the toxin, but is also functionally affected. This loss is compensated for by an increase in the concentration of the CryIC receptor after several generations of selection.

Strategies for resistance management are needed to extend the lifetime of chemical insecticides. It is equally important to implement such strategies with *B. thuringiensis* to maintain its usefulness as a safe and environmentally sound insect control agent. Our results provide a basis on which to build such strategies. When different ICPs are available with activity against the same insect species, resistance to one ICP does not always imply resistance against other ICPs. ICPs with different binding properties could be used in combination or sequentially for greater effectiveness or to delay resistance.

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18. Cubes (2 mm) were cut from dehydrated apple slices and placed singly into each well of a 24-well tissue culture plate. Toxin solution (2 μl) (dilutions ranging from 0.001 mg/ml to 10 mg/ml) was applied to each apple cube, which was then allowed to dry. A single second or third instar larva was placed into each well and the plates were covered and incubated at 25°C with 60% relative humidity. Larvae that consumed the toxin-coated apple cubes were subsequently fed normal diet (*2*) and were maintained until death or pupation. Larval mortality was scored every 4 to 5 days over a period of 20 to 25 days. Twelve or more larvae were used per dose. $LD_{50}$ and $CI_{95}$ for each toxin were calculated by a probit analysis program (written by G. A. Milliken, Kansas State University, Manhattan, KS). Protoxin purification and activation was performed as described in (*13*). Purification of the toxic fragment was done according to the method described by Hofmann *et al.* (*9*).
19. Duplicate samples of $^{125}$I-labeled toxin (*20*), either alone or in combination with varying concentrations of unlabeled toxin, were incubated at room temperature with brush border membrane vesicles in a total volume of 100 μl tris buffer (20 m*M* tris and 150 m*M* NaCl, *p*H 7.4) with 0.1% bovine serum albumin (BSA). Vesicles were prepared according to the method of M. Wolfersberger *et al.* [*Comp. Biochem. Physiol.* **86A**, 301 (1987)]. In experiments with CryIA(b) toxin on the S strain, mixtures were incubated for 60 min; all other experiments were done with a 90-min incubation period. In experiments with $^{125}$I-labeled CryIA(b) toxin (0.37 n*M*, S strain; 32 n*M*, R strain), vesicle concentrations of 100 and 200 μg of membrane protein per milliliter of S and R strain, respectively, were used. Experiments were set up with 12 concentrations of unlabeled ligand (from 0 to 10 μg/ml, S strain; 0 to 300 μg/ml, R strain). In experiments with $^{125}$I-labeled CryIC toxin (0.15 n*M*), vesicle concentrations of 150 and 70 μg of membrane protein per milliliter of the S and R strain, respectively, were used. Twenty-one concentrations of unlabeled ligand were used (from 0 to 100 μg/ml). Bound toxin was quantitated by ultrafiltration through Whatman GF/F glass-fiber filters. Each filter was rapidly washed with 5 ml of ice-cold tris buffer with 0.1% BSA. Binding data were analyzed with the LIGAND computer program (*17*). Molar $R_t$ values were transformed to picomoles per milligram of protein, on the basis of the amount of vesicle protein [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)]. Two or three binding experiments were performed on each of three independently prepared batches of membrane vesicles from both S and R strains. No significant differences in $K_d$ or $R_t$ were found between batches (one-way analysis of variance). Therefore, only the mean values and the standard deviations for the total set of repeat experiments are presented.
20. CryIA(b) toxin was labeled with chloramin T, essentially as described by W. M. Hunter and F. C. Greenwood [*Nature* **194**, 495 (1962)] to a specific radioactivity of 155,100 Ci/mol. CryIC-toxin was labeled with the Iodogen method (*9*); a specific radioactivity of 462,990 Ci/mol was obtained. Specific activity of iodinated toxins was determined with a "sandwich" enzyme-linked immunosorbent assay (ELISA) technique [A. Voller, D. E. Bidwell, A. Barlett in *Manual of Clinical Immunology*, N. R. Rose and H. Friedman, Eds. (American Society of Microbiology, Washington, DC, 1976), pp. 506–512].


