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Azurocidin, a Natural Antibiotic from Human Neutrophils: Expression, Antimicrobial Activity, and Secretion

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The azurophil granules of human PMN contain four antibiotic proteins, the serprocidins, which have extensive homology to one another and to serine proteases. Azurocidin, a member of this family, is a 29-kDa glycoprotein with broad spectrum antimicrobial activity and chemotactic activity toward monocytes. Insect cells transfected with a baculovirus vector carrying azurocidin cDNA produced a recombinant azurocidin protein. We purified the recombinant azurocidin protein from the culture medium of the infected cells and showed that it retained the antimicrobial activity of the native neutrophil-derived molecule. In addition, we present evidence that a 49-amino-acid region of the recombinant azurocidin protein is required for its secretion from insect cells.

The azurophil granule, a specialized lysosome of human neutrophils, contains a family of antimicrobial proteins with structural homology to serine proteases, the serprocidins (1,2). Three members of this family, cathepsin G, elastase, and proteinase 3 (PR-3),[5] are serine proteases while the fourth one, azurocidin, is a proteolytically inactive homolog (3,4). The cDNA for all four proteins have been isolated and sequenced (1,5–7) and the genomic structure of the cluster formed by azurocidin, elastase, and PR-3 has been reported (8). Azurocidin [also known as cationic antimicrobial protein 37 (9), or human heparin-binding protein (10)] is a 29-kDa glycoprotein with broad spectrum antimicrobial activity (3,11). Azurocidin has potent antibiotic activity against gram-negative bacteria, which may depend on an initial interaction with the negatively charged lipopolysaccharide present on the gram-negative bacterial cell surface (12). Azurocidin was also able to kill the gram-positive bacterium *Streptococcus faecalis* and the fungus *Candida albicans* (3). In addition to its antimicrobial activity, azurocidin is a strong chemotactic agent for monocytes *in vitro* (13) and stimulates their survival (14). It can also mediate reversible contraction of fibroblasts and endothelial cell monolayers (14). Neither the mechanism(s) nor the structural features necessary for the various activities of this natural antibiotic are known. To facilitate the structure–function analysis of this protein, we developed an expression system that leads to the synthesis of a recombinant azurocidin molecule. Insect cells infected with a baculovirus vector carrying the azurocidin cDNA secreted the protein into their culture medium. The purified recombinant azurocidin had an activity comparable to that of the native neutrophil-derived antibiotic protein. In addition, we present evidence that a determinant(s) located within 49 residues of the molecule is required for its secretion.

MATERIAL AND METHODS

Reagents

Restriction endonucleases were from New England Biolabs (Beverly, MA). T4 DNA ligase and calf intestine

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[5] Abbreviations used: Azu, azurocidin; PR-3, proteinase 3; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; rpHPLC, reverse phase high-performance liquid chromatography; PVDF, polyvinylidene difluoride membrane; CAPS, 3-(cyclohexylamino)-1-propane-sulfonic acid; DTT, dithiothreitol; PCR, polymerase chain reaction; dNTP, deoxyribonucleoside triphosphates; endo F, endoglycosydase F; GNA lectin, *Galanthus nivalis* lectin; LD_{50}, lethal dose 50; MBP, maltose binding protein; *his J*, histidine binding protein.

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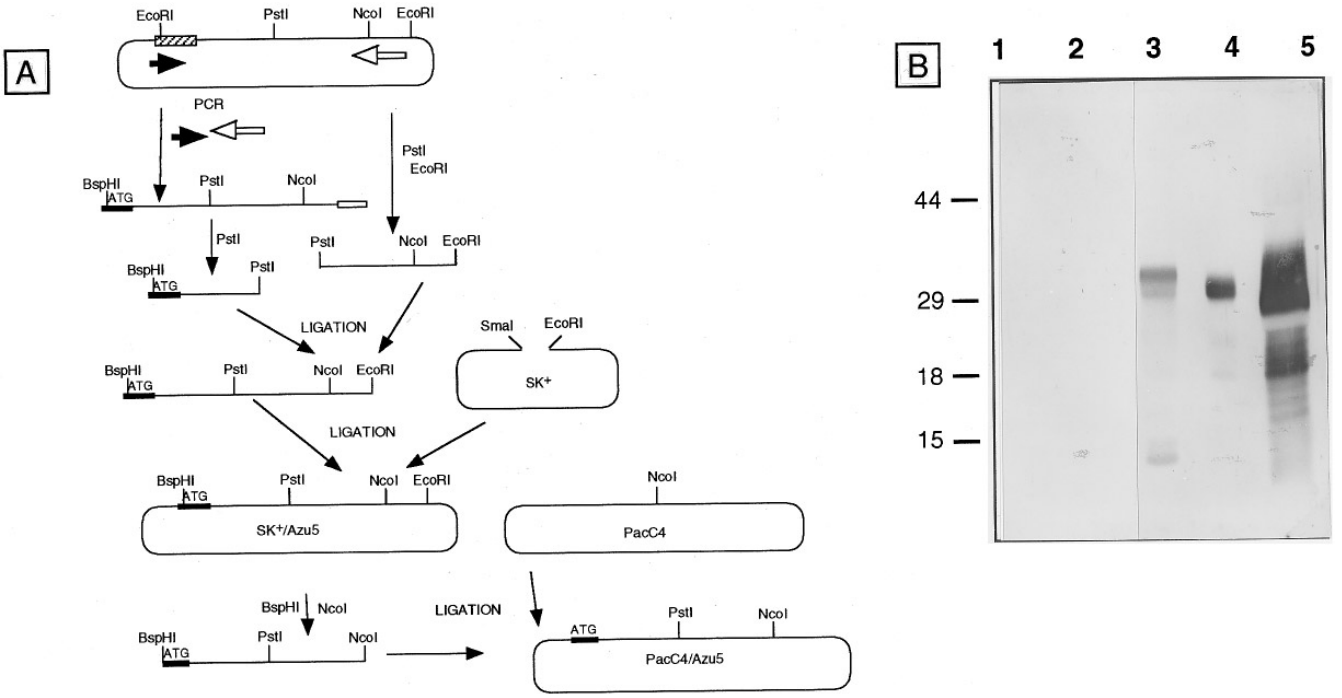

FIG. 1. (A) Construction of transfer vector pAcC4-Azu5. (B) Expression of azurocidin in Azu5-AcMNPV-infected insect cells. Insect cells were either untreated (lanes 1 and 2) or infected with Azu5-AcMNPV (lanes 3 and 4). Cells (lanes 1 and 3) and culture medium (lanes 2 and 4) from infected or control Sf9 were subjected to SDS–PAGE, electroblotted onto nitrocellulose membrane, and probed with rabbit IgG anti-azurocidin and alkaline-phosphatase conjugate secondary antibody. Human neutrophil azurophil granule extract (lane 5) was probed identically.

alkaline phosphatase were from Boehringer Mannheim (Indianapolis, IN). Grace's medium, fetal calf serum, and SF900 were from GIBCO (Gaithersburg, MD). Lactoalbumin hydrolysate and yeastolate were from Difco (Detroit, MI). Cationic liposomes used in the transfection mixes were from Invitrogen (San Diego, CA). All other chemicals were reagent grade.

