Ca^{2+} entry (*14*), but activation of phospholipase A_2 (which occurs well after DAG generation) (*33*) and membrane fusion (the final step in the sequence) require higher concentrations of intracellular Ca^{2+} (*14*, *22*). We therefore propose that small amounts of progesterone may trigger intracellular Ca^{2+} rises (*5*) that are sufficient for DAG generation, but that ZP action is necessary for a further rise in Ca^{2+} and for activation of late processes in the sequence underlying acrosomal exocytosis.

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11. Cauda epididymal spermatozoa from outbred TO mice (Harlan-Olac, UK), capacitated in vitro in a modified Tyrode's medium (*23*) for 1.5 hours at 37°C under 5% CO_2 and 95% air, were stimulated with progesterone (in dimethylsulfoxide) or heat-solubilized ZP (*2*, *3*). Acrosomal exocytosis (expressed as percent of AR pattern) was monitored by means of a chlortetracycline staining method (*2*) and phase contrast microscopy. For lipid studies, spermatozoa were capacitated in vitro without a label or with 150 μCi of [^{32}P]P_i per milliliter (*34*) or 2 μCi of [^{3}H]alkyl-lysoPC per milliliter (*35*) and were then diluted 1:1 with Hepes-buffered Tyrode's medium, centrifuged for 10 min at 600*g*, resuspended in the latter medium, and stimulated with progesterone or ZP or both for various times. Lipids were extracted, separated, and quantified as previously described (*13*, *18*, *34*, *35*). Departures from these procedures or use of other agonists or antagonists are indicated where applicable. Data were transformed [arcsin $\sqrt{(\text{percent of AR cells} \div 100)}$; $\log_{10}$ of metabolites] and analyzed with Student's *t* test, or were analyzed with the Mann-Whitney U test or a two-factor analysis of variance.
12. Only capacitated spermatozoa underwent exocytosis in response to progesterone; spermatozoa so treated exhibited a motility that was similar to that seen in untreated controls (inclusion of solvent alone). Neither 17β-estradiol nor testosterone (15 μM) induced acrosomal exocytosis or generation of DAG; the steroids did not affect cell motility.
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27. GABA exerted maximal stimulation at 0.5 μM; higher concentrations showed lesser increases in the number of cells undergoing exocytosis. The use of half-maximal concentrations of GABA (0.1 μM) and progesterone (2.5 μM) showed additive effects, which is indicative of a GABA-like receptor on the sperm surface. However, the maximized response seen with 0.5 μM GABA was further enhanced by inclusion of progesterone (Table 3), which suggests that the steroid may act on an additional membrane site.
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Stimulation and Inhibition of Angiogenesis by Placental Proliferin and Proliferin-Related Protein

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In many mammalian species, the placenta is the site of synthesis of proteins in the prolactin and growth hormone family. Analysis of two such proteins, proliferin (PLF) and proliferin-related protein (PRP), revealed that they are potent regulators of angiogenesis; PLF stimulated and PRP inhibited endothelial cell migration in cell culture and neovascularization in vivo. The mouse placenta secretes an angiogenic activity during the middle of pregnancy that corresponds primarily to PLF, but later in gestation releases a factor that inhibits angiogenesis, which was identified as PRP. Incubation of placental tissue with PLF led to the specific binding of this hormone to capillary endothelial cells. Thus PLF and PRP may regulate the initiation and then the cessation of placental neovascularization.

In mammalian reproduction, the placenta mediates the implantation of the embryo and exchange of nutrients and wastes between the mother and the fetus, prevents attack on the fetus by the maternal immune system, and regulates both maternal and fetal physiology through the secretion of hormones (*1*). Among the hormones secreted by the placenta are proteins in the prolactin and growth hormone family (*2*). In the mouse, these placental proteins include placental lactogen I and II, which bind to the prolactin receptor and have prolactin-like bioactivity, and two other proteins, PLF (*3*, *4*) and PRP (*5*, *6*). PLF was discovered as a serum growth factor–inducible mRNA (*7*, *8*) and protein (*9*) in mouse fibroblasts, and expression of PLF in muscle cells can inhibit muscle cell–specific gene expression and differentiation (*10*, *11*). The PRP mRNA was detected as a placental complementary DNA (cDNA) clone that cross-hybridized to the PLF cDNA (*5*), but the secreted forms of the encoded proteins share only 30% amino acid sequence identity.

