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An Ancient Role for a Prokineticin Domain in Invertebrate Hematopoiesis

Irene Söderhäll, Young-A Kim, Pikul Jiravanichpaisal, So-Young Lee, and Kenneth Söderhäll

Hematopoietic development requires firm control of cell proliferation and differentiation. Although recent research has revealed conserved function of transcription factors and signaling pathways regulating lineage commitment in hematopoietic development in *Drosophila melanogaster* and vertebrates, little is known about hematopoietic cytokines among the invertebrate phyla. In the present study, we show that differentiation and growth of hematopoietic stem cells in vitro from an invertebrate, *Pacifastacus leniusculus*, require an endogenous cytokine-like factor, astakine, containing a prokineticin (PK) domain. Astakine induces a strong hematopoiesis response in live animals. An astakine homologue was also found in the shrimp, *Penaeus monodon*. So far, PK domains are only identified in vertebrates, in which they, for example, direct angiogenic growth. Our finding of the first PK-like cytokine characterized from any invertebrate provides novel information concerning the evolution of growth factors and blood cell development. *The Journal of Immunology*, 2005, 174: 6153–6160.

Of all existing animal species at least 95% are included in the invertebrate taxa. These animals have evolved a variety of different strategies to defend their bodies against intruding pathogens. Lacking the memory of vertebrate immunity, invertebrate animals have to rely solely on innate immune mechanisms in their host defense. These include clotting and coagulation reaction (1, 2), phagocytosis and cellular encapsulation (3), antimicrobial peptides (4), lectins and pattern recognition proteins, and components of the prophenoloxidase (proPO) activating cascade (3). Most of these processes are conducted by or originate from the blood cells, the so-called hemocytes, and hence hematopoiesis is vital for invertebrate animals. There are several parallels between vertebrate and invertebrate innate immune defense, and some immune-related genes such as TLR are found in most animal phyla (5). In vertebrates, cytokines act as important coordinators of innate as well as adaptive immune responses. These soluble intercellular signaling molecules, which are secreted by a variety of cell types, form a complex network for regulation of immunity. Accordingly, the need for regulation of cellular immunity, as well as of hematopoietic development, has led to an intense search for invertebrate cytokines during the past decades. Functional homologues to vertebrate cytokines have been described mainly based upon immunocytochemical methods showing cross-reactivity of invertebrate cytokine-like molecules with Abs against vertebrate cytokines (6). Recent progress in genome-wide sequence analysis has enabled more detailed information on immune-related genes in invertebrates. Domain-based protein analysis of worm (Caenorhabditis elegans), fruit fly (*Drosophila melanogaster*), and ascidian (*Ciona intestinalis*) genomes have not revealed the presence of IL-like domains in any of these species (7). Evidence for cytokine-like activity was indicated for PDGF/VEGF family 2, a platelet-derived growth factor/vascular endothelial growth factor (VEGF)-like growth factor in *D. melanogaster*, because overexpression of this gene influenced the number of plasmocytes in the larvae (8). One TNF-like and three TNFR-like genes were detected in *C. intestinalis*, and also IL-1R-like genes have been found, three in *C. intestinalis* and one in the *D. melanogaster* genome (7). A cytokine-like molecule (coelomic cytolytic factor) found in the earlworm, *Eisenia fetida*, with lectin-like activities similar to TNF instead suggests a convergent evolution of cytokines (9). Similarly, the ENF peptides found in some lepidopteran insects have cytokine-like activity, but they lack any sequence homology to vertebrate proteins (10, 11).

Recent progress has revealed conserved function of transcription factors and signaling pathways regulating proliferation and lineage commitment in hematopoietic development in *D. melanogaster* and vertebrates (12). One such conserved signaling pathway is the JAK-STAT pathway, and in *Drosophila*, the JAK and STAT homologues, hop and Dstat92E, respectively, have been identified and appear to function in a similar manner to their mammalian counterparts. However, the only known JAK/STAT ligands in flies, upd, upd2, and upd3, lack any similarity to the various cytokines and growth factors that initiate activation of this system in mammals (13). The expression of *upd* is triggered in hemocytes by septic injury (13), and this cytokine is speculated to be released into the hemolymph and then activates the JAK/STAT-signaling pathway in the fat body (14). This activation was dependent of the *Drosophila* homologue of vertebrate class I cytokine receptor Dome, which is suggested to be the receptor for upd3 (14).

