Insect Cytokine Growth-blocking Peptide Triggers a Termination System of Cellular Immunity by Inducing Its Binding Protein*

Received for publication, June 6, 2003, and in revised form, July 15, 2003
Published, JBC Papers in Press, July 18, 2003, DOI 10.1074/jbc.M305986200

Yasuko Matsumoto, Yasunori Oda, Masahide Uryu, and Yoichi Hayakawa‡
From the Institute of Low Temperature Science, Hokkaido University, Sapporo 060-0819, Japan

Growth-blocking peptide (GBP) is a 25-amino acid cytokine found in lepidopteran insects that possesses diverse biological activities such as stimulation of immune cells (plasmatocytes), cell proliferation, and larval growth regulation. We found another novel function of GBP that induces a hemolysis of another class of blood cells (oenocytoids). In the lysate of oenocytoids we identified a GBP-binding protein that shows a specific affinity for GBP. The characterization of purified GBP-binding protein and its cDNA demonstrated it as a 49.5-kDa novel protein with a C-terminal region displaying limited homology to several insect lipoproteins. Results of Northern and Western blotting indicated that the GBP-binding protein should be synthesized only in blood cells. Immunoelectron microscopic analyses confirmed that indirect immunoreactive signals were mostly localized in oenocytoids. Kinetic and biological analyses of interaction between the GBP and the binding protein showed their strong binding was followed by clearance of GBP from hemolymph, thus indicating that this protein might function as an inhibitory factor against GBP. Based on these results, we propose that insect cytokine GBP shows multifunctions even in cellular immunity: it serves to stimulate immune cells and afterward silences its own action by inducing the binding protein through specific hemolysis.

Cytokines found in mammals are of central importance in the regulation of many physiological events such as immunity, inflammation, tissue remodeling, and embryonic development. They comprise a group of low molecular weight proteins that are produced by a variety of cell types and generally act in a paracrine or autocrine fashion. In insects, however, no cytokines had been identified until quite recently. The first family of cytokines identified in insects is now referred to as the ENF family of peptides; their functions are diverse. In vertebrates, most cytokines are produced by a variety of cell types and generally act in a paracrine or autocrine fashion. Insects, however, no cytokines had been identified until quite recently. The first family of cytokines identified in insects is now referred to as the ENF family of peptides; their functions are diverse. Cytokines found in mammals are of central importance in the regulation of many physiological events such as immunity, inflammation, tissue remodeling, and embryonic development. They comprise a group of low molecular weight proteins that are produced by a variety of cell types and generally act in a paracrine or autocrine fashion. In insects, however, no cytokines had been identified until quite recently. The first family of cytokines identified in insects is now referred to as the ENF family of peptides; their functions are diverse.

MATERIALS AND METHODS

Animals—Pseudaletia separata larvae were reared on an artificial diet at 25 ± 1 °C with a photoperiod of 16 h light:8 h dark. Penultimate larvae undergoing ecdisis between 4 and 4.5 h after lights on were designated as day 0 last instar larvae (3).

Hemocyte Preparation and Assay—Hemocytes were collected from larvae on day 4 of the last instar (sixth instar) according to the method of Pech et al. (15). Separation of hemocyte morphotypes was performed according to the slightly modified method of Clark et al. (16). Plasmatocytes and oenocytoids were isolated by loading hemocytes collected from 2–3 larvae onto Percoll gradients from 40 to 60% in Ex-cell 400 medium (JRH Bioscience). Plasmatocytes with an average purity of 70% and 90% and oenocytoids with a purity of ~90% were collected from the 45–49% and 52–56% Percoll interface, respectively. Oenocytoids were further purified by Percoll gradients from 0 to 60% in the same medium. By the second density gradient centrifugation, oenocytoids with an average purity of 70% and 90% and 12–14). Although the cytokine involved in the early immune reactions has been identified, we are still ignorant about the molecular mechanisms controlling the overall process in the cellular immune response. For example, is the effect of the cytokine specific only for early process of the immune reactions? How is the cytokine inactivated after stimulation of plasmatocytes?

To address these questions, we focused our attention upon hemocytes to assess their morphological and behavioral changes under the influence of GBP. We found that GBP induced lysis of a particular morphotype of hemocytes, oenocytoids. Further, in the lysate we identified the inhibitory factor against GBP action. Through the characterization of this GBP inhibitory factor, we propose here a novel mechanism by which the action of the insect cytokine is terminated in vivo.
Fig. 1. Time course of GBP-induced hemolysis in oenocytoids. A, phase-contrast micrographs of oenocytoid lysis. a, normal oenocytoid ~6 min after addition of GBP. Insert, this type of cell stained darkly by incubation with Dopa after fixation with glutaraldehyde. b-f, lysing oenocytoid within 100 s after a 26-min lag time. c-e, cytosolic contents spouted out of the lysing oenocytoid are indicated by arrowheads. e, oenocytoid ~38 after addition of GBP. Number in each photograph indicates time after addition of GBP. B, quantification of spread plasmatocytes and lysed oenocytoids in response to GBP. Mix suspension of ~1 × 10^6 isolated plasmatocytes (○, with GBP; ▲, with bovine serum albumin) and 1 × 10^6 oenocytoids (□, with GBP; ■, with bovine serum albumin) were plated into a 96-well plate (2 × 10^3 cell/well). In response to 1 nM GBP, plasmatocytes and oenocytoids were spread and lysed, respectively. Each point represents the mean ± S.D. for five independent determinations. Note that plasmatocyte spreading began ~20 min earlier than lysis of oenocytoid.