A proteolytically cleaved form of prolactin, designated 16-kD prolactin, can bind to endothelial cell receptors distinct from the prolactin receptor and inhibit angiogenesis (*12*). Among the members of the prolactin and growth hormone family, PLF and PRP are the most efficient for competing with 16-kD prolactin for binding to endothelial cells (*12*). In addition, although PLF has no prolactin-like effect in stimulating the growth of the pigeon crop sac, it does result in inflammation and vascularization at the site of injection (*13*). Thus, PLF and PRP might participate in the regulation of an-

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giogenesis. We report here that both of these molecules modulate the activity of endothelial cells in vitro and neovascularization in vivo.

PLF (*14*) stimulated the migration of bovine capillary endothelial cell cultures (*15*) in a Boyden chamber assay in a dose-dependent manner (Fig. 1A). The concentration of PLF required for half-maximal stimulation was approximately 600 ng/ml (27 nM), a concentration less than the highest concentration of PLF found in maternal serum (3 μg/ml, or 136 nM) (*4*). In contrast, PRP (*14*) failed to stimulate endothelial cell migration but did prevent cell migration induced either by basic fibroblast growth factor (bFGF) (Fig. 1B) or by PLF (Fig. 1C). The inhibitory activity was half-maximal at a PRP concentration of 100 ng/ml (4 nM), which is less than the maximal concentration of PRP present in maternal serum (3 μg/ml, or 125 nM) (*16*). In a rat cornea assay (*17*), both proteins gave results consistent with those from the cell migration assay (Table 1): PRP alone had no effect on blood vessels in the cornea (Fig. 2A); both bFGF (Fig. 2B) and PLF (Fig. 2C) stimulated blood vessel growth; and PRP inhibited neovascularization caused by bFGF (Fig. 2D) or PLF. Thus, PLF stimulated angiogenesis in vivo and activated endothelial cells directly in culture in the absence of any accessory cells, whereas PRP inhibited angiogenesis.

In the pregnant mouse, placental synthesis and secretion of PLF are maximal at midgestation (day 10) and decline by days 13 to 14 (*4*). Synthesis and secretion of PRP peak later, on day 12 or 13, but the concentration of this hormone remains above 2 μg/ml in serum until birth (*16*). Analysis of short-term cultures of placental tissue (*18*) collected from day 8 through day 18 of the 19- to 20-day mouse gestational period revealed a peak of secreted angiogenic activity at day 10 (Fig. 3A). From day 12 through day 18, this stimulatory activity was not evident, and instead a factor that inhibited endothelial cell response (anti-angiogenic) to bFGF was secreted (Fig. 3A). The angiogenic and anti-angiogenic activities secreted from tissue isolated from animals on day 10 or 12 of pregnancy were also detected in the in vivo assay of the rat cornea (Table 1).

The midpregnancy (day 10) angiogenic activity could be attributed primarily to PLF; a monoclonal antibody to PLF that neutralized the activity of the purified PLF protein (Fig. 3B) also reduced the activity released into the culture medium by placental tissue isolated on day 10 (Fig. 3D). The residual angiogenic activity was blocked with an antibody to scatter factor (hepatocyte growth factor) (*19*), which indicates that PLF and scatter factor, together, comprise most of the angiogenic activity detected in the day 10 sample. Similarly, the anti-angiogenic factor secreted from placental tissue isolated on day 12 of pregnancy could be attributed to PRP, because an affinity-purified antiserum to PRP (*20*) blocked the inhibitory effect of the purified hormone (Fig. 3C) and of the medium in which placental tissue isolated on day 12 was cultured (Fig. 3E). These data do not rule out the possibility that other angiogenic or anti-angiogenic factors are also released by the placenta, but indicate that any other secreted, soluble proteins that act directly on endothelial cells are likely to represent relatively minor activities.

