Polydnaviruses are essential for the survival of many Ichnuemonid endoparasitoids, providing active immune suppression of the host in which parasitoid larvae develop. The *Cotesia rubecula* bracovirus is unique among polydnaviruses in that only four major genes are detected in parasitized host (*Pieris rapae*) tissues, and gene expression is transient. Here we describe a novel *C. rubecula* bracovirus gene (CrV3) encoding a lectin monomer composed of 159 amino acids, which has conserved residues consistent with invertebrate and mammalian C-type lectins. Bacterially expressed CrV3 agglutinated sheep red blood cells in a divalent ion-dependent but Ca\(^{2+}\)-independent manner. Agglutination was inhibited by EDTA but not by biological concentrations of any saccharides tested. Two monomers of \(~14\) and \(~17\) kDa in size were identified on SDS-PAGE in parasitized *P. rapae* larvae. The 17-kDa monomer was found to be an N-glycosylated form of the 14-kDa monomer. CrV3 is produced in infected hemocytes and fat body cells and subsequently secreted into hemolymph. We propose that CrV3 is a novel lectin, the first characterized from an invertebrate virus. CrV3 shows over 60% homology with hypothetical proteins isolated from polydnaviruses in two other *Cotesia* wasps, indicating that these proteins may also be C-type lectins and that a novel polydnavirus lectin family exists in *Cotesia*-associated bracoviruses. CrV3 is probably interacting with components in host hemolymph, resulting in suppression of the *Pieris* immune response. The high similarity of CrV3 with invertebrate lectins, as opposed to those from viruses, may indicate that some bracovirus functions were acquired from their hosts.

Polydnaviruses are particles specifically associated with the ovaries of certain Braconid and Ichnuemonid endoparasitoids (1). They are divided into genera, *Ichnovirus* and *Bracovirus*, based on differing host range and morphology (2). Polydnavirus genomes exist as a series of different circular DNA segments (3), which are packaged singly or in groups into individual polydnavirus particles (1). Particle-associated DNA segments are known to originate from wasp chromosomal DNA and are transferred in their integrated form to subsequent generations of wasps (4). Thus, polydnaviruses from different wasps are genetically isolated from each other and considered as separate "species" (5). "Transmission" of particles is exclusively vertical (4, 6), and particles are therefore not detected in males, although episomal polydnavirus DNA may exist (7).

Production of particles is restricted to specialized ovarian calyx cells (1) and is initiated in the pupal phase, soon after the onset of cuticular melanization, and continues in female adult wasps (8–11). Although the replication mechanism is not completely understood, recent evidence suggests that controlled localized chromosomal amplification occurs before excision of the particle segments (10). Larger chromosomal segments may have smaller segments nested within (12). Particles accumulate in the oviduct and are injected into the host hemocoel, together with the parasitoid egg and various maternal secretions, at oviposition. The presence of polydnavirus particles is essential for survival of the egg and/or developing parasitoid larva (13–15).

Polydnavirus DNA segments do not contain genes for particle replication, so no particles are produced in the lepidopteran host (1, 16). Particles enter most host cell types (17, 18), and viral transcripts are produced in the first few hours after parasitization. Transcripts are generated either transiently (19) or persistently (17) during parasitism. Relative levels of *Campedium sonorensis* ichnovirus gene expression in *Helicoverpa virescens* larvae depend largely on gene copy number (16); therefore, segment nesting could conceivably function to increase the copy number of genes essential for parasitoid survival. Such genes presumably would encode abundantly expressed, secreted proteins rather than intracellular proteins (16).

*Cotesia rubecula* bracovirus (CrBV)\(^1\) genes are expressed in the host larvae, *Pieris rapae*, over a relatively short time period, from 4 to 12 h after parasitization (19). CrBV appears to express only 4 major genes, which differs from other systems, such as *C. sonorensis* ichnovirus, which is suspected of expressing over 35 genes comprising several gene families (20). The products of particle-associated genes act to suppress the host immune response (19, 21–25), most often by targeting hemocytes. Gene products may also lead to physiological disorders (e.g., arrested development) by interfering with the host endocrine system (26–29).