Cells and DNA

The insect cell line from *Spodoptera frugiperda* (Sf9, Invitrogen) was propagated at 27°C in Grace's medium supplemented with 10% fetal calf serum and 3.3 g/liter each of lactoalbumin hydrolysate and yeastolate. High Five cells from *Trichoplusia ni* egg cell homogenate (Invitrogen) were grown and infected in a serum-free medium (SF900, GIBCO). A linearized baculovirus DNA containing a lethal deletion (Baculogold, Pharmingen, San Diego, CA) was used for transfection. The pAcC4 baculovirus transfer vector (gift of Dr. R. Stevens, Frederick, MD) was used for the cloning of azurocidin wild-type and mutant DNAs.

Construction of a Transfer Vector Containing Azurocidin DNA (pAcC4-Azu5)

All molecular biology techniques were carried out as described (15). The construction of Azu-containing vector is outlined in Fig. 1A. The PCR amplification of azurocidin cDNA using the azu5 and azu3′ end primers allowed us to recreate the two N-terminal amino acids missing from the signal sequence in our original Azu cDNA (5,16). The PCR fragment was digested with *Pst*I and the 5′ end of the DNA (420-bp fragment) was isolated and cloned after gel purification into the shuttle vector SK^+ at the *Sma*I–*Pst*I site. The 3′ end of Azu DNA was obtained by digestion of authentic Azu DNA (5) with *Pst*I and *Eco*RI, and the 3′ end fragment (500 bp) was cloned into *Pst*I–*Eco*RI-digested SK^+ containing the 5′ end of Azu DNA, thus yielding a full-length azurocidin DNA (SK^+–Azu5). The final cloning of the Azu DNA into the pAcC4 transfer vector was performed by digestion of the SK^+–Azu5 construct with *Bsp*HI and *Nco*I and ligation of the purified 774-bp Azu DNA into *Nco*I-digested, dephosphorylated pAcC4 plasmid DNA. The orientation and integrity of the azurocidin DNA in the final construct were verified by restriction analysis and DNA sequencing (17). Purification of plasmid DNA was performed by CsCl gradients or by anion-exchange chromatography (Qiagen kit).

Generation and Purification of Recombinant Virus

Insect cells (2.5×10^6) were transfected with a mixture of 2 μg of pAcC4-Azu5 and 0.5 μg of purified linear-

ized baculovirus DNA in the presence of cationic liposomes (18). After 2 days in culture, the culture medium was removed and served as a source of virus for amplification. This viral suspension was used to infect a fresh monolayer of insect cells and after 4 days, total cell lysate and culture medium were tested for the presence of recombinant azurocidin by Western blot analysis. Recombinant virus present in culture supernatants of infected cells in which the Azu protein was detected were purified by limiting dilution (19) or by plaquing using agarose overlays (18). Recombinant virus containing foreign DNA unrelated to the Azu DNA was kindly provided by Dr. Thomas Leto (NIH, Bethesda, MD) and used as control in subsequent experiments.

Immunodetection of Protein Produced by Recombinant Virus

The Sf9 or High Five cells were infected with purified recombinant virus and after 4 days the culture medium and total cell lysate were tested for the presence of expressed protein by SDS–PAGE and Western blot analysis. Proteins were electrophoretically separated by SDS–PAGE, using a 15% slab gel, and either directly stained with silver or transferred onto a nitrocellulose membrane. Transfer to the nitrocellulose membrane was conducted in a Transfor Electrophoresis unit (Hoeffer) at room temperature for 2 h at 0.8 mA. Free binding sites on the membrane were blocked with 5% nonfat milk, 0.05% Tween 20, PBS, pH 7.4, and the membrane was incubated overnight at 4°C with rabbit polyclonal anti-azurocidin antibody and revealed as previously described (3). For dot blot analysis the antigen was directly spotted on the membrane, and the membrane let dry and processed as described above.

Purification of Recombinant Azurocidin Protein

Recombinant azurocidin was purified from the culture medium of insect cells infected with Azu5-containing *Autographa californica* nuclear polyhedrosis virus (Azu5-AcMNPV)-infected insect cells. Because attempts to purify azurocidin from serum-containing medium were hampered by the presence of albumin and other serum proteins, we decided to culture the insect cells in serum-free media (Sf900, GIBCO). In addition, High Five cells were used for purification purposes instead of the Sf9 cells tested initially because these cells have been reported to produce higher amounts of recombinant protein and to adapt rapidly to serum-free conditions (20).

High Five cells were infected with purified recombinant azurocidin virus and after 4 days incubation at 27°C, the culture medium was centrifuged at 4000 rpm at 4°C and dialyzed against 100 times its volume of PBS + 0.05% Tween 20 at 4°C for 16 h (Spectrapor dialysis tubing, 8000 MW cutoff). This dialyzed medium was then applied to Sepharose 4B containing rabbit IgG and subsequently to a similar matrix containing anti-azurocidin IgG. Columns were washed in PBS–0.05% Tween 20 followed by 10 mM phosphate, pH 6.3. The protein was eluted from the antiazurocidin column with 100 mM glycine, pH 2.5, and azurocidin-containing fractions were monitored by dot blot analysis. These fractions were pooled and subjected to rpHPLC using a Vydac (250 × 4-mm) C4 column (Rainin Instruments, Emeryville, CA) equilibrated in 0.1% TFA/10% acetonitrile. Proteins bound to the Vydac C4 column were eluted at 1 ml/min with a linear gradient of 10–60% acetonitrile in 0.1% TFA monitored for absorbance at 214 nm. Fractions were dried in a speed-vac concentrator (Savant Instruments, Farmingdale, NY), rinsed with 0.1% acetic acid, dried, and resuspended in 10 mM sodium phosphate, pH 5.5. Protein amounts for each step of the purification are indicated in Table 1.

