ate integrin-specific signals, such as the enhanced growth response to insulin. A 190-kD protein that associates with α_v integrins in platelet-derived growth factor (PDGF)–stimulated cells (*21*) may serve a similar function in linking the PDGF pathway to specific integrins. The signal transduction pathways of other growth factor and cell adhesion receptors are likely to be integrated by related mechanisms.

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9. Monoclonal antibody (mAb) LM609 against the $\alpha_v\beta_3$ integrin complex and mAb 147 against the α_v integrin subunit coprecipitated p185, whereas mAb P4C10 against the β_1 subunit and mAb P1D6 against the α_5 subunit did not (*22*).
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19. K. Vuori and E. Ruoslahti, unpublished data. The $\alpha_v\beta_3$-expressing lines were HIRcB, NIH 3T3, and NRK cells, and the lines expressing $\alpha_v\beta_5$ but not $\alpha_v\beta_3$ were HT29 colon carcinoma, UCLA-P3 lung adenocarcinoma, and Panc-1 pancreatic carcinoma cells. The $\alpha_v\beta_3$ associated with IRS-1 in all of the $\alpha_v\beta_3$-expressing cells after insulin stimulation. All of the cells attached equally well to vitronectin. All of the cell lines attached to laminin, collagen, or both, and this was inhibited by antibodies to β_1.
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24. Anti-$\alpha_v\beta_3$ 237 immunocomplexes from insulin-treated cells were resuspended in 10 mM tris-HCl (pH 7.5), 2% SDS, 1 mM sodium orthovanadate, and 50 mM sodium fluoride and heated at 95°C for 5 min. The supernatant was diluted 10-fold in 10 mM tris-HCl (pH 7.5) and reprecipitated with anti-$\alpha_v\beta_3$ 237. Similar results were obtained with anti-$\alpha_v\beta_3$ 1343 and anti-$\alpha_{IIb}\beta_3$ [E. Dejana *et al.*, *J. Cell Biol.* **107**, 1215 (1988)].
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26. After insulin stimulation, HIRcB cells (5×10^8) were lysed in 100 mM octyl-β-D-glucopyranoside in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and the phosphatase and protease inhibitors in (*23*). Chromatography was performed as in R. Pytela, M. D. Pierschbacher, S. Argraves, S. Suzuki, and E. Ruoslahti [*Methods Enzymol.* **144**, 475 (1987)].
27. Plates were coated with vitronectin (10 μg/ml) or type I collagen for 12 hours and then treated with 0.5% BSA for 2 hours. Adhesion to vitronectin was inhibited by anti-$\alpha_v\beta_3$ but not anti-$\alpha_5\beta_1$, and adhesion to collagen was inhibited by anti-β_1. Cells were greater than or equal to 85% viable on both vitronectin and collagen after 36 hours of incubation.

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Exocytosis in Spermatozoa in Response to Progesterone and Zona Pellucida

Eduardo R. S. Roldan,* Tetsuma Murase, Qi-Xian Shi

Exocytosis in mammalian spermatozoa (the acrosome reaction) is a process essential for fertilization. Both progesterone and zona pellucida induce exocytosis in spermatozoa, which may encounter both during penetration of the oocyte's vestments. When mouse spermatozoa were exposed first to progesterone and then to zona pellucida, exocytosis was enhanced to a greater degree than that seen when the agonists were presented together or in the inverse order, which suggests that the steroid exerts a priming effect. Progesterone similarly primed the generation of intracellular messengers evoked by zona pellucida. The effects triggered by progesterone were mimicked by γ-aminobutyric acid (GABA) and were blocked by bicuculline, which indicates that the steroid acts on a $GABA_A$ receptor.

At fertilization, the spermatozoon undergoes exocytosis in response to an oocyte-derived signal or signals (*1*). However, two conflicting views exist regarding the agonist responsible for triggering exocytosis. The generally accepted view is that, after traversing the cumulus oophorus, the spermatozoon is stimulated by the zona pellucida (ZP) (*1*), and exposure to ZP leads to tyrosine phosphorylation of p95 (*2*) and activation of a guanosine triphosphate (GTP)–binding protein (G_i class) (*3*). However, targets for these transducing mechanisms have not been identified. An alternative view states that progesterone, trapped in or produced by the cumulus oophorus, can also initiate exocytosis (*4*). Progesterone action is specific (*5–7*), is mediated by a surface receptor (*8*), and also leads to phosphorylation of p95 (*9*); G protein activation, however, does not take place (*10*). We tested whether these agonists interact at an early stage during exocytosis and examined which messengers are elicited by progesterone or ZP and whether these agonists activate different pathways for the generation of such messengers.