Suppression of the host immune response appears to be the primary function of most polydnavirus genes expressed in lepidopteran larvae and is considered an important evolutionary

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\(^{b}\) The abbreviations used are: CrBV, *Cotesia rubecula* bracovirus; CTL, C-type lectin; CRD, carbohydrate recognition domain; PBS, phosphate-buffered saline; RT, reverse transcription; ORBC, ovine red blood cell.
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adaptation for an organism directly exposed to the immune system of its host. One of the four major CrBV genes, CrV1, encodes a glycoprotein that is abundantly expressed in host tissues and inactivates hemocytes by destabilizing the cytoskeleton (19, 30). As a result, infected hemocytes are unable to encapsulate the parasitoid egg. A 32-kDa wapw-speciﬁc protein (Crp32) produced in calyx cells is associated with particles and also covers the parasitoid’s eggs, providing passive immune protection for the developing embryo (31). Whereas Crp32 appears to provide passive protection for the parasitoid, polydnavirus particles are resuspended in 1% triton X-100, safely passing through host immune function. Both elements are required for survival and development of the C. rubecula parasitoid (31).

C-type lectins (CTls) are proteins that bind to speciﬁc glycodeterminants and require the presence of divalent metal ions, most commonly Ca2+, to exhibit binding (32). CTls are deﬁned by a series of conserved residues in their carbohydrate recognition domains (CRDs) (33). Amino acid sequence differences in various CRDs produce a range of carbohydrate binding specificities. CTls are extremely diverse and have been subdivided into seven groups based on gene structure and nature of non-lectin domains (32). One class, simple CTls, has been isolated from invertebrates and appears to function as part of induced humoral immune responses (32), presumably binding to carbohydrates on the surface of foreign bodies or damaged tissue. These lectins are generally multimeric, with each monomer containing one CRD, and most often bind galactose as the primary ligand (33). Here we report on a novel CrBV gene, CrV3, the product of which shows divalent ion-dependent lectin activity and has a conserved CTL domain similar to those isolated from invertebrates and mammals. Although CTls have been isolated from a range of invertebrates, this is the ﬁrst report of a CTL associated with invertebrate viruses.

EXPERIMENTAL PROCEDURES

Insect Cultures—C. rubecula (Hymenoptera: Braconidae) endoparasitoid wasps were reared on cabbage-fed P. rapae (Lepidoptera: Pieridae) as described previously (34). Virus and Genomic DNA Isolation—Calyx ﬂuid from 50 female wasps was collected in PBS (138 mM NaCl, 2.7 mM KCl, 1.47 mM KH2PO4, and 7.3 mM Na2HPO4, pH 7.6) by homogenization of ovaries. The suspension was passed through a 0.45 μm syringe ﬁlter (Minisart®) and centrifuged at 15,800 × g for 15 min (35). Pelleted virus particles were resuspended in 80 μl of PBS, and DNA was isolated from this suspension as described previously (4). DNA was isolated from ovaries and female and male wasps by homogenizing them in a buffer made up of 10 mM Tris, 1 mM EDTA, and 1% SDS, pH 8.0. Protease K was added to a ﬁnal concentration of 0.25 μg/μl, and the samples were incubated at 40 °C overnight. Samples were treated with RNase A (125 μg/μl) at 37 °C for 30 min and then extracted with phenol/chloroform. DNA was precipitated by adding 2 volumes of ethagene and centrifuged at 15,800 × g for 20 min. Pellet was washed with 70% ethanol, dried at 37 °C, and resuspended in water.

Southern and Northern Hybridization—DNA samples were run on a 1% agarose gel and transferred to a nylon membrane (Amersham Biosciences) as described previously (36). Total RNA was isolated from 6 h parasitized P. rapae caterpillars according to Chomczynski and Sacchi (37). RNA samples were run on 1% agarose gels under denaturing conditions, using formaldehyde, and transferred to nylon membranes as described previously (36).