TABLE 1

Purification of Recombinant Azurocidin from Insect Cells

Purification step	Volume (ml)	Total protein mg/ml	Total protein mg	Protein yield (%)
Insect cell medium	300	0.05	15	100
Affinity chromatography	3.6	0.3	1	7
rpHPLC	0.3	1.7	0.5	3.4

Microbicidal Assay

Bactericidal activity was tested against *Escherichia coli* K12 (strain MC4100) and *S. faecalis* (ATCC 8043) as previously described (3). Fungicidal activity was tested against *C. albicans* (clinical isolate from Columbia Presbyterian Hospital, NY). Standard buffers used for the microbicidal assay were 50 mM sodium citrate, pH 5.5 (*E. coli*, 2×10^5 CFU/ml), and 10 mM sodium phosphate, pH 5.5 (*S. faecalis*, 2×10^4 CFU/ml, and *C. albicans*, 2×10^4 CFU/ml). Microbicidal activity was determined by calculating the decrease in colony-forming units for microorganisms incubated with the antimicrobial protein compared to that for microorganisms incubated in buffer alone at 37°C.

N-Terminal Protein Sequence

One microgram of purified recombinant azurocidin was added to sample buffer (0.125 mM Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol) and applied onto an SDS–10% polyacrylamide gel. The gel was prepared with the highest quality chemicals and allowed to polymerize overnight. Thioglycolic acid (0.1 mM) was added to the anode buffer. After electrophoresis proteins were transfered to PVDF membrane (Milli-

pore) in CAPS/DTT buffer at 50 V for 45 min (21). The PVDF membrane was stained with 0.1% amido black in 10% acetic acid, destained with 10% acetic acid, and the azurocidin band(s) excised. A Western blot was done on a separate lane in the same PVDF membrane to confirm the identity of the proteins stained with amido black. The N-terminal amino acid sequences were determined with an Applied Biosystems Gas Phase Sequencer at the Protein Sequencing Facility at the Rockefeller University.

Glycosylation of Azurocidin

Hydrolysis with peptide *N*-glycosidase F (*N*-glycanase; Genzyme, Boston, MA) was performed using 200 ng of purified recombinant or native azurocidin. The proteins were first denatured by boiling for 3 min in 0.5% SDS and 0.1 M β-mercaptoethanol. The samples were diluted in 0.2 M sodium phosphate, pH 8.6, 10 mM 1,10 phenantroline hydrate, 1.25% NP-40 and *N*-glycanase was added at 10 units/ml. The mixture was incubated at 37°C for 16 h (22). The samples were then boiled in SDS–PAGE sample buffer and subjected to electrophoresis followed by Western blot.

To compare the specific carbohydrate moieties present in recombinant and native azurocidin, we used the DIG glycan differentiation kit [Boehringer Mannheim, (23)]. This kit contains specific lectins that bind to carbohydrate moieties of glycoproteins such as terminal mannose, galactose, and sialic acid. Recombinant and native azurocidin (200–500 ng) were used and the reactions were performed according to the manufacturer's instruction.

High Five insect cells (0.5×10^6/well) were infected with Azu5-AcMNPV (0.5×10^5 PFU) and at 1 h postinfection, tunicamycin (4 μg/ml), an inhibitor of N-glycosylation, was added. Infected cells and culture medium were harvested at 3–4 days after treatment to analyze the protein expressed by the cells in the presence or absence of the inhibitor (18).

DNA Sequencing

The Azu DNA was sequenced by the dideoxynucleotide chain termination method using double-stranded plasmid DNA as template with a kit from U.S. Biochemical Corp. (Cleveland, OH) and ^{35}S-dATP. Alternatively, asymmetric PCR (to produce predominantly single-stranded fragments) was carried out on some of the Azu-pAcC4 templates using 1 pmol of the first oligonucleotide and 100 pmol of the second (15). Amplifications were carried out for 30 cycles. The majority of the unincorporated deoxynucleotides was removed by four successive washes in Centricon-30 and the single strands produced were sequenced using the USB kit as per the manufacturer's instruction. Sequencing reactions were analyzed by electrophoresis on 8% polyacrylamide gels, 0.4 mm thick containing 7 M urea. All oligonucleotide primers used in this study are shown in Table 2.

Azu Constructs

The Δ1 deletion was produced by digestion of the pAcC4-Azu5 template with *Pst*I and *Bam*HI, followed by T4 DNA polymerase treatment in the presence of dNTPs. After purification, the plasmid was allowed to religate itself in the presence of ligase.

The construct Δ2 was obtained by a three-step PCR amplification as follows: The first PCR fragment comprising the 5′ end of the Azu DNA was generated by amplification of the pAcC4-Azu5 template using the two primers Azu1 and Azu 121 A2. In the second PCR reaction, yielding the 3′ end of the molecule, a deletion was created by use of azudel3 and BVRP2 primers on a PacC4-Azu5 template. The products of first and second PCRs were purified as previously described and served as templates together with the Azu1 and BVRP2 primers to generate the Δ2 molecule. This last PCR fragment was purified, digested by *Pst*I and *Eco*RI, and cloned into *Pst*I–*Eco*RI-digested PacC4-Azu5.

The construct Δ3 was generated by digestion of pAcC4-Azu5 with *Dra*III and *Ecl*1364 and gel purification of the large fragment product of the digest, the extremities of which were then blunt ended by T4 DNA polymerase and dNTPs. The blunt-ended fragment was purified and allowed to religate in the presence of T4 ligase.

To generate the R1 construct, the pAcC4-PR3 vector (23a) was digested with *Pst*I and *Eco*RI and a 518-bp *Pst*I–*Eco*RI fragment was isolated and purified from agarose gel and cloned into *Pst*I–*Eco*RI-digested PAcc4-Azu5 plasmid DNA.

For the R2 construct, the pAcC4-PR3 vector (23a) was digested with *Pst*I and *Eco*RI and both the *Pst*I–*Eco*RI-cut pAcC4-PR3 vector and the 370-bp *Pst*I–*Pst*I fragment were gel purified. An Azu5-derived *Pst*I–*Eco*RI fragment was obtained by *Pst*I–*Eco*RI digestion of pAcC4-Azu5 and this fragment was cloned into *Pst*I–*Eco*RI-digested pAcC4-PR3 plasmid. The plasmid was reopened at the *Pst*I site in order to insert the 370-bp *Pst*I–*Pst*I fragment from the PR-3 sequence.