Mouse spermatozoa undergo exocytosis of the acrosome when exposed to ZP (Fig. 1) (*1*). Mouse spermatozoa capacitated in vitro (*11*) also undergo exocytosis when exposed to progesterone (Fig. 1) (*12*). To investigate how the actions of these ago-

Department of Development and Signalling, The Babraham Institute, Biotechnology and Biological Sciences Research Council, Cambridge CB2 4AT, UK.

*To whom correspondence should be addressed.

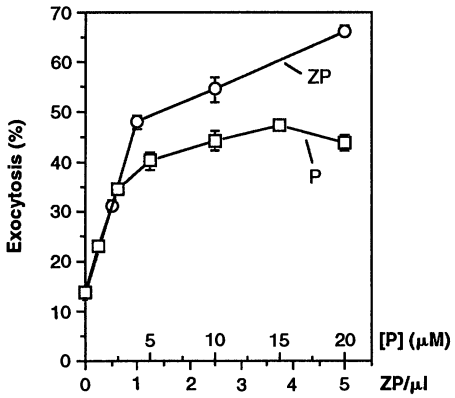

Fig. 1. Exocytosis stimulated by progesterone (P) and zona pellucida (ZP) in mouse spermatozoa. Spermatozoa capacitated in vitro were stimulated for 15 min with P or ZP, and acrosomal exocytosis was monitored as described (*11*). Results are means ± SEM (n = 3).

nists are mediated, we first quantified changes in the total mass of diacylglycerol (DAG) (*11*), the messenger presumed to play a central role in sperm exocytosis (*13*, *14*). Stimulation with either progesterone or ZP triggered considerable formation of DAG (Table 1) (*12*).

DAG can be generated by several routes. To test whether phosphoinositidase C–mediated polyphosphoinositide hydrolysis (*15*) is a source of DAG, spermatozoa were labeled with [^{32}P]orthophosphate (P_i) and then stimulated with agonists (*11*). Treatment with progesterone or ZP led to hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) (Table 1) and phosphatidylinositol 4-phosphate (PIP) (*16*). To explore whether phospholipase C (PLC) or the phospholipase D (PLD)–phosphatidate-phosphohydrolase pathway are additional mechanisms of DAG generation (*17*), the phosphatidylcholine (PC) pool was labeled with [^{3}H]alkyl-lysoPC, and changes in metabolites after stimulation were followed (*11*). Treatment with progesterone or ZP resulted in a rise in alkyl-diglyceride (Table 1). However, no rise in alkyl-phosphatidate was noticed (Table 1), which suggests either lack of PLD activation or no phosphorylation of alkyl-diglyceride. To discriminate between these options, spermatozoa were preincubated for 5 min with 1% ethanol (v/v) and then treated with progesterone or ZP. Formation of alkyl-phosphatidylethanol was not seen after these treatments (Table 1), which suggests an absence of PLD activation (generation of phosphatidylethanol is an unequivocal indication of PLD activity) (*18*). It follows that the generation of DAG evoked by progesterone and ZP occurs via similar pathways, involving phosphoinositidase C and PLC. Because the mass of phosphoinositides in cell membranes is very small, most DAG is likely to be generated by PLC. This is very different from the mechanisms of DAG generation operating in sea urchin sperm, where PLD–phosphatidate-phosphohydrolase represents the main pathway and PLC activity is not detectable (*19*), and it suggests that invertebrate spermatozoa may not be adequate models for mammalian sperm function.

