is essential for the antimitogenic effect of cicaprost.

Our data also indicate that prostacyclin regulates p27Kip1 by controlling the expression of the F-box protein, Skp2. Cicaprost blocked Skp2 gene expression, and forced expression of Skp2 rescued p27Kip1 down-regulation and S phase entry in cicaprost-treated cells. In addition to p27Kip1, others have reported that Skp2 targets cyclin E, p21Cip1/p130, c-Myc, and p57Kip2 for proteolysis (26–30). We saw no effect of cicaprost on p21Cip1 or cyclin E under conditions in which the induction of Skp2 was clearly inhibited. Although the basis for these different results is not completely clear, SCFSkp2 primarily targets free cyclin E for degradation. In our established MEFs (26), most of the cyclin E may be complexed to Cdk2. Moreover, the effect of SCFSkp2 on p21Cip1 degradation was studied in S phase (28); our study focuses on G1 phase regulation.

Ubiquitin-mediated proteolysis appears to be the most common mechanism regulating p27Kip1 levels during G1 phase progression (31), but p27Kip1 levels can also be regulated by an
Prostaglandins Regulate Skp2 Gene Expression

8-Bromo-cAMP does not block Skp2 induction in MEFs. Quiescent MEFs were trypsinized, reseeded at subconfluence into 100-mm dishes containing coverslips, and stimulated with 10% FBS in 100-mm dishes containing coverslips, and stimulated with 10% FBS in

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