The construct R3 was obtained by a four-step PCR amplification as follows: The first PCR was performed using prazu1 and prazu2 and the SK$^+$–PR-3 (1) template. The PCR1 fragment comprised the PR-3 sequence targetted for replacement flanked at both ends by a short Azu sequence. The second PCR used the PCR1 fragment and the Az121A primers on the original pAcC4-Azu5 template DNA to elongate the PCR1 fragment toward the 3′ end of the azurocidin DNA. The third PCR was performed with Azupepdel2 and Azu 7.1 on a pAcC4-Azu5 template. The third PCR fragment generated in this manner contained a sequence at the 3′ end that

TABLE 2

List of Oligonucleotides Used in this Study

Name	Sequence
	5′ → 3′
1. Azu5	GGTCATGACCCGGCTGACAGTCCTGGCCCTG
2. Azu3′ end	GGGAATTCAGAGGAGAGATCGGCTT
3. Azu1	ATCGTTGGCGGCCGGAAGGCGAGG
4. Az121A-2	CGGCTTCCACCGTGGCGTTC
5. Azudel3	GCCACGGTGGAAGCCGGCCGAGGCCCTGACTTC
6. BVRP2	GACCAGTGAACAGAGGTGCG
7. Prazu1	GGTGGCCGGCTGGGGGCGCGTGGGTGCCCAC
8. Prazu2	GCACACGTTGTTGGGGCGACAGAAGAAGGTGACCACGG
9. Az121A	CGGTCCCGGGTTGTTGAG
10. Azupepdel2	AGCCAGAACCCTGGGGTTAGC
11. Az7-1	GCCGGCCACCTGGCATCTG
12. Azu H3	GATGGTGTTCTCAACAACCCG

allowed it to hybridize to the PCR2 fragment in such a way as to cover the full length of the Azu DNA in the fourth PCR reaction which was performed using PCR2 and PCR3 as templates and the azupepdel2 and az121A as primers. The final PCR product (PCR4) was purified from agarose gel using the QIAEX gel extraction kit, digested to completion by *Dra*III and partially by *Pst*I. A 220-bp fragment *Pst*I–*Dra*III was isolated and cloned into *Pst*I–*Dra*III-digested pACC4-Azu5.

The RΔ4 construct was obtained by *Pst*I digestion of R3, purification from agarose gel of the *Pst*I–*Pst*I-digested pAcC4-R3 construct, and religation. Orientation and integrity of the DNA construct were verified by restriction analysis and DNA sequence as described previously.

RESULTS

Expression of Recombinant Azurocidin in Sf9-Infected Cells

Recombinant baculovirus was generated by homologous recombination between the PacC4-Azu5 vector (Fig. 1A) and the linear baculovirus DNA in transfected Sf9 cells.

Infected insect cells and culture medium were examined for the presence of azurocidin by Western blot analysis. As shown in Fig. 1B, in cells infected with Azu5-AcMNPV, two polypeptides were recognized by anti-azurocidin antibodies; the major one migrated at approximately 32.5 kDa while the minor species migrated slightly lower, at approximately 30 kDa. In the culture supernatant of these cells, a single polypeptide was recognized of the size of the lower cell-associated molecular species (30 kDa). The protein was absent from the cells or culture medium of Sf9 infected with a control recombinant virus containing unrelated DNA.

Purification of Recombinant Azurocidin from Azu5-AcMNPV-Infected Insect Cells

To facilitate the purification of secreted azurocidin, insect cells were cultured and infected in serum-free medium. High Five cells infected with the Azu5-AcMNPV construct behaved identically to the infected Sf9 cells in that they secreted azurocidin in culture medium. In order to optimize the yields of recombinant protein, we monitored the expression of azurocidin at 24, 48, 72, and 96 h postinfection. Optimum production was found to occur at 72 and 96 h and one of these two time points was selected for the collection of azurocidin containing supernatants (data not shown). As shown in Fig. 2, recombinant azurocidin was purified by immuno adsorption onto antiazurocidin antibodies coupled to CNBr–Sepharose (Fig. 2A) followed by rpHPLC (Fig. 2B). The fraction obtained by rpHPLC contained a single polypeptide species of MW 30 kDa, as assessed by SDS–PAGE followed by silver stain (inset, Fig. 2B). The identity of the 30-kDa species was confirmed by Western blot analysis. Recombinant azurocidin migrated at a MW slightly higher than that of neutrophil-derived azurocidin ($M_r \approx 29$).

Characterization of Recombinant Azurocidin

To further confirm the identity of the protein produced by the Azu5-AcMNPV-infected insect cells, we performed sequence analysis of two contiguous sections of the nitrocellulose membrane onto which the protein was transfered and of two lower MW species found in some recombinant azurocidin preparations (Fig. 1B, lane 4). As shown in Fig. 2C, recombinant azurocidin exhibited a single N-terminal sequence: It shared all 20 N-terminal amino acid residues with native azurocidin but exhibited one additional residue, Asp, at the first position in its N-terminal sequence. An Asp residue is