Acrosomal exocytosis relies on internalization of extracellular Ca^{2+}. It is not clear, however, which processes in the sequence leading to membrane fusion take place before and which after the rise in intracellular Ca^{2+} that is triggered by progesterone (*5*, *20*) or ZP (*21*). It has been hypothesized that generation of DAG takes place after Ca^{2+} influx, which is contrary to the situation seen in many somatic cells, and that such influx could occur via Ca^{2+} channels (*14*). Five results confirmed this hypothesis (Table 2). First, mouse spermatozoa stimulated with the Ca^{2+} ionophore A23187 (to provoke Ca^{2+} entry) experienced a rise in DAG. Second, when capacitated spermatozoa were suspended in a medium with little extracellular Ca^{2+} available for influx (no Ca^{2+} added; residual Ca^{2+} ~25 μM) (*22*) and were stimulated with progesterone or ZP, generation of DAG was less than that seen in medium containing millimolar Ca^{2+}. Third, if the residual Ca^{2+} was chelated with EGTA before stimulation, DAG formation was also low. Four, if capacitated spermatozoa in medium with millimolar Ca^{2+} were stimulated with progesterone or ZP in the presence of La^{3+}, a Ca^{2+} channel blocker, DAG formation was reduced. Five, when capacitated spermatozoa were exposed to the Ca^{2+} channel agonist Bay K 8644, formation of DAG and exocytosis (*16*) followed, which suggests the involvement of voltage-operated channels in Ca^{2+} entry. We investigated whether nifedipine, an inhibitor of voltage-operated Ca^{2+} channels, would interfere with DAG formation. Unfortunately, this was not a useful approach, because nifedipine had a stimulatory effect on DAG formation (*16*), which is consistent with its stimulatory action on acrosomal exocytosis (*23*). Taken together, these results not only demonstrate that activation of phosphoinositidase C and PLC elicited by progesterone or ZP occurs after Ca^{2+} entry via Ca^{2+} channels, but also that the parallel formation of inositol phosphates triggered by these two agonists probably has no relevance in Ca^{2+} modulation (*24*).

Table 1. Lipid changes in spermatozoa stimulated with progesterone (P) or zona pellucida (ZP). Mouse spermatozoa were capacitated in vitro in the absence or presence of a label (150 μCi of [^{32}P]P_i per milliliter or 2 μCi of [^{3}H]alkyl-lysoPC per milliliter), washed, and exposed for various times to 15 μM P or 1 ZP per microliter or solvent controls; lipids were then extracted, separated, and quantified (*11*). For measurements of [^{3}H]alkyl-phosphatidylethanol ([^{3}H]alkyl-PEt), 1% ethanol (v/v) was added 5 min before stimulation (*11*, *18*). Treatment times shown were optima for detection of maximal changes in each metabolite, as determined from previous time-course studies. Results are means ± SEM (n = 3).

Metabolite	Time (min)	Treatment		
		Control	P	ZP
DAG mass*	2.5	1.77 ± 0.11	3.61 ± 0.15‡	3.09 ± 0.08‡
[^{32}P]PIP_2†	5	27,030 ± 2,400	14,866 ± 1,106‡	15,200 ± 1,064‡
[^{3}H]alkyl-diglyceride†	5	6,644 ± 250	7,973 ± 298‡	7,774 ± 398‡
	15	6,754 ± 213	7,840 ± 332‡	6,910 ± 358
[^{3}H]alkyl-phosphatidate†	5	4,258 ± 180	4,308 ± 298	3,947 ± 296
	15	4,194 ± 207	3,800 ± 325	3,819 ± 308
[^{3}H]alkyl-PEt†	5	3,890 ± 158	4,068 ± 208	3,699 ± 196
	15	3,954 ± 171	4,106 ± 225	3,972 ± 182

*Measured in micrograms per 10^9 cells. †Measured in cpm per 10^8 cells. ‡Different from control, $P < 0.001$.

Table 2. The generation of DAG triggered by progesterone (P) and zona pellucida (ZP) takes place after Ca^{2+} influx via Ca^{2+} channels. Mouse spermatozoa were capacitated in vitro, washed, resuspended in Hepes-buffered Tyrode's medium (*11*) without or with 1.8 mM Ca^{2+}, and stimulated with 15 μM A23187, 15 μM P, 1 ZP per microliter, or the Ca^{2+} channel agonist Bay K 8644 for 2.5 min. EGTA (1 mM) or the Ca^{2+} channel blocker La^{3+} (250 μM) were added in some experiments (La^{3+} did not affect sperm viability). Lipids were extracted and separated, and DAG was quantified as described (*13*). Means ± SEM of at least three experiments are shown.