PLF is a circulating hormone and therefore has the potential to act at numerous target tissues. However, it is possible that PLF also acts locally as a paracrine regulator of the extensive neovascularization that occurs in the developing placenta. Indeed, the timing of PLF expression coincides with the development of the labyrinth, the region of the placenta in which blood vessels form (*1*). Support for a direct role of PLF in placental angiogenesis was obtained by incubation of ^{125}I-labeled PLF with placental sections (*21*), which revealed binding sites for PLF on labyrinth blood vessels (Fig. 4). The binding was specific and saturable; binding of radiolabeled PLF was blocked by the simultaneous incubation of sections with excess, unlabeled PLF (Fig. 4, A and B).

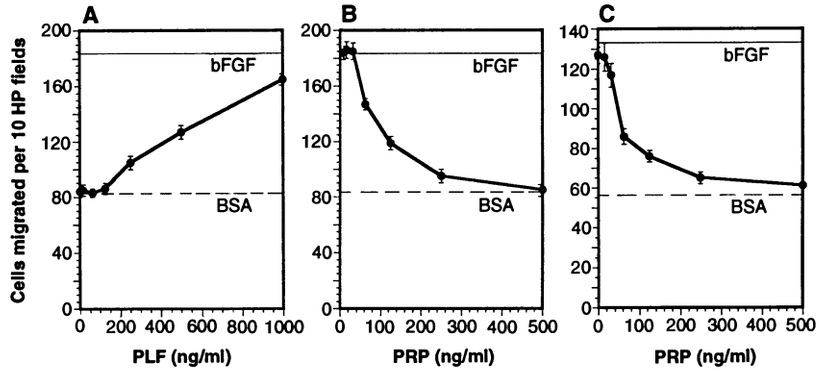

Fig. 1. Effects of PLF and PRP on bovine capillary endothelial cell migration. (**A**) Migration of endothelial cells in response to various concentrations of PLF. (**B**) Migration of endothelial cells in response to bFGF (10 ng/ml) combined with various concentrations of PRP. (**C**) Migration of endothelial cells in response to PLF (2 μg/ml) combined with various concentrations of PRP. In (A) through (C), the total number of migrating cells detected in 10 randomly selected high-power (HP) microscope fields was determined. The level of background migration [in response to 0.1% bovine serum albumin (BSA)] is shown by the dashed line, and the response to bFGF (10 ng/ml) is shown by the solid line. Data shown are the mean ± SD from four independent cell cultures in one experiment. The concentrations of PLF and PRP that gave half-maximal activities varied less than 20% between experiments.

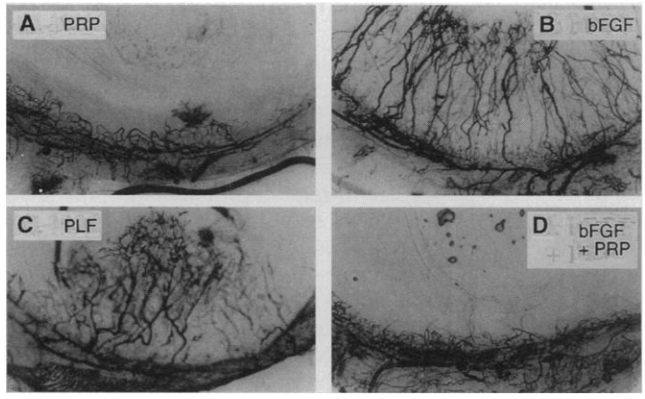

Fig. 2. Effects of PLF and PRP on angiogenesis in the rat cornea. Shown is a portion of a rat cornea implanted with a pellet containing (**A**) PRP, (**B**) bFGF, (**C**) PLF, or (**D**) PRP and bFGF, as described (*17*). Vessels were visualized with colloidal carbon.

Table 1. Activity of PLF and PRP in rat corneas.