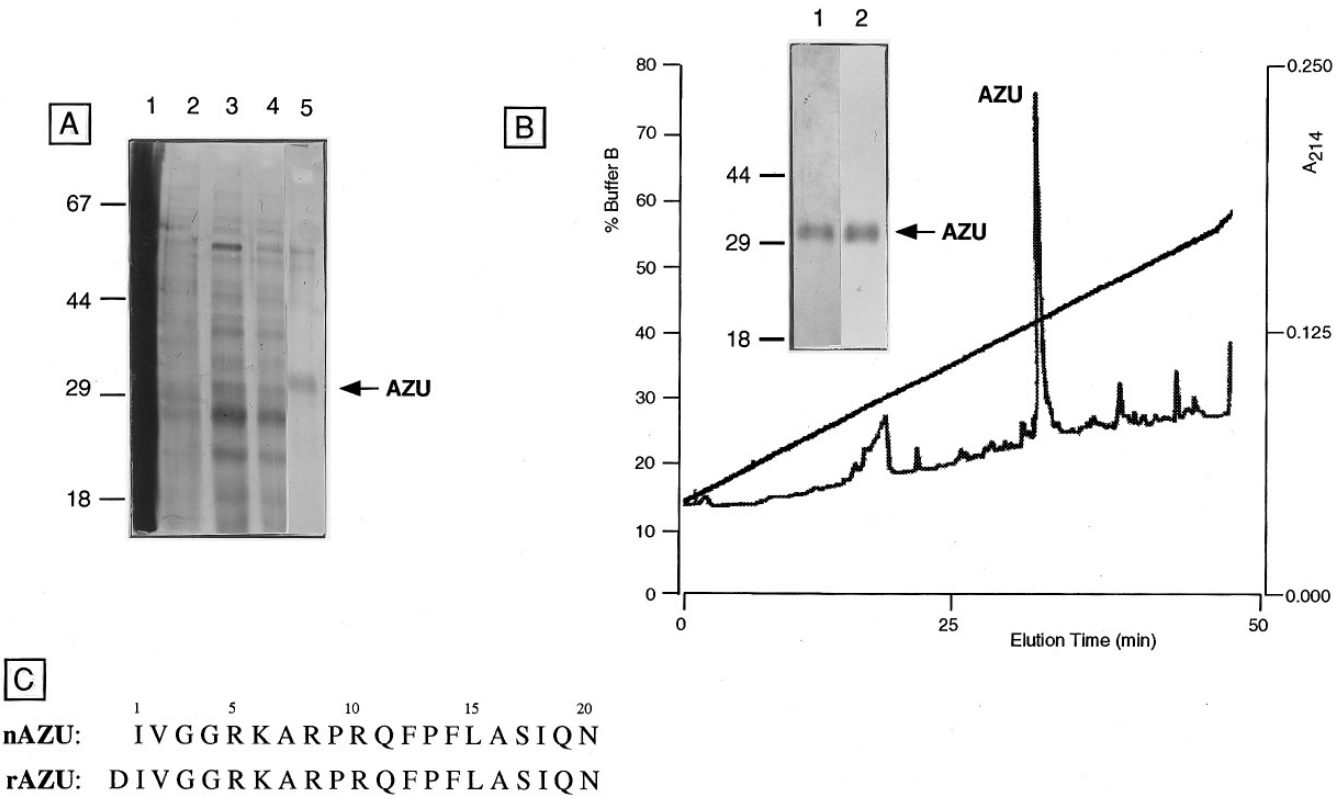

FIG. 2. Purification of recombinant azurocidin. (A) SDS–PAGE analysis of the affinity purification step; lanes 1 and 2, cells and supernatant from Azu5-AcMNPV-infected High Five cells; lane 3, concentrate of culture medium from infected insect cells applied onto the rabbit IgG anti-azurocidin Sepharose column; lane 4, flowthrough from the affinity chromatography column; lane 5, elution of proteins bound onto the column at low pH. Proteins are visualized by silver stain. Arrow points to the azurocidin protein. (B) rpHPLC profile of the affinity purified culture medium. Azurocidin-containing fractions eluted from the affinity column were concentrated 10-fold in a Centricon-10 ultrafiltration unit, made 0.1% in trifluoroacetic acid, and subjected to rpHPLC. rAzu elutes as a single peak with 40% acetonitrile. A single protein is present in this peak as shown by SDS–PAGE and silver stain (inset, lane 1) or Western blot (inset, lane 2). (C) N-terminal sequence analysis of native and recombinant azurocidin.

part of the prosequence predicted from the azurocidin cDNA (5,16) and immediately precedes Ileu, the first residue in the native neutrophil-derived azurocidin protein (11).

To examine the glycosylation of recombinant and native azurocidin, both molecules were treated with endo F, an enzyme that cleaves selectively high-mannose and N-linked carbohydrates. Figure 3A shows that recombinant and native azurocidins exhibit an increased electrophoretic mobility after endo F treatment. This difference in mobility corresponds to a decrease in MW of approximately 2 kDa for both proteins after glycosydase treatment and suggests that the two proteins are N-glycosylated.

When recombinant and native azurocidins were probed with five different digoxigenin-labeled lectins that recognize specific carbohydrate structures, the two molecules exhibited a positive reaction only with the GNA (*Galanthus nivalis*) lectin which recognizes terminal mannose (Fig. 3B) (23). To confirm that recombinant azurocidin was N-glycosylated, the Azu5-AcMNPV-infected insect cells were treated at 1 h postinfection with tunicamycin, an inhibitor of the first step of N-glycosylation (18). As shown in Fig. 3C, the protein produced by tunicamycin-treated insect cells migrated as a lower MW species (≈23.5 kDa) compared to the protein produced by untreated cells (≈30 kDa). In addition, the recombinant azurocidin from tunicamycin-treated cells was found to be exclusively cell-associated.

Antimicrobial Activity of Recombinant Azurocidin

The antimicrobial activities of native and recombinant azurocidin against *E. coli*, *S. faecalis*, and *C. albicans* were compared, as shown in Fig. 4. For each of the three microorganisms tested, the dose dependence of activity of recombinant azurocidin was very similar to that measured for native azurocidin. The LD_{50} of recombinant azurocidin against *E. coli*, *S. faecalis*, and *C. albicans* were 1.2 ± 0.1, 2.9 ± 0.4 and 8.2 ± 0.2 μg/ml, respectively, compared to 1.3 ± 0.05, 2.3 ± 0.2,

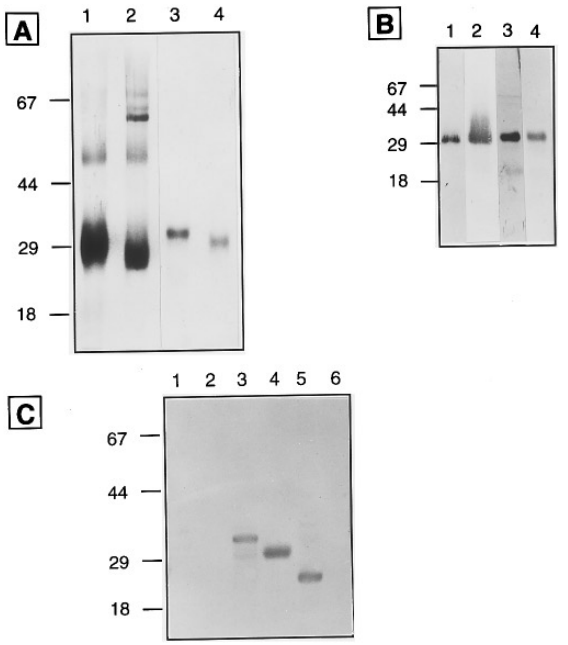

FIG. 3. N-glycosylation of recombinant and native azurocidin. (A) Treatment with endoglycosidase F. Purified recombinant (lanes 3 and 4) and native azurocidin (lanes 1 and 2) were incubated at 37°C for 16 h in the presence (lanes 2–4) or absence (lanes 1–3) of endoglycosidase F. Samples were subjected to SDS–PAGE and proteins visualized by silver stain. (B) Identification of rAzu and neutrophil-derived Azu terminal sugars by digoxigenin-labeled GNA lectin. Purified recombinant (lanes 1 and 2) and native azurocidin (lanes 3 and 4) (0.5 μg) were electroblotted onto nitrocellulose membrane and probed either with rabbit IgG against azurocidin and alkaline-phosphatase-conjugated secondary antibody (lanes 2 and 4) or with digoxigenin-labeled lectin GNA and anti-digoxigenin antibodies (lanes 1 and 3). (C) Inhibition of N-glycosylation by tunicamycin. Sf9 insect cells were infected with Azu5-AcMNPV and incubated in the presence (lanes 5 and 6) or absence of tunicamycin (lanes 3 and 4). Control cells (lane 1) and control medium (lane 2) or infected cells (lanes 3 and 5) and medium from infected cells (lane 4 and 6) were electroblotted and probed with rabbit IgG anti-azurocidin.