In this study, we for the first time show that a prokineticin (PK)-like cytokine is involved in hematopoiesis in an invertebrate, the freshwater crayfish, *Pacifastacus leniusculus*, and we also identified a similar homologue in another crustacean, the shrimp, *Penaeus monodon*.

Materials and Methods

Experimental animals

Freshwater crayfish, *P. leniusculus*, were purchased from Berga Kräftodling and were maintained in tanks with aerated running water at 10°C. Only intermolt crayfish were used.
Hemopoietic stem cells and culture

The hemopoietic stem cells were isolated as described previously (15). The cells were washed in cryash PBS and incubated in L15 medium (Sigma-Aldrich) supplemented with 5 μM 2-ME, 1 μM phenylthiourea, 60 μg/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml gentamicin (Sigma-Aldrich), and 2 mM L-glutamine at a density of 5 × 10^5 cell/ml.

Hpt cell-stimulating activity

The fractions to be tested were added to the hpt cell culture, and cell spreading activity was observed after 24 h. At different time intervals, total RNA was extracted from the cultured cells using Gene Elute Total Mammalian RNA extraction kit (Sigma-Aldrich) and expression of PI3K (GenBank accession no. AY630695), and proPO was analyzed by RT-PCR, using Thermoscript (Invitrogen Life Technologies) and the following primers: PI3K forward, 5'-CAGAGGAGGGAATTGTGAGG-3' and reverse, 5'-TCGTGATGTCAGTTGAAGC-3'; for proPO, forward, 5'-AGTAGAACAGCTCCTACACTGC-3' and reverse, 5'-ACTGATGTCTTATGAAATTCCGGC-3'. Different numbers of PCR cycles were performed to semiquantitatively compare the expression, and the expression of a 40S ribosomal protein was used as a control.

BrdU labeling and detection

DNA synthesis was determined by incubating the cultured cells with 10 μM BrdU. After 16 h, the cells were fixed with 70% ethanol in 50 mM glycine. BrdU incorporation was detected as described previously (15).

In situ hybridization

Cultured stem cells were analyzed by in situ hybridization using cDNA probes for proPO and crayfish 40S ribosomal protein as described previously (15). Briefly, cells were cultured attached to slides and fixed with 95% ethanol for 5 min at room temperature and stored in 70% ethanol at −20°C until used in hybridization experiments. Sequences corresponding to bp 550-1254 of proPO (GenBank accession no. X83494) and bp 359 of a 4OS ribosomal protein were labeled with digoxigenin (DIG) using Kle-

Purification and molecular cloning of astakine

All purification steps were performed at 4°C. Throughout the purification, fractions containing cell spreading activity were collected, and at the final stage of purification, BrdU incorporation experiments were performed to semiquantitatively compare the expression, and the expression of a 40S ribosomal protein was used as a control (15).