Treatment				DAG mass (μg/10^9 cells)
Ca^{2+}	EGTA	La^{3+}	Agonist	
+	–	–	–	1.89 ± 0.13
+	–	–	A23187	4.50 ± 0.08*
+	–	–	P	3.17 ± 0.02*
+	–	–	ZP	3.08 ± 0.17*
–	–	–	P	1.72 ± 0.15†
–	–	–	ZP	1.87 ± 0.18†
–	+	–	P	1.90 ± 0.21†
–	+	–	ZP	1.47 ± 0.31†
+	–	+	P	2.33 ± 0.18†
+	–	+	ZP	1.80 ± 0.25†
+	–	–	Bay K 8644 (1 nM)	3.19 ± 0.21*
+	–	–	Bay K 8644 (100 nM)	4.58 ± 0.26*

*Different from control, $P < 0.001$. †Different from Ca^{2+}/P or Ca^{2+}/ZP, $P < 0.001$.

Progesterone- and ZP-induced Ca^{2+} influx activate similar pathways for the generation of DAG. However, the two agonists may use different transducing pathways to prompt Ca^{2+} entry. Putative receptors for ZP3, the ZP glycoprotein with exocytosis-inducing activity (*1*), have been identified (*2, 25*), but their links with Ca^{2+}-modulating mechanisms are not well understood; the activation of a G protein–regulated Ca^{2+} channel by ZP has been proposed (*21*). Progesterone, on the other hand, acts on a membrane receptor (*8*) that might resemble the GABA receptor, because progesterone-stimulated exocytosis is inhibited by GABA antagonists (*7*). The findings that sperm membranes appear to have GABA binding sites (*26*) and a protein or proteins recognized by antibodies to the GABA receptor α subunit (*7*) are consistent with this idea.

To ascertain whether progesterone acts through a GABA receptor, the effects of progesterone and GABA on the generation of DAG (as an indicator of both phosphoinositidase C and PLC activation) and on exocytosis were examined. It was found (Table 3) that GABA triggered DAG generation and exocytosis in mouse spermatozoa and that, when used together, progesterone and GABA expressed additive stimulatory ability on both DAG generation and exocytosis (*27*). Further characterization revealed that GABA- and progesterone-stimulated generation of DAG was inhibited by bicuculline, a $GABA_A$-receptor antagonist (*28*); similarly, exocytosis elicited by these agonists was inhibited when bicuculline was included (Table 3). On the other hand, bicuculline did not inhibit DAG formation or exocytosis elicited by ZP (*16*), which is consistent with the idea that ZP is acting through a different receptor. We also verified that progesterone effects were not mediated by an intracellular receptor: Equimolar concentrations of the compound RU-486, a known antagonist of the intracellular progesterone receptor (*29*), did not inhibit progesterone-stimulated exocytosis (*16*). Taken together, these results demonstrate that progesterone-stimulated exocytosis occurs (at least in part) via a GABA-like receptor, a remarkable finding because GABA actions are thought to be essentially inhibitory. It is not known how activation of the sperm GABA receptor leads to activation of a Ca^{2+} channel and to Ca^{2+} entry, but this is not without a precedent, as reported for rat astrocytes (*30*). It is also possible that progesterone could act on a Ca^{2+} channel in a more direct manner, in addition to its action on the $GABA_A$ receptor (*31*); this idea is endorsed by the observation that the already-maximized GABA effect on DAG formation and exocytosis was enhanced by inclusion of half-maximal concentrations of progesterone (*27*) (Table 3).

Finally, we tested the possibility that initiation of exocytosis by progesterone or ZP are not irreconcilable alternatives but, rather, that both agonists interact sequentially during sperm activation. Penetration of spermatozoa through the cumulus oophorus could result in an acute exposure to low micromolar progesterone concentrations (*4*) (higher than those found in oviductal fluids) and initiation of exocytosis, whereas interaction with the ZP would trigger its completion. Because attachment and binding of spermatozoa to the ZP take several minutes (*32*), a temporal separation between agonists could be expected to enhance their actions. When spermatozoa were treated sequentially with half-maximal concentrations of progesterone and ZP (see Fig. 1), exocytosis was enhanced as compared with simultaneous presentation of both agonists or presentation of agonists in the inverse order (Table 4). Similarly, treatment with progesterone followed by ZP led to maximal generation of DAG and maximal breakdown of PIP_2 (Table 4) and PIP (*16*). These results demonstrate a priming role for progesterone in the initiation of exocytosis. In addition, they suggest that the effective concentrations of these agonists in vivo may be much lower than originally thought. Cross talk between pathways activated by each agonist is possible, although it is unlikely to relate to the generation of DAG, because similar pathways are activated by both agonists. It is more likely that cross talk exists between mechanisms underlying Ca^{2+} entry. Another more attractive possibility is that the sequential action of these agonists is responsible for the sequential activation of events requiring different concentrations of Ca^{2+} (*14, 22*). Activation of sperm phosphoinositidase C and PLC for DAG generation is known to take place even after limited