Construction and Screening of a 6 h Parasitized Larval P. rapae Library—Total RNA was extracted from P. rapae larvae at 6 h after parasitization by mated C. rubecula wasps (QuickPrep® total RNA extraction kit; Amersham Biosciences). mRNA was then isolated from total RNA (PolyATtract™ mRNA isolation system; Promega). The isolated mRNA was used for construction of the cDNA library containing cloned insert DNA (pBluescript® SK+ plasmids (cDNA synthesis kit, ZAP-cDNA synthesis kit, and ZAP-cDNA® Gigapak® III Gold cloning kit; Stratagene). The library was ampliﬁed and titered according to the manufacturer’s instructions before being probed with total CrBV DNA previously digested with BamHI and HindIII and labeled with 32P. Probes were prepared as described (Ready-To-GoTM DNA labeling beads; Amersham Biosciences). Positive clones were re-screened, resulting in isolation of the complete CrV3 coding region. CrV3 was sequenced using M13 forward and reverse primers directly from the phagemid vectors produced by the aforementioned protocols and subsequent automated sequencing (Applied Biosystems).

PCR Ampliﬁcations—Speciﬁc primers to the CrV3 open reading frame (5’ primer CrV3-F and 3’ primer CrV3-R; see Fig. 1A) were designed containing Sp61 and T71 restriction sites to allow for direct ampliﬁcation of the ampliﬁed fragment into the pQE30 expression vector (Qiagen). Primer sequences were as follows (restriction sites are underlined): CrV3-F: CGCGGATCCAAAAACATAAGCTCAGG; and CrV3-R, GCCGCTGCAGTCACCTCTCTTTGTCGAAAG. Approximately 500 ng of genomic DNA from C. rubecula was used as template in PCR reactions. A 50-μl reaction was prepared by mixing 5 μl of 10× reaction buffer, 3 μl of MgCl2 (Promega), 1 μl of CrV3-F primer (0.1 μg/μl), 1 μl of CrV3-R (0.1 μg/μl), 0.5 μl of deoxynucleotide triphosphates (15 mM), and 0.5 μl of Taq DNA polymerase (Promega) and template DNA. After 5 min at 94 °C, 30 ampliﬁcation cycles were run including denaturing at 94 °C for 1 min, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. Final extension was carried out for 5 min at 72 °C. Reaction products were electrophoresed on a 1.2% agarose gel at 110 mA and visualized using ethidium bromide.

Reverse Transcription-PCR (RT-PCR)—CrV3-F and CrV3-R primers were used in RT-PCR of RNA isolated from 6 h parasitized P. rapae larvae, utilizing avian myeloblastosis virus reverse transcriptase (Promega). 1.5 μg of RNA and 0.1 μg of CrV3-R primer, in a ﬁnal volume of 10.7 μl, were heated to 95 °C for 5 min to denature RNA, before being cooled on ice. Reverse transcription was performed by adding 3 μl of 5× RT buffer (Promega), 0.3 μl of RNAin (Promega), 0.5 μl of avian myeloblastosis virus reverse transcriptase, and 0.5 μl of deoxynucleotide triphosphates (15 mM) before heating at 42 °C for 1 h and then heating at 95 °C for 5 min. The total contents were then used in a PCR by adding 3.5 μl of 10× reaction buffer, 1 μl of CrV3-F primer (0.1 μg/μl), 1 μl of CrV3-R (0.1 μg/μl), 0.5 μl of deoxynucleotide triphosphates (15 mM), 0.5 μl of Taq DNA polymerase, and 29 μl of H2O. PCR ampliﬁcation, electrophoresis, and visualization protocols were as performed for standard PCR of CrV3.