Expression and purification of recombinant protein

The entire open reading frame of mature astakine was amplified by PCR using forward primer, 5'-GGCCTTTGTAACAGGCCAGGAC-3' and reverse primer, 5'-TTATCTGAGGCTTGTAGTGAGGCTGACG-3'. The PCR product was inserted into the pET42a expression vector (Novagen). *Escherichia coli* strain BL21 (DE3) (Novagen) was transformed by the pET42-astakine plasmid, and the recombinant protein was purified on Ni-NTA agarose (Qiagen). The detailed protocols for production of fusion proteins are as follows: E. coli cells were grown to absorbance 0.6 at 600 nm, and then, expression was induced with 1 mM isopropyl-1-thiogalactoside for 16 h at 23°C. The bacterial cells were harvested, and the cell pellet was suspended in 50 ml of lysis buffer (50 mM sodium phosphate (pH 8.0) and 300 mM NaCl) per 1 liter of culture volume. The cells were sonicated three times for 10 s (Sonifier cell disruptor B-30; Branson Sonic Power), followed by centrifugation at 13,000 × g for 30 min at 4°C. The pellet was resuspended in a 6 M urea buffer A (6 M urea, 100 mM sodium phosphate, 10 mM Tris, and 1 mM 2-ME (pH 8)). Fusion proteins were allowed to bind onto Ni-NTA beads and then washed ex- tensively with buffer B (6 M urea, 0.5 M NaCl, 10 mM Tris, and 1 mM 2-ME (pH 8)) and then washed by a gradient of buffers B and C (6 M urea, 0.5 M NaCl, 10 mM Tris, 1 mM 2-ME (pH 8), and 20 mM imidazole). To remove urea, the Ni-NTA column was washed with a gradient of buffers C and D (6 M urea, 1 M NaCl, 10 mM Tris, 1 mM 2-ME, and 0.5 mM 2-ME (pH 8), and 20 mM imidazole). Fusion protein-bound beads were equilibrated with digestion buffer (50 mM Tris, 150 mM NaCl, and 1 mM CaCl2 (pH 7.5)). Digestion was performed overnight at room temperature with 34 U of protease factor Xa (Novagen) per 20 ml of reaction volume (10 ng of factor Xa/μl of fusion protein). The cleaved recombinant astakine was collected in the flow through. The remaining protease factor Xa was removed by binding to a X-arrest agarose slurry (Novagen). Trifluoroacetic acid (TFA) was added to the recombinant astakine (0.05% final concentration), and the acidified sample was loaded onto Sep-Pak C18 cartridge (Waters) equilibrated with 0.05% TFA. The cartridge was washed with 0.05% TFA and eluted with 80% acetonitrile containing 0.05% TFA. The eluted fractions were vacuum-dried and stored at −20°C until use. The refolding buffer (1 mM oxidized glutathione, 0.2 mM reduced glutathione, 0.02% Tween 20, 10% glycerol, 10 mM Tris (pH 8), 150 mM NaCl, and 100 mM sodium phosphate (pH 8)) was added to the dried sample and incubated overnight at 4°C. Finally, the buffer was changed into modified L15 medium by passing it over a PD-10 (Amersham Biosciences) column according to the manufacturer’s instructions.

Abs and Western blot analysis

Recombinant astakine was used for production of a rabbit antisem. The amount of protein for injection in each rabbit was 150 μg. Abs were affinity purified on a column containing recombinant astakine coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). Recombinant astakine (1.5 mg) was used to couple to 0.14 g of CNBr-activated Sepharose 4B, according to the manufacturer’s instruction. Astakine rabbit antisem (0.75 ml) was diluted 1:1 with TBS (10 mM Tris (pH 7.5) and 0.9% NaCl) and passed through the affinity column twice. After washing the column with 1 ml of 50 mM Tris (pH 7.5) and 50% ethanol, the antiserum (0.75 ml) was eluted with 0.5 M urea (pH 2.5). The eluted fractions were neutralized by adding 1 M Tris (pH 7.5). For immunoblotting, the plasma proteins were subjected to 15% SDS-PAGE under reducing conditions and then electrotransferred to Hybond-C

strong reducing conditions (16) for determination of purity and amino acid sequences.

For amino acid sequence determination, the protein bands were excised from gels and cleaved with trypsin by in-gel digestion. The peptides were analyzed by electrospray ionization mass spectrometry on a Q-tof mass spectrometer (Waters) using the Mass Lynx software.

For cDNA cloning, two nested degenerate primers were designed for 5'-end according to the amino acid sequence 5'-APLGEMGNYFQPVK-3', 5'-ACRTTTAALKTGTIGT-3', and 5'-TRCCAAART GITYGCG-3'. Total RNA was isolated from hemocytes using Gene Elute Mammalian Total RNA extraction kit (Sigma-Aldrich), and 5'-RACE was performed using 5'-RACE System for Rapid Amplification of cDNA ends (Invitrogen Life Technologies). All PCR products were subcloned into a pCR2.1-TOPO (Invitrogen Life Technologies), according to the manufacturer’s instructions, and sequenced by ABI PRISM 377 DNA sequencer. The primers used for the sequencing reaction were designed to the deduced amino acid sequence.