Test substance	Vascularized corneas/total corneas
PRP	0/4
PLF	4/4
PRP + bFGF	0/4
PRP + PLF	0/3
Day 10 PCM	3/3
Day 12 PCM	0/3
1:1 mixture of day 10 and day 12 media	0/3

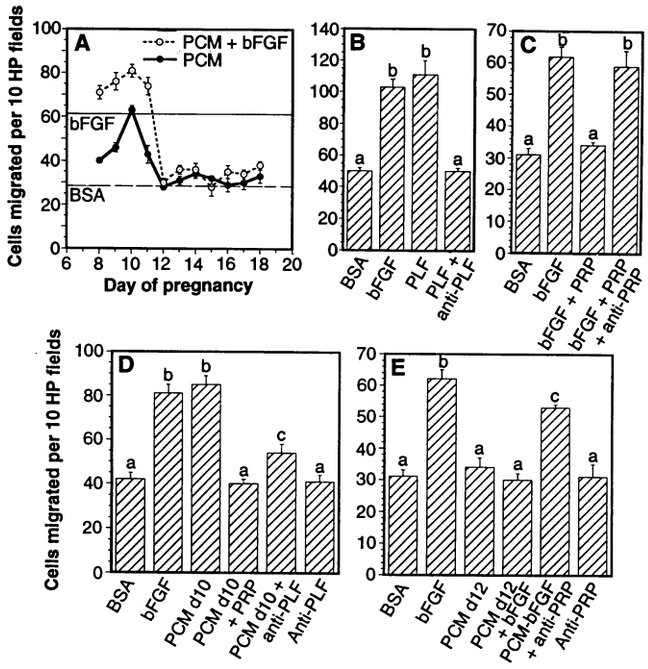

Fig. 3. Identification of PLF and PRP as the major angiogenic and anti-angiogenic proteins secreted from the mouse placenta. (**A**) Angiogenic activity of medium in which placental tissue had been cultured (placental conditioned medium or PCM). PCM from cultured tissue collected on days 8 to 18 of gestation was assayed for the stimulation of bovine capillary endothelial cell migration (solid thick line) and for the inhibition of a response to bFGF (10 ng/ml) (dashed line). The effects of bFGF (10 ng/ml) and 0.1% BSA are indicated by the horizontal lines. The same pattern was obtained with two independent sets of PCM, including maximum stimulatory activity on day 10 and inhibitory activity evident from day 12 to day 18. (**B**) Blocking activity of a PLF monoclonal antibody. The migration of endothelial cells was measured in response to 0.1% BSA, bFGF (10 ng/ml), PLF (1 μg/ml), or PLF (1 μg/ml) supplemented with monoclonal antibody 209 to PLF (20 μg/ml) (anti-PLF). (**C**) Blocking activity of antibodies to PRP (anti-PRP). Endothelial cell migration was measured in response to 0.1% BSA, bFGF (10 ng/ml), bFGF (10 ng/ml) supplemented with PRP (500 ng/ml), or a combination of bFGF (10 ng/ml), PRP (500 ng/ml), and affinity-purified antibodies to PRP (5 μg/ml). (**D**) Effect of anti-PLF on the angiogenic activity of day 10 PCM. Cell migration was assayed in the presence of 0.1% BSA, bFGF (10 ng/ml), day 10 (d10) PCM, day 10 PCM supplemented with PRP (500 ng/ml), day 10 PCM supplemented with anti-PLF, or anti-PLF alone. (**E**) Effect of anti-PRP on the anti-angiogenic activity of day 12 PCM. Endothelial cells were treated with 0.1% BSA, bFGF (10 ng/ml), day 12 PCM, day 12 PCM supplemented with bFGF (10 ng/ml), day 12 PCM combined with bFGF (10 ng/ml) and anti-PRP, or anti-PRP alone. Data in all panels represent the mean ± SD from four independent cell cultures. Data in (B) through (E) were subjected to a one-way analysis of variance and analysis by Tukey's test; in each case, bars marked "a," "b," and "c" differed significantly from each other with $P < 0.01$.