Collection of Protein Samples and Western Blotting—P. rapae larvae were bled into PBS saturated with phenylthiourea via removal of a proleg, and the hemolymph was centrifuged at 2300 × g for 5 min at room temperature. Supernatant (cell-free hemolymph) was removed, and the cellular pellet was resuspended in PBS. Gut tissue and head capsule were removed, and the fat body was washed and then homogenized in PBS before centrifugation (9300 × g for 10 min) and removal of supernatant (fat body proteins). Protein samples were stored at −20 °C and electrophoresed on denaturing 15% SDS-polyacrylamide gels as described by Laemmli (38). Proteins were generally not heated during the electrophoresis unless the effect of heating on protein expression was desired. Samples were run in conjunction with SeeBlue™ pre-stained standard protein markers (Novex) to allow subsequent estimation of sample protein sizes. Proteins were either stained within the gels using Coomassie Blue (Sigma) or, alternatively, transferred to a nitrocellulose membrane (Amersham Biosciences) as described previously (36). Before obtaining anti-CrV3, blots were probed with a 1:10,000 dilution of an alkaline phosphatase-conjugated monoclonal anti-polyHistidine antibody (clone His-1; Sigma). Anti-CrV3 was used at a dilution of 1:5000 (see below).

Expression of CrV3 in Bacteria—Gene-speciﬁc primers were designed (CrV3-F and CrV3-R) to amplify the open reading frame of the CrV3 gene, excluding a putative signal sequence corresponding to the ﬁrst 14 amino acids of the protein (see Fig. 1). These primers were used in PCR of phagemid vector produced during library screening to obtain the required fragment for ligation into the pQE30 bacterial expression vector (Qiagen). The desired PCR product was puriﬁed (Perfectprep® Gel Cleanup Kit; Eppendorf), precipitated, and digested with Sp61 and T71, as was pQE30, before ligation of the digested DNAs using T4 DNA ligase (Promega). M15 strain of Escherichia coli (Qiagen) was transformed with the ligation reaction contents, using heat shock. Colonies containing desired recombinant vectors were identiﬁed by PCR of bacterial cells using vector-speciﬁc primers. Production of bacterial CrV3 (containing 6 additional vector-derived histidine residues) was induced by the addition of 1 mM isopropyl-1-thio-galactopyranoside to bacterial culture at 37 °C. The target protein was identiﬁed by Western blotting and contained mainly in the insoluble fraction of total bacterial proteins, with only a small amount being soluble.

Puriﬁcation of Insoluble Bacterial CrV3 Protein—50 ml of induced
FIG. 1. A, DNA nucleotide sequence and deduced amino acid sequence for CrV3 from the *C. rubecula* bracovirus (GenBank™ accession number AY234855). The putative signal peptide is boxed and contains a predicted methionine start codon as the first amino acid. The predicted signal peptide cleavage point is denoted with an arrowhead. Amino acids representing putative N-glycosylation sites are underlined. The stop codon is indicated by an asterisk, and the polyadenylation signal is in bold. CrV3-F and CrV3-R primer binding sites are double underlined and located at the 5' and 3' ends of the open reading frame, respectively. Intron nucleotides are in lower case letters. B, hydrophobicity profile of CrV3. Distribution of hydrophobic and hydrophilic domains was determined using ProtScale software. Amino acid positions are represented on the x axis, and a score of relative amino acid hydrophobicity is represented on the y axis. Positive scores indicate hydrophobic residues, and negative scores indicate hydrophilic residues. A highly hydrophobic domain predicted at the N terminus of CrV3 is consistent with a putative signal peptide at the same region.
bacterial culture was centrifuged at ~7700 × g for 10 min at 4 °C. Cells were then resuspended in a lysis buffer (6 M GuHCl, 0.1 M NaH₂PO₄, and 0.01 M Tris, pH 8.0) and gently rocked for 1 h. The sample was centrifuged at 12,000 × g for 15 min at 4 °C before incubation (1 h, RT) of the supernatant with 300 μl of nickel-nitrotriacetic acid resin beads (Qiagen) previously equilibrated in 8 M urea with (0.1 M NaH₂PO₄ and 0.01 M Tris, pH 8.0). Non-bound proteins were removed with buffers containing 8 M urea with pH > 8.3, and bound proteins were eluted with buffers containing 8 M urea with pH < 6.0. Samples were diluted with 2 volumes of water before being dialyzed overnight in Tris-buffered saline (0.15 M NaCl and 0.01 mTris, pH 8.0) at 4 °C to remove excess urea to renature the protein. Protein was concentrated by vacuum drying.