The nucleotide positions of the synthesized primers were according to Ref. 17. The deduced amino acid sequence was analyzed using the Basic Alignment Search Tool search program (National Center for Biotechnology Information), and multiple sequence alignment was performed according to Ref. 17.

For amino acid sequence determination, the protein bands were excised from gels and cleaved with trypsin by in-gel digestion. The peptides were analyzed by electrospray ionization mass spectrometry on a Q-tof mass spectrometer (Waters) using the Mass Lynx software.

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extra membranes (Amersham Biosciences) in transfer buffer (25 mM Tris-HCl, 190 mM glycine, and 20% methanol) for 2 h at 280 mA on ice. All the following steps were performed at room temperature. The membrane was blocked subsequently in TTBS (1% Tween 20 in 20 mM Tris-HCl and 150 mM NaCl (pH 7.4)) containing 3% gelatin for 1 h and washed briefly three times and then incubated with astakine Ab (0.2 ng/ml) in TTBS overnight at 4°C. Then, the membrane was washed with TTBS for 20 min, a procedure that was repeated three times. The ECL anti-rabbit IgG peroxidase-linked, species-specific whole Ab from donkey (Amersham Biosciences) diluted 1/5000 with TTBS was incubated for 1 h and washed with TTBS for 20 min three times. For detection, the ECL Western blotting reagent kit (Amersham Biosciences) was used, according to the manufacturer’s instructions.

cDNA cloning of shrimp astakine

Degenerate primers were designed from the conserved region of astakine cDNA in both directions: forward-1, 5'-GTYYTGTTGTTGCGTATGTA-3', forward-2, 5'-CATGCCGGCGGIGITSYGAY-3', forward-3, 5'-GAY GGGCCTWIGARTGYTGY-3', and reverse, 5'-IARICCIICCICRKCAIG GRCACAT-3'. Each pair of forward primer and T7 or one-pair reverse primer and T3 was used to amplify DNA fragment from a shrimp (P. monodon) hemocyte cDNA library (UNI-ZAP XR cDNA) by using PCR. The resulting PCR products from the reverse primer and T3 were purified and then cloned into a TOPO vector (Invitrogen Life Technologies) and sequenced as above. The resulting PCR product contained a sequence encoding the anticipated conserved amino acids CLPLDGL. Based on this sequence, specific oligonucleotide primers were designed from both plus and minus strands: forward-1, 5'-TTGTCATGGTGCTTGCTTG-3'; reverse-1, 5'-TGCCGAGGTTAAGAGTCGGT-3'; forward-2, 5'-ATCAGGCCGCTCCTGTT-3'; forward-3, 5'-GACGACCGCCTTGGGC GACCTGGG-3'; and reverse-2, 5'-CCACGTTCGCAAGGGG-3'. The UNI-ZAP XR cDNA library from hemocytes of shrimp was amplified by PCR, using the different specific primers in combination with T3 or T7, and then cloned into a TOPO vector (Invitrogen Life Technologies) and sequenced as above. The resulting PCR products from the reverse primer and T3 or T7, and the resulting PCR products were cloned and sequenced as above.

Injections and bleeding

Crayfish were injected at the base of a walking leg with 50 μl of 1 mg/ml LPS (E. coli 055:B5; Sigma-Aldrich) or CFS. Injections were also made with 50 μg of dsRNAa arranged in crayfish saline, was injected into the base of a walking leg. The injection was repeated after 24 h, and at the same time, an injection was made with 50 μl of 1 mg/ml LPS (E. coli 055:B5; Sigma-Aldrich). Control animals were injected with an equal volume of crayfish saline or GFP siRNA. THC values were determined for all crayfish at the start of the experiment and at different time intervals after RNAi treatment. Total RNA was extracted from hemocytes at different time intervals after RNAi treatment and analyzed by RT-PCR as above.