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# Derepression of Ferritin Messenger RNA Translation by Hemin in Vitro


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**Incubation of a 90-kilodalton ferritin repressor protein (FRP), either free or complexed with an L-ferritin transcript, with hemin or $Co^{3+}$-protoporphyrin IX prevented subsequent repression of ferritin synthesis in a wheat germ extract. Neither $FeCl_3$ in combinations with $H_2O_2$, nor $Fe^{3+}$ or $Fe^{2+}$ chelated with EDTA, nor $Zn^{2+}$-protoporphyrin IX, nor protoporphyrin IX caused significant inactivation of FRP. FRP that had been inactivated by hemin remained chemically intact, as revealed by SDS–polyacrylamide gel electrophoresis. Inclusion of chelators of iron or free radical scavengers did not alter the inactivation produced by hemin. These and other results indicate that hemin derepresses ferritin synthesis in vitro.**


FERRITINS ARE IRON STORAGE PROteins that are found in virtually all cells (*1*). Their synthesis in vertebrates is regulated in response to the extracellular iron supply at the translational level (*2*). Kinetic evidence suggests that a significant number of other vertebrate mRNAs are similarly regulated (*3*). The ferritin translational regulatory machinery consists of a conserved sequence of 28 nucleotides [the iron-responsive element (IRE)] in the mRNA 5′ untranslated region (5′ UTR), which makes translation responsive to iron (*4*), and a 90-kD ferritin repressor protein (FRP) that binds to the IRE in the paucity of iron (*5–9*).

We now describe properties of a third regulatory component, the "ferritin inducer." The ferritin inducer should be a metabolite whose concentration rises and falls in response to the rate of iron uptake, and which interacts with the FRP, either directly or indirectly, in such a way as to relieve the repression of ferritin mRNA translation. Evidence is presented in this report that hemin fulfills these criteria, and therefore may be a ferritin inducer.

To study the effect of iron compounds on ferritin synthesis, FRP was ordinarily incubated with hemin, or analogs thereof, in a redox buffer made up of glutathione plus oxidized glutathione (termed GSB) for various times and temperatures. Wheat germ lysate, L-ferritin and apolipoprotein A-1 transcripts, [$^{35}$S]methionine, and other


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components required for translation were then added, and the mixture was further incubated for 1 hour at 24°C. The labeled ferritin and apolipoprotein products synthesized during the second incubation step were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and fluorography (*10*). The repression of ferritin synthesis by partially purified FRP (*7*) (Fig. 1A, lanes 1 and 2) was partially relieved by the addition of hemin directly to the translation reaction mixture (Fig. 1A, lanes 3 and 4). This derepression was enhanced by first incubating FRP with hemin in the presence of GSB (lanes 7 to 12). Maximum relief of repression was obtained at 200 μ*M* hemin. Prior incubation with GSB alone had a small derepressive effect (compare lanes 2 and 7), reminiscent of the observation that sulfhydryl oxidation inactivates a partially purified IRE binding protein (*6*). However, the slight derepression caused by GSB did not increase with increasing concentrations (Fig. 1B), or with the ratio of oxidized to reduced glutathione in the buffer system (*11*). The omission of GSB often resulted in a general inhibition of translational activity by hemin (Fig. 1B; compare the apolipoprotein bands in lanes 2, 3 and 7 or 10). This nonspecific inhibition was probably due to the generation of peroxides and free radicals by hemin. GSB also prevented significant inhibition of other enzymes (for example, Eco RI, Hind III, Hpa II, Nar I, Sal I, and Xho I) by 200 μ*M* hemin under the preincubation conditions used in Fig. 1A, lane 11 (*11*).

The action of hemin was temperature-dependent, being more effective at 37°C than at 30°C; higher temperatures (for example, 42°C) inactivated FRP in the presence or absence of hemin or GSB (*11*). The derepression activity of hemin was not due to hydrogen peroxide formation, because $H_2O_2$ in the absence or presence of $FeCl_3$ did not prevent the action of FRP on ferritin mRNA translation (Fig. 1C).

Some partially purified FRP preparations contained a nonspecific inhibitory activity (compare the apolipoprotein synthesis in Fig. 1C, lanes 1 and 2). This contaminant was absent from highly purified preparations of FRP (*9*). These preparations were sensitive to hemin treatment but not to $Fe^{3+}$-EDTA or $Fe^{2+}$-EDTA (Fig. 1D). A threefold molar excess of the iron chelator, Desferal (desferrioxamine mesylate), did not interfere with the relief caused by hemin (Fig. 1D). EDTA also did not significantly reduce the relief activity of hemin (*12*). Thus the effect of hemin was not due to the release of chelatable iron. The results (Fig. 1, C and D) also suggest that FRP activity is relatively resistant to the generation of hydroxyl radicals or of superoxide radical ions (*13*).