and 8 ± 0.8 μg/ml for native azurocidin. In our assay conditions, both molecules were ≈7 times more active against *E. coli* (LD_{50}: 1.2 μg/ml) than against *C. albicans* (LD_{50}: 8 μg/ml). Because varying physiologic conditions such as pH and ionic strength affect the antimicrobial activity of native azurocidin, we examined the effect of pH and salt concentration on recombinant azurocidin activity. As in the case of native azurocidin, the killing of each of the target organisms by the recombinant azurocidin protein was detectable over the entire pH range tested (pH 5.5–7.8) but optimum at pH 5.5 (data not shown). The addition of NaCl markedly reduced the activity of recombinant azurocidin against all three organisms. A 77% reduction of activity against *E. coli* was observed upon addition of 75 mM NaCl, compared to the 80% reduction of activity observed with neutrophil-derived azurocidin (data not shown).

Identification of a Region of Azurocidin Necessary for Its Secretion from Azu5-AcMNPV-Infected Insect Cells

In the course of our studies on the expression of the Azu protein, we discovered that the C-terminal half of the molecule was necessary for secretion of azurocidin outside of the insect cell (construct Δ1, Fig. 6). Because this finding impacted on future goals such as generating various fragments of the molecule, we decided to locate the region(s) that might be important for secretion.

Two categories of Azu constructs were used; the first class of constructs carried various deletions in the Azu DNA, while the second class of constructs consisted of replacement of various Azu DNA sequences by PR-3 DNA sequences (Fig. 5). Replacement constructs with PR-3 sequences were used because PR-3, in constrast to Azu, was not secreted in the medium in the baculovirus–insect cell expression system (23a) and small differences in the nonhomologous regions of these two proteins might account for the difference in their abilities to be secreted from insect cells.

Various transfections of insect cells using each of the mutants Azu-pAcC4 DNAs together with baculovirus DNA were performed and the truncated or modified azurocidin polypeptides expressed by the transfected cells were localized by Western blot analysis (Fig. 6). Neither the azurocidin protein product of AzuΔ1 nor the product of AzuΔ2 was secreted in the culture medium of the insect cells. In contrast, a significant proportion of the truncated AzuΔ3 molecule was secreted, suggesting that the deleted region between amino acids 187 and 225 was not necessary for secretion.

The product of the Azu replacement 1 (R1), in which the C-terminal half of azurocidin was replaced by the C-terminal half of PR-3, remained cell-associated. In contrast, the hybrid molecule Azu replacement 2 (R2), in which the N-terminal half of azurocidin was replaced by the N-terminal half of PR-3, was exported. Thus, the azurocidin sequence between amino acids 114 and 225 present in R2 and absent from R1 appeared necessary for secretion. The Azu replacement 3 (R3) construct encoded for the entire azurocidin molecule except for a stretch of PR-3-derived sequence between residues 130 and 153 corresponding to a nonhomologous region in the C-terminal half of the molecule. Like its original Azu5 counterpart, the Azu R3 molecule was secreted from transfected insect cells. Taken together, these results suggested that the sequence required for azurocidin secretion outside of the Azu5-AcMNPV-infected insect cells is located between residues 114 and 129 or/and between residues 154 and 186. When a deletion was created to remove residues 114 to 129 (construct RΔ4), the product remained cell-associated. This suggests that the region between residues 114 and 129

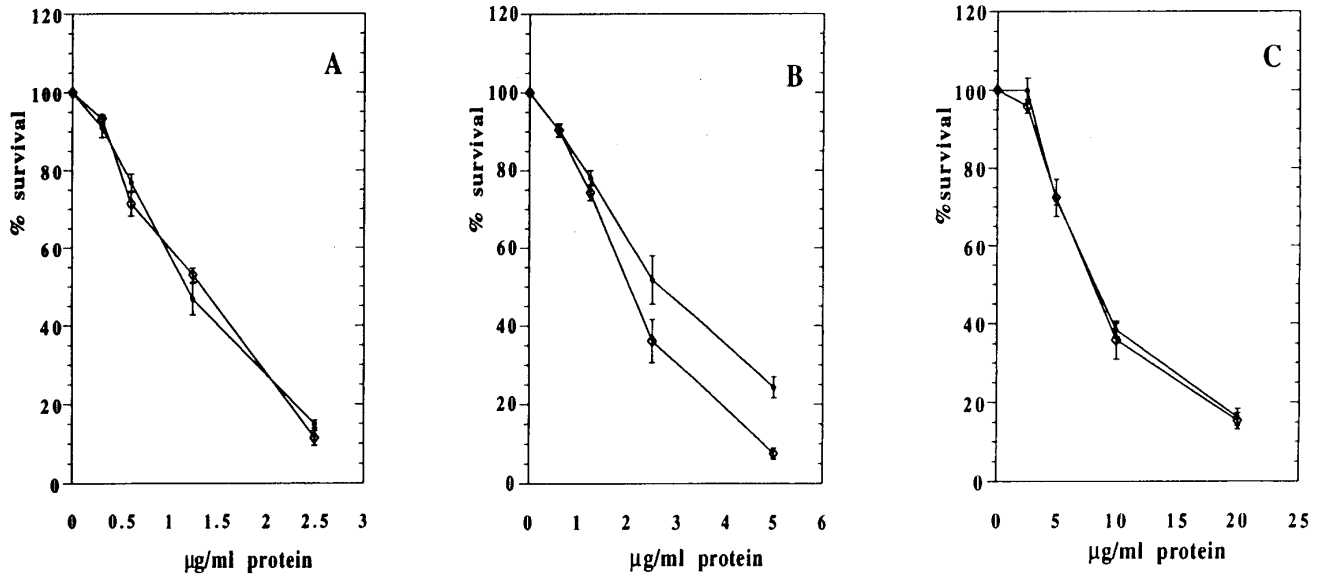

FIG. 4. Dependence of antimicrobial activity on azurocidin concentration. Microbicidal assays were conducted in the standard buffers against three microorganisms: (A) *Escherichia coli;* (B) *Streptococcus faecalis;* and (C) *Candida albicans.* Results are expressed as means ± SEM for three experiments, each performed with material from a separate purification. Each assay point was done in triplicate. Open and solid circles correspond, respectively, to native and recombinant azurocidin.

contains a determinant(s) necessary for azurocidin secretion (Fig. 7).