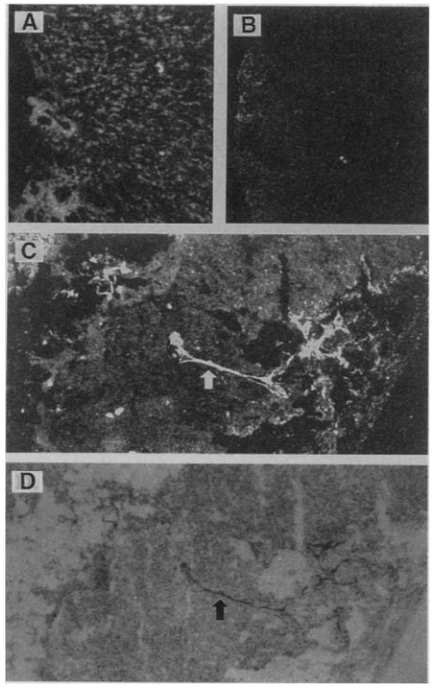

Fig. 4. Binding of PLF to placental endothelial cells. Shown are dark-field photomicrographs of adjacent placental sections incubated with ^{125}I-labeled PLF without (**A**) or with (**B**) excess unlabeled PLF as competitor; bright regions indicate binding by PLF. (**C**) Dark-field and (**D**) bright-field views of another placental section incubated with ^{125}I-labeled PLF and stained with the BL4 lectin that binds to capillary endothelial cells (*24*); the arrows point to a blood vessel.

Thus, PLF may be a primary regulator of placental angiogenesis in the mouse between days 8 and 12 of gestation. The timing of PRP secretion suggests that the anti-angiogenic activity of PRP may be responsible for the later restriction of placental angiogenesis, which may be critical in limiting the growth and invasiveness of the placenta. PRP is synthesized within the giant cell and junctional zones surrounding the labyrinth (*6*, *22*), where it would be well positioned to restrict the spread of blood vessels. PLF has been detected in amniotic fluid (*4*) and thus may also stimulate fetal angiogenesis directly. In contrast, PRP is excluded from the fetal circulation (*16*); this may be important in preventing its anti-angiogenic activity from affecting fetal development. Because the PLF gene is also expressed in immortalized fibroblasts (*7–9*) in response to the transcription factor AP-1 (*23*), it is also possible that PLF may be an angiogenic factor synthesized and secreted by Fos- or Jun-dependent tumors.

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14. PLF and PRP were purified from the conditioned medium of CHO cell cultures that were stably transfected with a PLF [S.-J. Lee and D. Nathans, *J. Biol. Chem.* **263**, 3521 (1988)] or PRP (*6*) expression construct. These preparations may include both biologically active and inactive forms, and therefore the concentrations of active protein required for half-maximal effects may actually be lower than those determined here.
15. Primary cultures of bovine adrenal capillary endothelial cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum and endothelial cell mitogen (100 μg/ml) (Biomedical Technologies, Stoughton, MA), and used at passage 15. Endothelial cell migration assays were done as described [S. S. Tolsma *et al.*, *J. Cell Biol.* **122**, 497 (1993)]. Cells used for migration assays were incubated for 24 hours in DMEM without serum supplemented with 0.1% bovine serum albumin (BSA) and then collected and transferred to gelatinized 0.5-μm filters (Nucleopore Corporation, Pleasanton, CA) in an inverted modified Boyden chamber at 1.75×10^4 cells per well in DMEM with 0.1% BSA. Cells were allowed to adhere to the bottom side of the filter during a 1- to 2-hour incubation; the chamber was then placed upright and test proteins were added on the top side of the filter. After a 3- to 4-hour incubation at 37°C, the filters were removed from the chambers, fixed, and stained, and the number of cells that had migrated to the upper surface of each filter was determined. Each sample was tested in quadruplicate within an experiment, and each experiment was repeated at least twice. Each compound was monitored (in the range of concentrations used for the migration assays) by trypan blue exclusion, and no toxicity was seen.
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17. We monitored angiogenic or anti-angiogenic activity in vivo by implanting hydroxyethylmethacrylate (Hydron) pellets (Interferon Sciences, New Brunswick, NJ) containing the test compounds 1 to 1.5 mm from the limbus of the cornea in anesthetized rats and scored them at day 7 for vigorous ingrowth of vessels [F. Rastinejad, P. J. Polverini, N. P. Bouck, *Cell* **56**, 345 (1989); P. J. Polverini, N. P. Bouck, F. Ras-

tinejad, *Methods Enzymol.* **198**, 440 (1991)]. Pellets were prepared from a solution of PLF (10 μg/ml), PRP (2.5 μg/ml), bFGF (0.15 μM), or placental conditioned medium (100 μg/ml). A single pellet was implanted in each rat cornea. Vessels were visualized by perfusion with colloidal carbon.