A variety of heme analogs were tested for relief activity. This was done in the presence or absence of 10 m*M* mannitol, which effectively scavenges the hydroxyl radical (*14*). Mannitol did not affect the relief activity of hemin, again suggesting that the hemin effect is not due to the generation of hydroxyl radicals (*11*). Although $Zn^{2+}$-protoporphyrin IX had slight relief activity, protoporphyrin IX had none (*11*). However, $Co^{3+}$-protoporphyrin IX was as effective as hemin (Fig. 1E) in relieving repression (*15*). Low concentrations (10 to 25 μ*M*) of $Sn^{2+}$-protoporphyrin IX caused slight derepression, although higher concentrations were inhibitory to translation of all mRNAs (*11*).

We then addressed the question of whether an FRP-mRNA complex can be dissociated by hemin or related compounds. Highly purified FRP was incubated in the presence of ferritin and apolipoprotein transcripts for 10 min at 0°C, conditions that are sufficient for complete complex formation (*16*). Subsequent incubation steps, first containing $Co^{3+}$-protoporphyrin IX, and then translation components, were conducted as described above. Addition of $Co^{3+}$-protoporphyrin IX again allowed ferritin synthesis (Fig. 2A), which was probably due to dissociation of the FRP-mRNA complex (*17*). The time course of the derepression reaction was examined (Fig. 2B); the FRP-mRNA complexes appeared to form rapidly, as repression was not increased by incubating the transcripts for 10 or 20 min. In contrast, the derepression of translation induced by $Co^{3+}$-protoporphyrin IX was a relatively slow process, requiring at least 30 min for completion.

Because of the propensity of heavy metals and their complexes to generate free radicals and peroxides, we have examined whether the FRP molecule was either proteolyzed or cross-linked (*18, 19*) by hemin treatment. To test for these possible effects, FRP was incubated with GSB, or a combination of GSB and hemin, and then analyzed by SDS-PAGE under nonreducing conditions (*19*) (Fig. 3). Neither proteolysis nor cross-linking occurred under conditions sufficient for derepression of ferritin translation. A similar result was obtained when the same reaction products were analyzed by SDS-PAGE under reducing conditions (*11*). Of course, these results do not guarantee that FRP is completely inert to hemin-catalyzed peroxidation (*18*). Nevertheless, three free radical scavengers that inhibit these degradative re-