DISCUSSION

Because of the emergence of new infectious agents and of antibiotic resistance in important pathogenic microorganisms, it is important to identify new classes of antibiotics. Possible candidates are the family of antimicrobial proteins and peptides present in the azurophil granules of human neutrophils (4,11,24–27). Little information exists concerning the structure–function relationship or mechanism of action of these proteins. The availability of complementary cDNA clones in expression systems would facilitate such studies, particularly for the larger molecules (28,29).

Initially, we had selected two types of bacterial systems for the expression of recombinant azurocidin. The first one consisted of fusing the azurocidin DNA to *malE*, the gene coding for the maltose binding protein (a periplasmic protein) (30,31). This construct resulted in the expression of an MBP–Azu hybrid protein, which remained in the bacterial cell cytoplasm. While the fusion protein could be purified through its MBP tag and remained soluble, the azurocidin part of the hybrid became highly insoluble once removed by proteolytic cleavage. No activity could be detected in either the hybrid or the cleaved Azu (Almeida *et al.*, unpublished results).

In a further attempt to target the authentic mature Azu to the periplasmic compartment of *E. coli*, a nonreducing environment where disulfide bonds may form, Azu DNA was fused to the signal sequence for *his J* (a periplasmic protein). This strategy has been successful for the expression of functional mammalian trypsin in the periplasm of *E. coli* (32). The Azu product of this construct was found to be largely insoluble, membrane associated, and functionally inactive (Almeida *et al.*, unpublished results).

The lack of success with bacterial expression systems prompted us to develop the baculovirus–insect cell expression system for the synthesis of recombinant azurocidin. We discovered that the 30-kDa protein produced by the Azu5-AcMNPV-infected insect cells was predominantly located in the culture medium of these cells while a small amount could be found cell-associated. Infected cells but not culture medium also contained a higher MW species (approximately 2 kDa larger) with Azu reactivity and which likely represents unprocessed azurocidin. Purified recombinant azurocidin exhibited an antibiotic activity very similar to that of neutrophil-derived protein against gram-negative, gram-positive bacteria and fungi.

In fitting with the conservation of function, close similarities in structure were observed between recombinant and neutrophil-derived azurocidin. Recombinant and neutrophil-derived azurocidin have an identical N-terminal sequence over 20 residues, except that recombinant azurocidin exhibits an additional amino acid residue Asp which preceeds Ileu, the first residue in native azurocidin (11,13). The Asp residue presumably