RNA interference (RNAi) experiments

Small interference RNA (siRNA) molecules were designed from the astakine nucleotide sequence using Qiagen siRNA design software (Qiagen), and the sequences were as follows: 5'-AACAGCCAGGAGCG CCGACTGT-3' (a75) and 5'-CGGCGCCGTTAAGGGATT-3' (a192). siRNA for GFP was used in transfection experiments as a control. dsRNA for astakine was constructed containing 350 bp using Megascript T7 (Ambion). Semigranular (SG) hemocytes were isolated as previously described (16) and were attached to the surface of tissue culture plates and incubated in modified L15 medium. The SG cells were treated with siRNA (a75 or GFP) and Effectene transfection reagent (Qiagen), according to the instructions from the manufacturer, and incubated at 16°C. After 24 h, the medium was replaced with fresh medium containing siRNA and Effectene. The spent medium after the second 24-h period was used to induce spread- ing hpt cells, and we named this protein astakine (Fig. 2C).

Purified astakine stimulated hpt cells at concentrations ranging from 0.1 to 6 μg/ml (Fig. 3, A–C). Mature 5G and granular (G) hemocytes express the transcription factor PiRunt as well as proPO. However, in hpt cells, normally <4% of the cells express the proPO transcript, and PiRunt can only be found at a low level (15). In hpt cells cultured in vitro, PiRunt (data not shown), as well as proPO mRNA (Fig. 3, D and E), could be detected by in situ hybridization as well as by RT-PCR, 30–60 min after addition of astakine to the cultured cells (Fig. 3F). Both transcripts were found mainly in spread cells that by their morphology were judged as most similar to SG cells.

Astakine is a prokineticin homologue

To further confirm that astakine was the responsible molecule, we determined the partial amino acid sequences of three trypsin-digested fragments as follows: APLGEAGSSCNNVTQPQVPK, YLGFCPCR, and QLPSQDNTLDSSYY. We used the first sequence to design degenerate primers, and by 5'-RACE technique, we were able to isolate a 265-bp cDNA fragment from the hemocyte mRNA. To obtain a full-length cDNA, this fragment was used for designing primers used in 5'- and 3'-RACE, and finally, a cDNA encompassing 1.017 kb encoding a 104-aa residue protein with a putative signal peptide of 22 aa and a mature protein of 82 aa was identified (Fig. 4A). The calculated molecular mass for astakine is 10,948 Da and after cleavage of the signal peptide is 8,798 Da. Sequence analysis of the cDNA for astakine shows that it contains a conserved PK domain (Fig. 4, A and B), with 10 cysteine residues in the mature protein that potentially can form five disulfide bridges. The deduced protein sequence of astakine cDNA shows some homology with the PK domain of the Bv8 peptides from the frog Bombina sp., as well as of prokineticins from mammals (31% identity, 54% similarity with Bombina variegata GenBank accession no. AAD45816, and 36% identity, 50% similarity with Homo
sapiens PK2, GenBank accession no. NP068754) (Fig. 4B). Interestingly, the termination codon is followed by an untranslated region containing a high number of repeats. Using degenerate primers for astakine, a cDNA coding for a similar protein was isolated from a P. monodon hemocyte cDNA library. The deduced amino acid sequence of this shrimp protein is 124 aa with a signal peptide of 21 aa (Fig. 4C). The shrimp protein shares 40% identity and 52% similarity with astakine, and the protein has an insert of 13 aa after residue 44 that is similar to PK2 from bull (Bos Taurus, NP955516) that has a similar insert together with an additional arginine-rich part. Construction of a recombinant astakine was performed in the pET-42 system, and the recombinant protein showed stimulating effect on hpt cell spreading, attachment, and proliferation. Both recombinant and purified astakine moved in SDS-PAGE as a 15-kDa protein under reducing as well as nonreducing conditions (Fig. 2C). A similar pattern in SDS-PAGE was found for the recombinant human PK with a calculated mass of 9667 Da (18), and therefore, we assume that astakine moves as a monomeric nonglobular molecule.