**Anti-CrV3 Antibody Production**—Purified bacterial CrV3 was visualized on 15% SDS-acrylamide gels by staining with water-dissolved Coomassie Blue. CrV3 protein bands were excised from the gel with sterile blades and crushed. One rabbit was used to produce anti-CrV3 by an initial injection of the purified CrV3 (~5 μg) mixed with Freund’s complete adjuvant (Sigma), followed by two booster injections with purified CrV3 with Freund’s incomplete adjuvant (Sigma) at 2 and 4 weeks, respectively, after the initial injection. Antisera was obtained 2 weeks after the final injection and used to probe Western blot membranes at a dilution of 1:5,000. Bound anti-CrV3 was then visualized by alkaline phosphatase-labeled secondary anti-rabbit antibody (1:10,000).

**N-Glycosidase Digestion of CrV3**—Total proteins from cell-free hemolymph of 6 h parasitized *P. rapae* larvae were mixed with SDS-PAGE loading buffer containing β-mercaptoethanol. Igepal CA-630 nonionic detergent (Sigma) was added to a final concentration of 0.8% before addition of 2 units of recombinant N-glycosidase F (Roche Diagnostics) and incubation for 18 h at 37 °C.

**Characterization of CrV3-mediated Hemagglutination**—Lectin activity was measured by mixing 25 μl of serially diluted bacterial CrV3 extract with 25 μl of 2% trypsinized and gluteraldehyde-stabilized ovine red blood cells (ORBCs; Sigma) in PBS containing 2% bovine serum albumin. Samples were mixed well in U-bottomed microtiter wells before incubation at 37 °C for 1 h. Complete agglutination caused ORBCs to form a diffuse layer over the bottom of the wells, whereas unagglutinated cells formed a small dot at the center of the wells. Lectin titer was measured by mixing 25 μl of 2% trypsinized and gluteraldehyde-stabilized *E. coli* or *S. typhimurium* with 50 μl of serially diluted bacterial CrV3 (CrV3~5 μg) mixed with Freund’s complete adjuvant (Sigma) and incubated for 18 h at 37 °C. To test for dependence of lectin activity on divalent cations, 25 μl of serial dilutions causing 50% inhibition of lectin activity was made for all sugars tested. To test for inhibitory ligands, 5 μl of sugar solution (various concentrations) in PBS was added before incubation in place of the 5 μl of PBS used to dilute ORBCs in the standard assay. Lipopolysaccharide (E. coli, serotype 055:B5A; Sigma) and Laminari tetrose were added as described for the other sugars and up to a maximum concentration of 1 mg/ml. Comparison of concentrations causing 50% inhibition of lectin activity was made for all sugars tested. To test for dependence of lectin activity on divalent cations, 25 μl of serial CrV3 sample dilutions were prepared in 1 ml divalent cations (Mg, Mn, and Ca) or 1 ml EDTA and mixed with 25 μl of 2% ORBCs as described above. Increasing concentrations of divalent cations were also added to EDTA-inhibited CrV3 to restore lectin activity.

**RESULTS AND DISCUSSION**

**Molecular Characterization and Expression of CrV3**—*C. rubecula* parasitoid wasps inject polydnavirus particles into the hemocoe of *P. rapae* larvae at oviposition, leading to infection of host tissues by the particles and transient expression of particle-associated genes (19). CrV1 was previously isolated by screening a cDNA library constructed from 6 h parasitized caterpillars using total CrBV DNA as a probe (19). The same method was used here to isolate a ~700-bp cDNA encompassing the coding region of a putative CrBV gene and including a poly(A) tail (Fig. L). To confirm the cDNA as particle-derived, the fragment was cloned and used as a probe in both a Southern blot of digested CrBV DNA (Fig. 2A) and a Northern blot of RNA from unparasitized and 6 h parasitized larvae (Fig. 2B). Hybridization occurred to a CrBV restriction fragment of ~4 kb and to a parasitism-specific transcript of ~1.1 kb. These data and the fact that the same probe bound to genomic DNA from female wasps but not to that of *P. rapae* (data not shown) indicate that the cDNA originated from particles introduced to the oviposition.