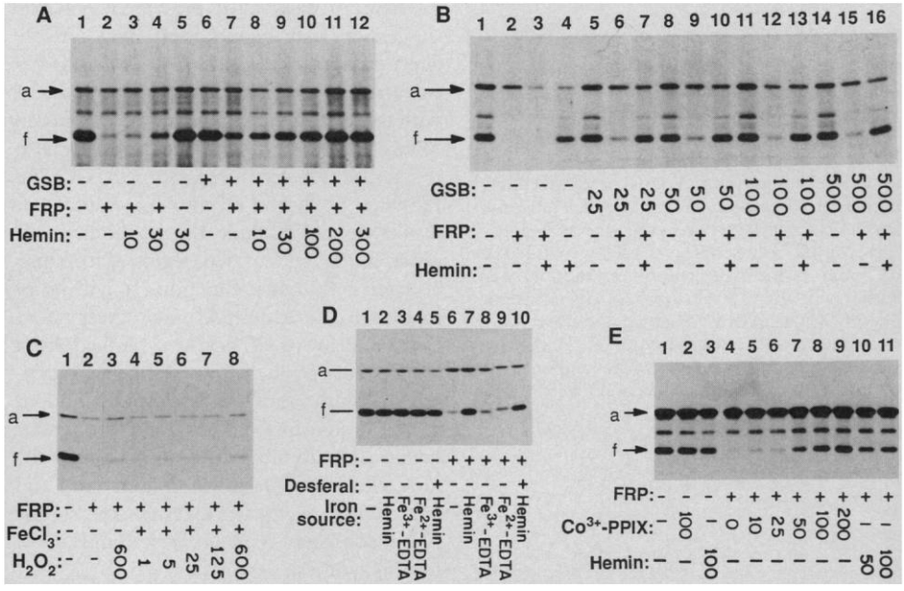


**Fig. 1.** Inhibition by hemin of ferritin mRNA translational repression. (**A**) In lanes 1 to 5, reticulocyte FRP and varying hemin (in micromolar concentrations), where indicated, were included directly in the translation reaction. In lanes 6 to 12, reticulocyte FRP, varying hemin, and GSB were first incubated in a 3.2-μl volume for 10 min at 37°C before the subsequent translation reaction. Ferritin and apolipoprotein products analyzed by SDS-PAGE and fluorography are indicated by "f" and "a," respectively. (**B**) All translation reactions were preceded by a 30-min incubation step at 37°C, which contained, where indicated, reticulocyte FRP, 200 μ*M* hemin, and varying GSB. Subsequent translation reactions and product analyses were conducted as described in (A) and (*10*). (**C**) The two-step protocol described in (B) was used. The first incubation contained reticulocyte FRP, 200 μ*M* $FeCl_3$ where indicated, and varying $H_2O_2$. (**D**) The two-step protocol described in (B) was used. The first incubation contained highly purified liver FRP, 200 μ*M* hemin, or 200 μ*M* FeNaEDTA, or 200 μ*M* $FeSO_4$ plus 200 μ*M* $Na_2H_2EDTA$, or 200 μ*M* hemin plus 600 μ*M* Desferal, where indicated, and GSB. (**E**) The two-step protocol described in (B) was used. The first incubation contained reticulocyte FRP, varying hemin or $Co^{3+}$-protoporphyrin IX, and GSB.

actions (*18*)—desferal (at 600 μ*M*), glutathione (at 2.5 m*M*), and protoporphyrin IX (at 200 μ*M*)—failed to influence the derepression of ferritin synthesis by hemin or its cobalt analog. Thus it seems unlikely that derepression by hemin is due to the nonspecific degradation of FRP.

That $Co^{3+}$ but not $Zn^{2+}$ can effectively replace $Fe^{3+}$ in the porphyrin complex suggests that a specific binding site for hemin may exist on the FRP molecule. Indeed, competition studies revealed that neither protoporphyrin IX nor $Zn^{2+}$-protoporphyrin IX at high concentrations significantly reduced the derepressive effect of hemin (*11*). The concentrations of hemin and $Co^{3+}$-protoporphyrin IX that produced half-maximal derepression of reticulocyte FRP were on the order of 50 μ*M*, as measured by densitomeric scanning of Fig. 1, A and E. This compares to the value of 5 to 20 μ*M* hemin that is required to inhibit the hemin-regulated inhibitor in reticulocytes (*20*). These numbers seem to define, at least in vitro, a relatively narrow concentration range for hemin in reticulocytes. At the upper end of this range hemin would induce ferritin synthesis for the storage of excess iron, whereas at the lower end it would activate the phosphorylation of eukaryotic initiation factor 2 for the shutoff of protein synthesis (*21*).

Our results show that hemin can derepress ferritin synthesis in vitro. However, it is not clear whether hemin plays a comparable role in vivo. Some investigators have argued that the agent that causes destabilization of transferrin receptor mRNA (*22*) and the induction of ferritin mRNA translation (*23*) is a chelatable form of iron, and therefore is not hemin or a close relative thereof. However, others have presented evidence suggesting that hemin itself may directly affect the former reaction (*24*) and, by inference, the latter. Our results are consistent with the second view; that is, that hemin can induce ferritin synthesis and probably also destabilize transferrin receptor mRNA (*25*). However, it should be emphasized that our results do not exclude the possibility that other, as yet unidentified, forms of iron may also serve the same function. It will be of interest to determine whether these other chelatable forms of iron are derived from hemin through the action of heme oxygenase, the synthesis of which is rapidly induced at the transcriptional level by exogenous hemin (*26*). In any event, the fact that the intracellular hemin concentration rises and falls in response to the external iron supply (*27*) is consistent with a role as an inducer of ferritin synthesis.