Table 3. GABA stimulates generation of DAG and acrosomal exocytosis, mimics progesterone (P) actions, and shows additive effects with P; bicuculline (a $GABA_A$-receptor antagonist) inhibits both GABA and P actions. Mouse spermatozoa were capacitated in vitro and stimulated for 15 min, and acrosomal exocytosis (percent of AR pattern) was monitored as described (*11*). For determination of DAG mass, capacitated sperm were washed, resuspended in Hepes-buffered Tyrode's medium (*11*), and stimulated for 2.5 min. Lipids were extracted and separated, and DAG was quantified as described (*13*). GABA = 0.5 μM; bicuculline = 10 μM (preincubated with sperm for 10 min before agonist added; bicuculline did not affect sperm viability). Means ± SEM of at least four experiments are shown.

Treatment			DAG mass ($\mu g/10^9$ cells)	Exocytosis (%)
P	GABA	Bicuculline		
–	–	–	1.83 ± 0.11	19.8 ± 1.21
2.5 μM	–	–	2.90 ± 0.19*	36.4 ± 1.81*
15 μM	–	–	3.29 ± 0.05*	50.8 ± 0.80*
–	+	–	2.83 ± 0.14*	48.0 ± 2.64*
2.5 μM	+	–	3.36 ± 0.21*†	66.3 ± 1.52*†
15 μM	–	+	2.24 ± 0.18‡	16.3 ± 1.15‡
–	+	+	1.95 ± 0.08‡	15.7 ± 1.35‡

*Different from control, $P < 0.001$. †Different from 2.5 μM P or GABA, $P < 0.01$. ‡Different from 15 μM P or GABA, $P < 0.001$.

Table 4. The sequential exposure of spermatozoa to progesterone (P) and zona pellucida (ZP) results in maximal generation of DAG, hydrolysis of PIP_2, and exocytosis. For lipid changes, mouse spermatozoa were capacitated in vitro without or with 150 μCi $[^{32}P]P_i$/ml, washed, resuspended in Hepes-buffered Tyrode's medium (*11*), and exposed to P, ZP, or both for 5 min; to P for 3 min, followed by a 2-min exposure to ZP; to the same agonists in the inverse order (ZP for 3 min, followed by P for 2 min); or to solvent controls (added from the beginning of incubation or in sequence). Lipids were extracted and separated, and DAG or PIP_2 were quantified (*11*). For exocytosis, spermatozoa were capacitated (*11*) and were stimulated with P, ZP, or both for 15 min; or they were exposed first to P or ZP for 3 min, then the other agonist was added for the rest of the incubation period (controls as above). Exocytosis (percent of AR pattern) was monitored as described (*11*). Results are means ± SEM ($n = 4$).

Treatment	DAG mass ($\mu g/10^9$ cells)	$[^{32}P]PIP_2$ (cpm/10^8 cells)	Exocytosis (%)
Control	1.65 ± 0.04	28,028 ± 1,506	13.9 ± 0.86
2.5 μM P	2.30 ± 0.05*	16,956 ± 1,846*	34.14 ± 1.24*
0.5 ZP/μl	2:81 ± 0.18*	15,976 ± 1,189*	37.17 ± 1.14*
P + ZP	2.74 ± 0.05*	12,555 ± 950*	41.14 ± 2.65*
P → ZP	4.13 ± 0.11*†	8,906 ± 835*†	55.43 ± 1.31*†
ZP → P	2.32 ± 0.25*	13,930 ± 841*	44.57 ± 3.07*

*Different from control, $P < 0.001$. †Different from 2.5 μM P, 0.5 ZP/μl, P + ZP, or ZP → P; $P < 0.001$.

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 $\sqrt{}$(percent of AR cells ÷ 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

Dowdy Jackson, Olga V. Volpert, Noël Bouck, Daniel I. H. Linzer*

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-

D. Jackson and D. I. H. Linzer, Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208, USA.
O. V. Volpert and N. Bouck, Department of Microbiology-Immunology and the R. H. Lurie Cancer Center, Northwestern University Medical School, Chicago, IL 60611, USA.

*To whom correspondence should be addressed.