**Binding of the cDNA to only one site in the Northern blot reveals that CrV3 shows no significant nucleotide sequence homology with other CrBV-related genes.** The cDNA was subsequently sequenced with data showing an open reading frame of 480 bp (Fig. 1A). A methionine codon (ATG) at the beginning of the open reading frame was identified as the only possible codon with a nucleotide sequence environment predicted for functional initiation codons (39). The predicted molecular mass of CrV3 is 17.6 kDa, with a pl of 9.13. Computer analyses (PSORT II; psort.nibb.ac.jp/form2.html) of the deduced amino acid sequence revealed a putative signal peptide encompassing the first 14 amino acids of the protein, with a cleavage point predicted at the end of the signal peptide (Fig. 1A), indicating that CrV3 protein is probably secreted from cells of origin. A hydrophobicity plot (Fig. 1B) was produced using ProtScale software (40). Highly hydrophobic residues near the N terminus support predictions of signal sequence composed of N-terminal amino acids. Three putative N-glycosylation sites were found in the open reading frame, as well as a polyadenyl-
Proteins are also glycosylated.

Analysis of cell-free hemolymph from 6 h P. rapae (D) treated (N) and N-glycosidase F-treated (C) CrV3 tetramer bands. Analysis of cell-free hemolymph from 6 h parasitized P. rapae larvae, which was either left at room temperature (PRT) or boiled for 10 min (P100) before electrophoresis. Heating resulted in dimer (lower arrowhead) denaturation into monomers and release of tetramer (upper arrowhead) from a putative larger complex. E and F, analysis of purified extract from induced bacteria containing empty plasmid or recombinant plasmid with CrV3 open reading frame. No CrV3-related molecules were detected in non-recombinant cells that were left at room temperature (BRT) or heated to 65 °C for 10 min (B65). CrV3 monomer and a range of multimers were detected in recombinant cells that were left at room temperature (CRT). Multimers were denatured into components when recombinant cells were heated at 65 °C for 10 min (CRT65). Boiling of CrV3 sample (CRT100) resulted in denaturation of a larger CrV3 complex, allowing detection of multimers not seen in the same amount of protein left at room temperature (CRT).

RT-PCR, utilizing primers from the CrV3 open reading frame, was used to test for production of CrV3 transcript in fat body and hemocytes from 6 h parasitized larvae (see Fig. 2F). These data indicate that CrV3 is produced by hemocytes and fat body cells. Western blot analysis, using anti-CrV3 antibodies, was performed on total proteins from larval fat body, hemocytes, and cell-free hemolymph at 6 h after parasitization (Fig. 2F). The presence of a large amount of CrV3 in the cell-free hemolymph compared with fat body or hemocytes confirms that the protein is secreted and possibly interacts with soluble hemolymph components. It appears that the relative amount of each CrV3 monomer varies with its location within parasitized larvae (Fig. 2F). In cell-free hemolymph, the ratio of 17-kDa monomer to 14-kDa monomer is usually 2:1 (see Fig. 2, D and F), whereas in fat body, the ratio is reversed (Fig. 2F). These data are consistent with CrV3 being secreted from fat body (and/or hemocytes) into the hemolymph because this is where most of the 17-kDa monomer is detected. The smaller monomer detected in fat body and hemocytes is probably intracellular. Presumably, only glycosylated monomers are secreted from infected cells before wasp or host elements remove cellular. Presumably, only glycosylated monomers are secreted from fat body (and/or hemocytes) into the hemolymph because this is where most of the 17-kDa monomer is detected. The smaller monomer detected in fat body and hemocytes is probably intracellular. Presumably, only glycosylated monomers are secreted from infected cells before wasp or host elements remove cellular. Presumably, only glycosylated monomers are secreted from infected cells before wasp or host elements remove
**Pteris** hemolymph (30). CrV3 hemolymph concentration was at a maximum at 6 h parasitization but was almost undetectable in hemolymph by Western analysis at 24 h parasitization (data not shown), an observation consistent with the transient expression of CrV3 (19).