SG cells release astakine

Because astakine cDNA was isolated from a crayfish hemocyte library, we decided to use SG and G cells to further confirm the activity of this protein. Both cell types were found to express astakine; however, the level in SG cells was significantly higher. When SG cells were isolated and incubated in a modified L15 medium for 5–24 h, the spent medium was found to have hpt cell-stimulating activity. Purification of SG cell-conditioned medium for astakine RNAi-silenced SG cells (Fig. 5A), and the level of the astakine transcript was decreased significantly in the RNAi-silenced SG cells (Fig. 5B). In contrast, cells treated with siRNA for GFP (as a control) still secreted astakine. Thus, these experiments clearly show that the hpt cell-stimulating activity in SG cell-conditioned medium is mediated by astakine and that silencing of the astakine gene results in a lower level of astakine protein in the SG cell during culture.

Astakine influences hematopoiesis in vivo

Astakine present in the plasma was demonstrated to have in vitro cytokine-like activity, and therefore, we extended our study further...
FIGURE 4. Astakine cDNA, amino acid sequence, and alignment with vertebrate PK. 
A. The 1.017-kb crayfish astakine encodes a 104-aa residues protein with a signal peptide of 22 aa (underlined) and a mature protein of 82 aa. The PK domain is marked by italics from aa 6 to 86. The mature protein contains 10 cysteine residues that potentially can form five disulfide bridges. The termination codon is followed by an untranslated region, containing a high number of repeats. B. Sequence alignment of mature astakine with mature PK domain-containing proteins from different species. PI = P. leniusculus GenBank AY787656, FrPK2 = sequence is from Ref. 20, HsPK1 = human AAQ89046, BtPK2 = B. taurus NP955516, MmPK2 = mouse PK2 AAM4572, HsPK2 = human PK2 NP068754, and Bv8 = B. variegata AAD45816. Red boxes indicate identity, and blue-boxed residues show similarities. C. Sequence alignment of crayfish astakine (PI) with the shrimp (Pm, P. monodon; GenBank AY787657) PK domain-containing protein. Red boxes indicate identity, and blue-boxed residues show similarities.
to also include the function of astakine in vivo. Because LPS or β1,3-glucans induce increased release of hemocytes from the hemopoietic tissue in vivo (15), we first compared plasma from LPS and sham-injected crayfish regarding the content of astakine. As shown in Fig. 6A, the protein was hardly detectable in nonchallenged plasma (Fig. 6A, lane 1), whereas LPS-injected animals had a high concentration of astakine (Fig. 6A, lane 2). Western blot analysis similarly showed an increase in astakine plasma content after LPS injection (Fig. 6A, lanes 3 and 4). However, no significant difference was detected in gene expression of astakine after LPS injection (Fig. 6B), and this suggests that the increased amount of astakine in LPS-injected plasma is due to some other mechanism such as increased translation, stimulation of release of astakine from the hemocytes, decreased turnover of the protein, or a combination of these processes.

Injection of purified as well as recombinant astakine (0.02 μg/g weight of crayfish) into freshwater crayfish also induced rapid increase in the THC, whereas injection of a cysteine rich antibacterial peptide as a control (GenBank accession no. CF542616) did not affect the number of circulating hemocytes (Fig. 6C). This result shows that astakine induces an increased hemocyte synthesis and plays a critical role in the hemopoietic process also in vivo.

We also performed in vivo gene inactivation experiments to further confirm the requirement for astakine in hpt cell differentiation. dsRNA of 350 bp for astakine was found to efficiently silence astakine expression, whereas siRNA similarly decreased the expression level but to a lower extent. Injection of siRNA and large dsRNA was performed twice with 24-h intervals, and silencing of astakine expression lasted for 30–48 h (Fig. 7A). The THC in crayfish after astakine silencing did not change significantly during the short period of silencing. However, when LPS was injected into animals to cause a drop in hemocyte number in the circulation down to 10% of normal, then there was no recovery of hemocyte numbers (THC) in astakine-silenced animals, whereas in the control silenced animals the THC returned to normal levels soon after injection of LPS (Fig. 7B).