18. Placental tissue was isolated from Swiss-Webster mice (Harlan Breeding Laboratories, Indianapolis, IN) from day 8 through day 18 of gestation. Tissue minces were placed in culture in DMEM without serum, and the medium from each culture was collected 24 hours later. A culture contained tissue from several placentas obtained from one pregnant mouse, and two or three independent cultures were prepared for each time point. The samples of conditioned medium were centrifuged briefly to remove tissue fragments and then concentrated by centrifugation through a Centricon filter with a 10,000 molecular weight cutoff (Amicon, Beverly, MA). The samples were stored at −20°C until they were tested in the endothelial cell migration assay.
19. D. Jackson, O. Volpert, N. Bouck, D. I. H. Linzer, unpublished data.
20. Antibodies were affinity-purified from a polyclonal antiserum to PRP (*6*). Medium from cultured placental tissue obtained on day 12 of gestation was fractionated by nondenaturing polyacrylamide gel electrophoresis. After transfer to nitrocellulose, the region of the filter containing PRP was excised and incubated with the polyclonal antiserum. Bound antibodies were eluted in 0.2 M glycine-HCl (pH 2.8), neutralized, dialyzed against phosphate-buffered saline (PBS), and concentrated in a Centricon microconcentrator.
21. Mouse placentas from days 14 to 16 of gestation were isolated, rinsed in phosphate-buffered saline (PBS), and frozen in a dry ice–ethanol bath. The tissue was embedded in O.C.T. compound (Miles, Elkhart, IN), and 5-μm sagittal sections were mounted onto gelatin-coated slides. Sections were dessicated and stored at −80°C, transferred to 4°C 24 hours before use, and then dried at room temperature for 10 min. A Pap-pen (Research Products International, Mount Prospect, IL) was used to create fluid barriers around each section before the sections were washed once in 50 mM tris-HCl (pH 7.6), 2 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 140 mM NaCl, and 0.1% BSA (solution 1) for 15 min at room temperature, twice in PBS for 10 min at room temperature, and then incubated in solution 1 supplemented with 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 5 mM $MnCl_2$ (solution 2) for 2 hours at 4°C. After this incubation, 20 μl of ^{125}I-labeled PLF [6000 cpm of protein iodinated with Iodogen (Pierce, Rockford, IL) and separated from iodine by Sephadex G-100 column chromatography] in solution 2, with or without unlabeled PLF (1 μg/ml), was placed on each section and incubated for 24 to 48 hours at 4°C. Samples were washed three times in ice-cold PBS and then exposed to 2 mM bis (sulfosuccinimidyl) suberate (BS^3; Pierce) for 15 min at 4°C to cross-link proteins; the cross-linking reaction was quenched with 100 mM tris-HCl (pH 7.6). Sections were washed three times in PBS, fixed in 5% paraformaldehyde (pH 7.6) for 5 min at room temperature, and then washed again three times in PBS. Pap-pen barriers were removed by treatment with xylenes for 5 min, and the sections were dehydrated through an ethanol step series and dried in a vacuum with desiccant for 1 hour. The slides were then coated with Kodak NTB-2 photographic emulsion, allowed to dry, and stored at 4°C for 2 weeks until they were developed.
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24. Placental sections were stained to identify endothelial cells before coating with emulsion. To inactivate endogenous horseradish peroxidase (HRP) activity, we incubated sections in a solution of 70% methanol and 3% H_2O_2 in PBS for 30 min at 4°C and then rinsed them three times in PBS. The sections were then blocked in 5% BSA in PBS for 30 min at 4°C, incubated with the biotinylated lectin B4 from *Bandeiraea simplicifolia* [BSI-B4 from Sigma, St. Louis, MO; J. Coffin *et al.*, *Dev. Biol.* **148**, 51 (1991)] for 30 min at 4°C, washed three times in PBS, and finally incubated with a 1:500 dilution of streptavidin conjugated to HRP for 30 min at 4°C. The samples were again washed three times in PBS, and the HRP activity was visualized by addition of 1 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride and 0.02% H_2O_2 in PBS. After the sections were stained, they were processed as before for emulsion autoradiography.
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TECHNICAL COMMENTS