Dimer and tetramer CrV3 molecules were detected in small amounts under denaturing conditions in parasitized larvae and were both shown to contain glycosylated monomers (Fig. 3, A-C). The relative amount of different oligomers appeared to vary with individual larvae, and often only one type was detected (compare Figs. 2 D and 3, A and B). The significance of this phenomenon is not clear. Boiling of cell-free hemolymph proteins from 6 h parasitized larvae resulted in an increase in CrV3 tetramers and a decrease in dimers (Fig. 3 D). It seems likely that boiling denatures the dimers and releases the tetramers from a large complex formed with a soluble hemolymph component or CrV3 alone. CrV3 hexamers and smaller oligomers were detected in purified bacterial CrV3 under denaturing conditions (Fig. 3 E). Heating bacterial CrV3 to 65 °C resulted in a breakdown of smaller multimers into their components. However, boiling resulted in an increase of all detectable multimers (Fig. 3 F), indicating that the bacterial CrV3 is forming much larger homogeneous complexes that are denatured at temperatures near 100 °C. The observation that CrV3

![Fig. 4. A, comparison of partial amino acid sequences in lectin domains of polydnavirus, insect, and mammalian CTLs. Homologous residues with CrV3 are boxed in black, and residues conserved among various CTLs are indicated by asterisks. B, comparison of complete amino acid sequences from CrV3 and hypothetical proteins from C. ruficrus and C. karyai bracoviruses. Indicators are as described for A. Sequences are from CrV3 (CrV3; GenBank™ accession number AY234855), C. ruficrus bracovirus hypothetical protein (CrufPDV; GenBank™ accession number BAC55179), C. karyai bracovirus hypothetical protein (CkarPDV; GenBank™ accession number BAC55180), B. mori lipopolysaccharide-binding protein (Bm; GenBank™ accession number CAB38429), P. americana hemolymph lipopolysaccharide-binding protein (Pa; GenBank™ accession number BAA00616), and Homo sapiens asialoglycoprotein receptor of hepatic lectin H1 (Hs; GenBank™ accession number NP00162).](http://www.jbc.org/)

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**Fig. 4. A, comparison of partial amino acid sequences in lectin domains of polydnavirus, insect, and mammalian CTLs. Homologous residues with CrV3 are boxed in black, and residues conserved among various CTLs are indicated by asterisks. B, comparison of complete amino acid sequences from CrV3 and hypothetical proteins from C. ruficrus and C. karyai bracoviruses. Indicators are as described for A. Sequences are from CrV3 (CrV3; GenBank™ accession number AY234855), C. ruficrus bracovirus hypothetical protein (CrufPDV; GenBank™ accession number BAC55179), C. karyai bracovirus hypothetical protein (CkarPDV; GenBank™ accession number BAC55180), B. mori lipopolysaccharide-binding protein (Bm; GenBank™ accession number CAB38429), P. americana hemolymph lipopolysaccharide-binding protein (Pa; GenBank™ accession number BAA00616), and Homo sapiens asialoglycoprotein receptor of hepatic lectin H1 (Hs; GenBank™ accession number NP00162).**
appears to only form multimers that are multiples of dimers suggests that pre-formed CrV3 dimers are the minimum element required for polymerization. The fact that bacterial CrV3 forms multimers indicates that sugar residues are not required for dimerization/multimerization. These large complexes were probably not entering the acrylamide gel or were not transferred to the membrane. Formation of large multimers is characteristic of several of the invertebrate CTLs characterized previously (42–46).