**Discussion**

The results presented here demonstrate the presence of an invertebrate cytokine that is directly involved in hemopoiesis. This cytokine, astakine, has structural similarities with vertebrate PK, such as endocrine gland-derived VEGF (EG-VEGF) (19–23), and thus, our finding may shed new light upon the evolution of blood cells and vascular endothelial cells from invertebrates to vertebrates. In *Drosophila*, the VEGFR homologue is expressed in hemocytes and is involved in the early migration of plasmacytocytes into areas of ligand-expressing cells in the embryo (24, 25), in a similar manner as vertebrate VEGF (26). In addition, this receptor was shown to function in antiapoptotic hemocyte survival during embryogenesis (27). The recent discovery of EG-VEGF as a mitogen selective for endothelial cells of endocrine glands has provided a novel view of the regulation of angiogenesis (23, 28, 29), and our finding of an EG-VEGF homologue initiating a rapid differentiation of hemopoietic stem cells adds a novel function to this new family of angiogenetic factors.

**FIGURE 5.** Astakine is released from SG hemocytes. *A*, Cell spreading effect of conditioned medium (CM) from SG cells ( ), after treatment with siRNA_{control} as a control ( ), and with siRNA_{astakine} ( ). Mean ± SD for three experiments. *B*, Levels of astakine transcript in SG hemocytes as determined by RT-PCR. Lane 1 = silencing with siRNA a75, lane 2 = control silencing with siRNA GFP, and lane 3 = nontreated SG hemocytes.

**FIGURE 6.** Astakine affects hemopoiesis in vivo. *A*, LPS injection into live animals induces increased amount of astakine in the plasma; lane 1 = plasma from control animals, and lane 2 = plasma from LPS injected were subjected to ultracentrifugation (200,000 × g, 2 h), and then, 15 μg of proteins from the resulting supernatant were loaded on SDS-PAGE. Lanes 3 and 4: immunoblotting of plasma treated as above from control (lane 3) and LPS-injected animals (lane 4). *B*, Expression of astakine in hemocytes after LPS injection as detected by RT-PCR. One microgram of RNA was used in each reaction, and the PCR products were detected after different numbers of cycles, down to the detection limit at 15 cycles; (−) = sham injection, (+) = LPS injection. *C*, THC index after injection of purified (■) and recombinant astakine (r-astakine, •). THC index was calculated as total number of hemocytes after treatment/initial total number of hemocytes. Control injections were crayfish saline (□) or a cysteine rich 15-kDa crayfish antimicrobial peptide (cabp1, GenBank accession no. CF542616; □), and recombinant astakine with the fusion tag uncleaved (r-astakine + Tag; ◦). Mean ± SD for three experiments.
Runx transcription factors are involved in vertebrate hematopoiesis, Drosophila crystal cell development (12, 22), as well as SG and G cell differentiation in crayfish (15). Runx genes encoding a conserved family of DNA-binding proteins were discovered originally in Drosophila (30). The three mammalian Runx genes, which encode several isoforms due to alternative splicing or variable promoter usage, produce transcription factors that are involved in normal development and have tissue-specific patterns of expression. Recent evidence also point to a role for these genes as oncogenes and tumor suppressors (31). In addition to a role in embryonic hematopoiesis and differentiation of megakaryocytes and lymphocytes during adult hematopoiesis (32), recent reports also support a role for Runx genes in angiogenetic growth, and vascular endothelial cells express Runx genes activated by angiogenic factors (33). In vertebrates, the close association between embryonic hematopoiesis and developing blood vessels has indicated a common origin of vascular endothelial and hemopoietic cells (34), and recent findings in Drosophila (35) as well as in humans (36) have provided evidence for a common precursor, a hemangioblast, with endothelial as well as hemopoietic capacity. SG as well as G cells in crayfish express the PI Runt transcript, and in hpt cells, the expression of PI Runt is activated by astakine. These findings, taken together, may indicate that the PK (or EG-VEGF) originally functioned in primitive blood cells and later did evolve functions in tissue-specific angiogenetic. In a simplified way, one can postulate that because most invertebrates lack a closed circulatory system, the hemocytes can be considered as multifunctional, as immunocytes and as vascular endothelial cells, for example in wound healing, and that production of these cells resembles the synthesis of angiogenetic tissues in vertebrates. Astakine induces increased transcription of the PI Runt gene as well as of the proPO gene in vitro. An increased astakine content in plasma in vivo results in an increased transcription of PI Runt inside the hemopoietic tissue, whereas the proPO transcript is not detected until the hemocytes are released into the circulation (15).