Strontium Isotopes in Mid-Cretaceous Seawater

B. Lynn Ingram *et al.* (*1*) model a general decrease in mid-Cretaceous strontium (Sr) isotope ratios that occurred as a result of an increase in oceanic crustal production. Their model uses the emplacements of only three oceanic plateaus: the Ontong-Java, Manihiki, and Kerguelen. Ingram *et al.* conclude "that the crustal production rates proposed by Larson [*2*, *3*] . . . are far too large . . ." (p. 549) because I have previously proposed (*2*–*5*) that these events were part of a much larger increase in oceanic crustal production from other oceanic plateaus, and in particular, from increased spreading rates at oceanic ridges. The comparison by Ingram *et al.* of their model for these three oceanic plateaus to my more general compilations of oceanic crustal production (*2*–*5*) is incorrect because it is a comparison between different volumes, and also types, of oceanic crust. For this comparison they first calculated a conversion factor of 6×10^8 mol of hydrothermal Sr flux per 1 km^3 oceanic crustal production, which fits a Cretaceous "background" oceanic crustal production rate of 20 km^3 per year (Fig. 1). This rate includes all extrusive basalts, diabase dikes, and underlying intrusive gabbros in the entire crustal sections above the mantle for both oceanic plateaus and normal ridges. However, in their figure 3, Ingram *et al.* propose a model to match observed Sr isotope ratios based only on the extrusive basalt volumes (*6*) capping the Ontong-Java, Manihiki, and Kerguelen plateaus. Finally, they contrast these matches with a much larger Sr isotope anomaly calculated from my compiled volumes (Fig. 1) of both the extrusive and intrusive sections of both plateaus and ridges. In general, the ratio of extrusive to intrusive crust in both situations is about 1 to 4 or 1 to 5. Ingram *et al.* (*1*) use 10×10^6 km^3 and 8×10^6 km^3 for the extrusive basalt volumes of (i) Ontong-Java and Manihiki and (ii) Kerguelen plateaus, respectively. My estimates (included in Fig. 1) of the combined extrusive and intrusive volumes of these same features were 64×10^6 km^3 and 45×10^6 km^3 (*3*–*5*). Thus, one would expect that the model Sr isotope anomalies calculated using these two different sets of figures would be different, but the comparison is meaningless because Ingram *et al.* have compared apples to oranges, or more precisely, apples to apples *and* oranges.

It is unlikely that serious, systematic errors will reduce the mid-Cretaceous pulse in oceanic crustal production as shown in Fig. 1 because I (*2*) deliberately chose the radiometric time scale (*7*) with the longest mid-Cretaceous time interval to test rigorously for such a pulse. The choice of any other recently published time scale would increase the amplitude of this pulse, and the most recent time scale (*8*) would increase the amplitude by about 20% relative to the steady state baselevel. The total increase in mid-Cretaceous crustal production (Fig. 1) is about what is necessary to account for the 250 m of eustatic sealevel elevation (*9*) during that period.

The misuse of crustal volume data in the study by Ingram *et al.* (*1*) points out an interesting question regarding the source locations in the oceanic crust where Sr is leached by hot, hydrothermal fluids. Experimental results (*10*) suggest that fluid leaching diabase dike rock at 400°C matches the output of ridge crest hot springs and that basalts are much less susceptible to leaching. Field studies on ophiolites (*11*) confirm this result. Such source rocks and conditions probably are present directly above the 1.5 to 3 km deep (*12*) magma chambers at fast and intermediate spreading ridge crests. Fluid circulation must penetrate this deeply because convective cooling has depressed these magma chambers well below the 0.5-km level of neutral buoyancy (*12*–*14*). The situations for oceanic plateaus and