**Similarity of CrV3 and Known CTLs**—Comparison of the deduced amino acid sequence with those from the GenBankTM revealed that CrV3 shows significant conservation with various C-type lectins. Significantly, key amino acids are conserved in CTLs from invertebrates and mammals that are also found in CrV3 sequence (Fig. 4A). Thus, sequence similarities suggest that CrV3 is a lectin whose activity is dependent on the presence of divalent metal ions. Interestingly, the highest levels of similarity are with hypothetical proteins from *C. ruficrus* and *C. karyai* bracoviruses (67% and 61%, respectively) (Fig. 4A). This effect and the strong enhancement of agglutination by Mn<sup>2+</sup> may indicate that Mn<sup>2+</sup> may be important for tight regulation of CrV3 activity in vivo. It is possible that metal dependence of native CrV3 differs from that of recombinant protein.

CrV3-mediated agglutination was not significantly inhibited by any of over 20 sugars tested at 100 mM (Table II). None of the common mono- and disaccharides were significantly inhibitory, a result that was not expected, given the relatively simple specificities of the closest vertebrate lectins (commonly binding galactose). To test the assay, lectin from *Helix pomatia* was used to agglutinate cells and was completely inhibited by its hapten sugar, N-acetyl-d-galactosamine (49). It is possible that bacterial CrV3 has altered specificity compared with wild-type CrV3 due to differences in post-translational modifications; however, this is unlikely, given that lectin activity is readily demonstrated by red blood cell aggregation and dependence on divalent ions. It seems more likely that CrV3 requires a complex sugar and/or amino acid residues for its binding or is highly discerning in relation to which sugar anomer is encountered or what accessory elements are attached to the basic sugar monomer. Amino acid residues on each side of the complex sugar and/or amino acid residues for its binding or is demonstrated by red blood cell aggregation and dependence on divalent ions. It seems more likely that CrV3 requires a complex sugar and/or amino acid residues for its binding or is highly discerning in relation to which sugar anomer is encountered or what accessory elements are attached to the basic sugar monomer.

**CrV3 Lectin Activity: Hapten Sugars and Dependence on Divalent Metal Ions**—Purified bacterial CrV3 agglutinated trypsinized and gluteraldehyde-fixed ovine red blood cells. Lectin activity was shown to be enhanced in the presence of 1 mM Mg<sup>2+</sup> and Mn<sup>2+</sup> but was independent of Ca<sup>2+</sup> (Table I). Lectin activity was completely abolished in the presence of 1 mM EDTA and was restored by the addition of 0.5 mM Mg<sup>2+</sup> or 1 mM Mn<sup>2+</sup> but not by Ca<sup>2+</sup> concentrations up to 5 mM. Surprisingly, this is in contrast to other described CTLs, which are invariably Ca<sup>2+</sup>-dependent. The effect of Mn<sup>2+</sup> had a marked CrV3 concentration-dependent threshold, whereas the effect of Mg<sup>2+</sup> gradually decreased as CrV3 levels were reduced (data not shown). This effect and the strong enhancement of agglutination by Mn<sup>2+</sup> may indicate that Mn<sup>2+</sup> may be important for tight regulation of CrV3 activity in vivo. It is possible that metal dependence of native CrV3 differs from that of recombinant protein.
conserved proline (33), as do the hypothetical bracovirus lectins, the occurrence of the preceding lysine residue in CrV3 is rare among such lectins. Thus, the unusual CrV3 sequence may possibly explain its Ca\(^{2+}\) independence and why simple galactose-derived sugars do not inhibit CrV3-mediated agglutination as expected and may indicate that CrV3 specificity is atypical. It is perhaps intuitive that CrV3 may have highly specific binding requirements because it presumably targets an individual element associated with host immunity. Lipo-polysaccharide from *E. coli* (serotype 055:B5; Sigma) at 1 mg/ml also failed to significantly inhibit CrV3-mediated agglutination.

Preliminary in vitro experiments suggest that CrV3 may lessen the ability of healthy host hemocytes to spread on a foreign surface and may cause agglutination of these cells at high concentrations when present in the surrounding medium. Unlike CrV3, binding specificity, obtain purified native CrV3, and determine its mode of action.

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