Sequence analysis of crayfish astakine as well as of the shrimp homologue revealed that these proteins contain a conserved PK domain. The first member of this family of cysteine-rich secreted proteins to be sequenced was the black mamba venom protein (MIT1) isolated from Dendroaspis polyepilepse in 1998 (37). The mamba venom protein contains 80-aa residues and five disulfide bridges, and the solution structure of this protein is similar in its global fold to colipases involved in fatty acid digestion (38). A similar five-disulfide bridge domain was detected in the small protein Bv8 isolated from skin secretions of Bombina species, which stimulates contraction of guinea pig ileum (39). Receptors for Bv8 were detected in mammalian tissues (40), and mouse as well as human homologues of frog Bv8 have been characterized and found to be highly expressed during spermatogenesis (41). Interestingly, human PK2 is expressed rhythmically in the suprachiasmatic nucleus, which controls circadian rhythm of physiological and behavioral processes in mammals (42). A different function has been assigned recently to PK1 (EG-VEGF) when this molecule was shown to induce proliferation, migration, and fenestration in capillary endothelial cells (28, 29) and also as a stimulator of angiogenesis in testis (29). Expression of human Bv8 homologues is mainly found in testis and in ovary but also in peripheral blood leukocytes where its role still remains unclear (29).

PK contain 10 cysteine residues that in the black mamba venom protein MIT1 forms five disulfide bonds (38), and these cysteine residues are all conserved in the two crustacean astakines. The N-terminal sequence of all known vertebrate PK contains seven amino acids that are completely conserved (AVITGAC), and these are critical for the biological activity (19). This N-terminal sequence is not present in the crayfish astakine, although Gly5 and Cys7 are conserved in the mature protein, and the C-terminal cysteine-rich domain shares high similarity with the vertebrate PK. Also, a shrimp homologue of astakine was characterized, and the shrimp protein shares 40% identity and 52% similarity with astakine. The N-terminal sequence of the shrimp astakine is, as the crayfish astakine, different from its vertebrate homologues in that it lacks five of the seven conserved N-terminal amino acids. Therefore, the suggested naming of these proteins as AVIT protein family (19) may not be appropriate because it excludes at least the crustacean homologues of PK domain proteins from this group with similar biological activity. The shrimp astakine was found to be most similar to the B. Taurus PK2 and both sequences have an insert of 13 aa after residue 44 (in the bull followed by an additional arginine-rich part). The two crustacean astakines are also similar in having tyrosine near their C-terminal end, and a tyrosine rich C-terminal is also found in the Fugu as well as of the rainbow trout sequence (GenBank accession no. BX 859212), which may indicate a common function of the C-terminal end in lower animals.

Astakine as well as vertebrate PK also share some similarities with the Dickkopf (Dkk) family of cysteine-rich proteins and some weak similarity with colipases. Dkk proteins are implicated as critical signal molecules in developmental processes and are known to act as Wnt antagonists and are necessary for head development in
Xenopus (43). Dkk-1 is also known to be crucial for the proliferation of adult human mesenchymal stem cells from bone marrow stroma (44). These cells are implicated in having an important role in tissue repair throughout the body and are able to differentiate into numerous different mesenchymal cell lineages (45). The family contains two cysteine-rich domains among which the C-terminal part has similarity to PK domains. Recently, Dkk-related proteins have been found in the venom of a funnel-web spider (46) and in the freshwater polyp Hydra, where it was expressed in differentiating nematocytes (47). By searching expressed sequence tag and genome databases, Dkk-related sequences were identified in the genome of Ciona intestinalis, indicating an origin of this cysteine-rich domain at an earlier stage of evolution (47).

Astakine is a new cytokine in invertebrates containing a PK domain, and the isolated molecule is directly involved in hematopoiesis and blood cell differentiation. In conclusion, this finding of an invertebrate PK-like protein that can induce hemopoietic stem cells to differentiate provides novel information concerning the evolution of these secreted cysteine-rich developmental regulators.

Disclosures

The authors have no financial conflict of interest.

References