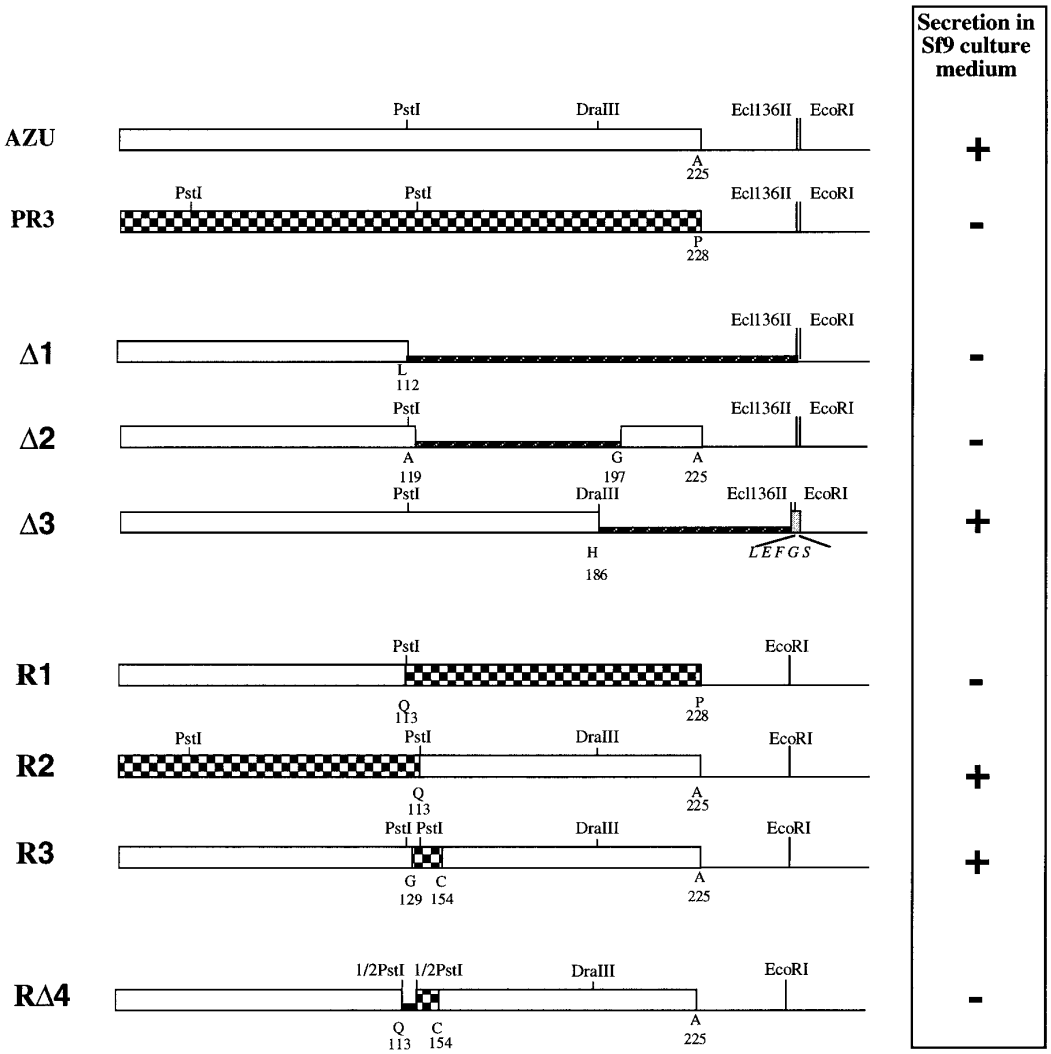

FIG. 5. Regions of the azurocidin polypeptide produced by various deletion or replacement (Azu–PR-3) constructs. The first line and the second line represent the full-length Azu and PR-3 constructs. Single restriction sites are indicated above. Amino acid residues with their position in the polypeptide sequence are indicated below. The remaining lines correspond to various Azu constructs encoding either for truncated Azu polypeptides (Δ1, Δ2, Δ3) or for Azu–PR-3 hybrid polypeptides without (R1, R2, R3) or with a deletion (RΔ4). The regions of the azurocidin DNA that are present are indicated as white area. Deletions are indicated by a thin black cross-hatched line. In Δ3, four amino acids that are not found in native azurocidin (italics) are added onto the C-terminus, as a result of the construction. The regions of azurocidin DNA found in the replacement constructs are indicated as white areas whereas the regions of PR-3 appear as checkered areas. The numbers below the replacement constructs refer to amino acid positions in the azurocidin polypeptide that are located at the fusion point with the PR-3 polypeptide and that are common to both Azu and PR-3 sequences.

originates from the predicted azurocidin prosequence, suggesting that processing of the recombinant molecule occurs in insect cells but stops short of the last amino acid of the Azu leader sequence (Asp) (5,16).

While the processing of the signal sequence is likely mediated by a signal peptidase at a consensus site, the mechanism(s) by which the putative prosequence is cleaved off in native azurocidin is unknown (16). Indeed, a processing that requires hydrolysis after an acidic residue such as Asp is unusual (33). Lysosomal dipeptidyl peptidase I has been implicated in the post-translational processing of prosequences found in other members of the family of granule serine proteases (34,35) and might also play a role in the processing of azurocidin. Since such an enzyme displays a unique specificity, it may be present in only certain cell types, particularly those of the hematopoietic lineage. Thus the fact that rAzu exhibits an Asp extension at the N-terminal could be due to the absence in insect cells of the enzyme that is required for the complete processing of the propeptide. It is interesting to note that the presence of an additional residue at the N-terminal does

not appear to interfere with the antimicrobial activity of this protein.

Previous work suggested that native azurocidin is N-glycosylated (36). A similar type of glycosylation was observed for rAzu (this work). When tunicamycin, an inhibitor of the first step of N-glycosylation (18), was added to insect cells infected by Azu5-AcMNPV, it reduced the size of the protein expressed by these cells, likely causing them to synthesize an unglycosylated form of rAzu. In addition, this inhibitor blocked Azu secretion by insect cells, thus indicating that transport through the endoplasmic reticulum and/or secretion depended on N-glycosylation. Both the treatment of neutrophil-derived azurocidin and rAzu with endo F (*N*-glycanase) and the probing with various lectins of the carbohydrates present on native and recombinant molecule indicated the existence of "high-mannose" *N*-glycan chains on both molecules (23). More detailed structural analysis would allow us to characterize and compare the exact oligosaccharide composition of the two molecules.

While most proteins synthetised in the baculovirus–insect cell system are directed to their appropriate subcellular location (18), azurocidin, a protein normally stored within the azurophil granules of human neutrophils, is secreted by the insect cells. The human lysosomal enzyme β-galactosidase is also secreted into the media when expressed in insect cells (37) while proteinase 3 which colocalizes with azurocidin in neutrophil granules exhibits an intracellular distribution (23a).

To map out the region(s) required for the secretion of rAzu in the baculovirus–insect cell system, we used deletions and replacements. The deletions examine whether particular sequences in Azu contribute to its localization outside of the insect cell. The replacements used nonhomologous sequences of PR-3. It is based on the fact that (i) PR-3, a structural homolog of Azu, is

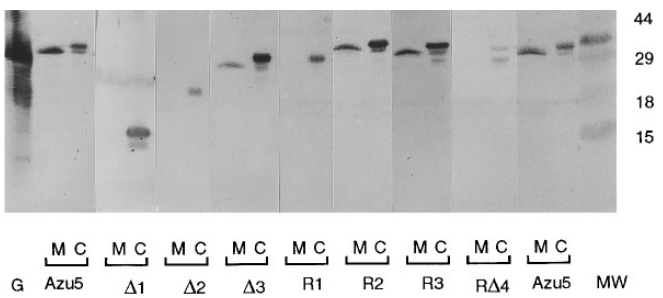

FIG. 6. Western blot analysis of azurocidin polypeptides produced by various deletion and Azu–PR-3 strains. Lane 1 corresponds to neutrophil azurophil granule extract (G). Culture media (M) and cells (C) from Sf9 infected with AcMNPV carrying the various Azu constructs indicated at the bottom of the figure were run on SDS–PAGE, transferred to nitrocellulose, and probed with anti-Azu rabbit IgG as described in Fig. 1. The last lane corresponds to MW standards (MW).

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          110         120         130
           |           |           |
AZU: LPLQNATVEA GTRCQVA GWGS
PR3: LPQQDQPV PHGTQC LAMGWGR
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FIG. 7. Sequence comparison between azurocidin and PR-3 in the region implicated for Azu secretion. Gaps were introduced to maximize alignment. The Azu sequence is from Ref. (5) and the PR-3 sequence from Ref. (1).

not secreted by insect cells and that (ii) sequence or structure differences between PR-3 and Azu may explain the functional differences of the two proteins. While the deletion constructs may change the overall conformation of the molecule, the replacement constructs are likely to introduce more discrete changes. The products of Δ3, R2, and R3 were secreted in the culture medium of the infected insect cells while the products of Δ1, Δ2, and R1 remained cell-associated. These results suggested that a critical determinant for secretion was located between residues 114–129 and/or residues 154–186. A deletion of the region 114–129 resulted in absence of secretion of the truncated product, further suggesting that this region may be important for export of Azu outside the insect cells. Secretion can be influenced by a number of factors which include glycosylation, cysteine pairing, and other conformational determinants (38–41). Native Azu contains three potential N-linked glycosylation sites (N100, N114, and N145) and eight cysteines which may form disulfide bonds (5,10,36). When cysteine residues are deleted (construct Δ2) or Asn and Cys residues removed (construct RΔ4), secretion no longer occurs. Thus, glycosylation and/or correct folding may account for the secretion of azurocidin from insect cells and its lack thereof in the case of PR-3. Further studies using single-site mutants at Asn and Cys residues may further address this issue.

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