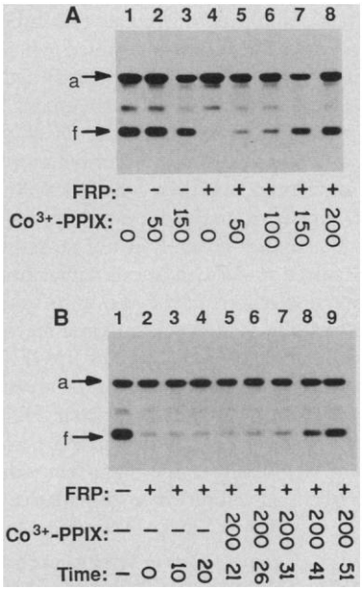


**Fig. 2.** Reversal of ferritin mRNA translational repression by a hemin analog. (**A**) Transcripts and FRP were incubated for 10 min at 0°C, and then varying concentrations of $Co^{3+}$-protoporphyrin IX were added, plus GSB. After incubation at 37°C for 30 min, the reactions were chilled, and translational components were added. Translation reactions and product analyses were as in Fig. 1 and (*10*). (**B**) Transcripts and FRP were mixed at 0°C, and at the indicated times, aliquots were withdrawn; after 20 min at 0°C, $Co^{3+}$-protoporphyrin IX plus GSB were added and the reaction temperature was increased to 37°C. Withdrawn aliquots were added to translation components at 0°C, and all translational reactions were started simultaneously.

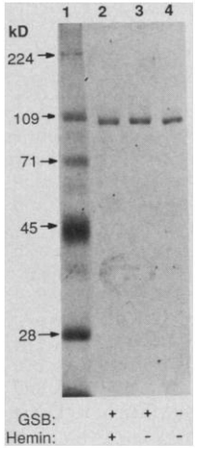


**Fig. 3.** Absence of chemical degradation or crosslinking of FRP by hemin. Highly purified liver FRP was either not incubated or incubated for 30 min at 37°C with GSB, or with GSB plus 200 μ*M* hemin, and analyzed in the absence of reducing agents by SDS-PAGE (8% acrylamide) and silver staining. Molecular size marker proteins were analyzed in lane 1.

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10. All porphyrin (Porphyrin Products, Logan, UT) solutions were made fresh immediately before use according to the manufacturer's instructions. Transcripts encoding rabbit L-ferritin and human apolipoprotein A-1 were prepared in vitro from plasmids with the use of T7 RNA polymerase (*8*). Homogenous FRP was from rabbit liver (*9*). A S3PI preparation of FRP was partially purified from rabbit reticulocytes (*7*) and then fractionated on heparin agarose (*9*). The peak fractions contained approximately 2% FRP. In most translation reactions, 9.4 μg of partially purified reticulocyte FRP or 0.1 μg of homogenous liver FRP were incubated for 30 min at 37°C with varying concentrations of hemin (or analogs thereof), a redox buffer (GSB) made up of a 5 to 1 mixture of glutathione to oxidized glutathione (usually at 2.5 and 0.5 m*M*, respectively), 20 m*M* Hepes-KOH, *p*H 7.4, and residual salts and buffers carried over from diluted stock solutions (these were approximately 70 m*M* KCl, 8 m*M* Hepes-KOH, *p*H 7.4, 7 m*M* tris-HCl, *p*H 7.4, 3% glycerol, 0.03 m*M* EDTA, 0.7 m*M* dithiothreitol and, in the case of liver FRP, 0.5 m*M* β-mercaptoethanol) in a final volume of 3.2 μl. The concentration of FRP in this first incubation reaction was 0.37 μ*M*. After the first incubation step the reactions were chilled, and other components necessary for translation were added, including 10 fmol of rabbit L-ferritin and 10 or 20 fmol of human apolipoprotein A-1 transcripts (final concentrations of 0.001 or 0.002 μ*M*, respectively) and other components as described (*8*). Translation reactions (final volume of 10 μl) were incubated at 24°C for 60 min, and products were analyzed by SDS-PAGE (12% acrylamide) and fluorography.
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# Inhibition of Angiogenesis by Recombinant Human Platelet Factor–4 and Related Peptides

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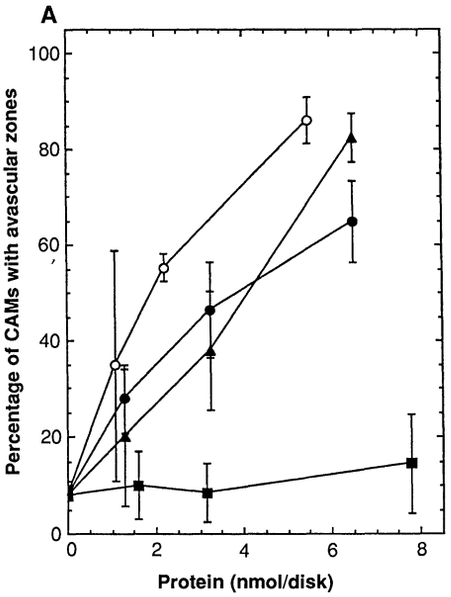
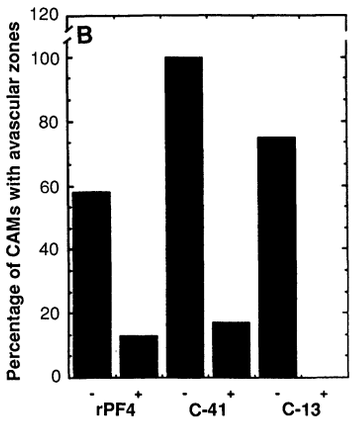
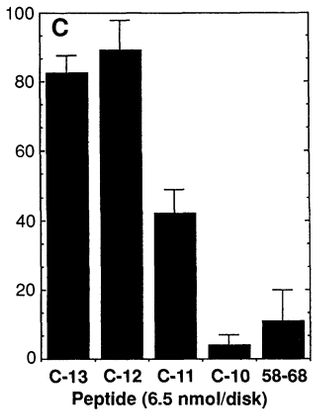
**Recombinant human platelet factor–4 (rhPF4), purified from *Escherichia coli*, inhibited blood vessel proliferation in the chicken chorioallantoic membrane in a dose-dependent manner. Treatment of several cell types with rhPF4 in vitro suggested that the angiostatic effect was due to specific inhibition of growth factor–stimulated endothelial cell proliferation. The inhibitory activities were associated with the carboxyl-terminal, heparin-binding region of the molecule and could be abrogated by including heparin in the test samples, an indication that sulfated polysaccharides might modulate the angiostatic activity of platelet factor–4 in vivo. Understanding of the mechanisms of control of angiogenesis by endogenous proteins should facilitate the development of effective treatments for diseases of pathogenic neovascularization such as Kaposi's sarcoma, diabetic retinopathy, and malignant tumor growth.**

PLATELET FACTOR–4 (PF4) IS A platelet α-granule protein originally characterized by its high affinity for heparin (*1, 2*). The protein is released from platelets during aggregation as a high molecular weight complex of a tetramer of the PF4 polypeptide and chondroitin sulfate (*3, 4*), which dissociates at high ionic strength. The complete primary structure of PF4 was determined by amino acid sequencing of proteolytic peptides (*5*) and later by isolation of the human gene (*6*). Although PF4 has several biological activities, including prevention of immunosuppression (*7*), chemotactic activity for neutrophils and monocytes (*8*) as well as for fibroblasts (*9*), inhibition of bone resorption (*10*), and inhibition of angiogenesis (*11*), its physiological significance is obscure. Of these activities, only the immunomodulatory effects have been confirmed with recombinant PF4 (*12*).

Angiogenesis inhibitors might be useful agents for treatment of solid tumors that require neovascularization for growth (*13*), and interest in this therapeutic approach has increased with the recognition that diseases




D

| Peptide | Sequence |
|---|---|
| | 58 60 65 70 |
| C-13 | P L Y K K I I K K L L E S |
| C-12 | L Y K K I I K K L L E S |
| C-11 | Y K K I I K K L L E S |
| C-10 | K K I I K K L L E S |
| 58-68 | P L Y K K I I K K L L |

**Fig. 1.** Inhibition of angiogenesis by recombinant PF4 and related peptides. Angiostatic activity was measured by blind assessment of the presence or absence of avascular zones in the chicken CAM as described (*14*), with minor modifications. Three-day-old fertilized eggs were carefully removed from their shells and placed into petri dishes in an incubator with 3% $CO_2$, test substances were implanted on day 6, and vessel growth was assessed on day 8. Implant disks were made by air-drying 10-μl aliquots of a protein solution containing 0.5% (w/v) methyl cellulose. (**A**) Inhibition of angiogenesis by rhPF4 (●), C-41 (○), C-13 (▲), and N-29 (■). Data represent the average inhibition from several assays; at least ten sample disks were implanted per assay. Error bars represent SEM. (**B**) Heparin at 50 μg/disk (Upjohn) was either included (+) or omitted (−) from test samples during implant preparation. Peptides were present at 6.5 nmol/disk. Methyl cellulose alone or with heparin was inactive in these assays. (**C**) Inhibition of CAM angiogenesis by short carboxyl-terminal peptides of human PF4. Each peptide was tested at 6.5 nmol/disk in a minimum of three assays (30 implants). Error bars represent SEM. (**D**) Sequences of short carboxyl-terminal peptides used in these studies.

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