Purity of ~80–90% were obtained. Phenoloxidase activity in oenocytoid cells was characterized by the method of Rizki and Rizki (17). GBP-induced plasmatocyte-spreading activity was assayed by methods similar to that of Strand et al. (1). Isolated plasmatocytes and lysed oenocytoids were washed twice in Ex-cell 400, then resuspended in the same medium and plated into 96-well culture plates (1 × 10^4 cells/well). In response to 1 nM GBP, plasmatocytes and oenocytoids were spread and lysed, respectively. Each point represents the mean ± S.D. for five independent determinations. Note that plasmatocyte spreading began ~20 min earlier than lysis of oenocytoid.
endoproteinase, peptide fragments were separated on a SDS-PAGE gel and electrically transferred to polyvinylidene difluoride membrane filter, essentially according to Burnette (20). The transferred peptide fragments of GBP-binding protein were sequenced using an automatic protein sequencer (PPSQ-21; Shimadzu Corp.).

**Construction of Hemocyte cDNA Library—**Total RNA was isolated from whole body of day 1 last instar larvae of the armyworm larvae by the method of Chomczynski and Sacchi (21). Polyadenylated RNA was purified using the Quick Prep micro mRNA purification kit (Amersham Biosciences). A cDNA library was constructed using the Zap-cDNA packaging kit (Stratagene) and Superdex 75 column (Stratagene) according to the manufacturer’s instructions.

**Sequence Analysis of GBP-binding Protein cDNA—**Based on the N-terminal and internal peptide sequences determined by the peptide mapping and microsequencing described above, several degenerate primers were synthesized. Among them, by two primers, 5'-GWIISYV-TIACIATHTTYTTI-3' and 5'-TCGCCATTRTTIACGCIGC-3', the DNA fragment was amplified using the cDNA library as a template. The PCR amplification reaction was conducted according to the method of Hayakawa and Noguchi (22). Six gene-specific primers were designed from the nucleotide sequence of the binding protein cDNA fragment. These primers were used in conjunction with the anchor primers in 5' and 3' rapid amplification of cDNA ends kits (Invitrogen) to amplify both ends of the binding protein gene from the cDNA library.

**PCR products were subcloned into pBluescript KS(-) (Stratagene) and sequenced using Thermo Sequenase II dye terminator cycle sequencing kits (Amersham Biosciences). All DNA segments were sequenced at least twice in both directions using an ABI PRISM TM377 DNA sequencing system (Applied Biosystem). Computer-assisted sequence analysis was done with GENETYX-MAC Ver 10.1 (Software Development Co., Tokyo, Japan).

**BLAcore Analysis—**Real-time surface plasmon resonance experiments were performed on a BLAcore biosensor X system (Pharmacia Biosensor AB, Uppsala, Sweden). All experiments were performed at 25 °C with a constant flow rate of 10 μl/min. [biotin-L-lysyl-GBP] was immobilized to the Streptavidin sensor chip (Sensor Chip SA; Pharmacia Biosensor). A reference surface, to which no ligand was bound, was included on the chip. Various concentrations (0.0001–1.0000 nM) of GBP were preincubated with 18 nm GBP-binding protein at 25 °C for 6 h, and then each mixture was injected during the association phase with running buffer (0.1% Tween 20, 0.02% CHAPS in PBS, pH 7.0). Samples were injected in duplicate in random order in at least two separate experiments. The concentration of the GBP-binding protein bound to the sensor chip was calculated using the standard curve that had been previously made by BLAcore evaluation software 3.0 using resonance unit values for the injected standard solutions of the GBP-binding proteins. Kinetic analysis was evaluated by the Resolution affinity program in BLAcore evaluation software 3.0.

**RESULTS**

GBP-induced Hemolysis in Oenocytoids—When hemocytes isolated from the armyworm *P. separata* larvae were incubated with 10 nM GBP, immune cells called plasmatocytes rapidly spread on the surface of the culture plate (1). During prolonged observation, we realized that another morphotype of hemocytes was lysed ~20 min after the initiation of the plasmatocyte spreading (Fig. 1A). They were thought to be morphologically classified as oenocytoids. Because it was reported that oenocytoids contain prophenoloxidase in insect larvae (15, 26), we confirmed this identification by showing that these hemocytes melanized when incubated in the medium containing Dopa (Fig. 1A). To assess the contribution of GBP to both the activation of plasmatocytes and the lysis of oenocytoids, time course studies were conducted for an 80-min period after exposure to 1 nM GBP. Plasmatocytes were stimulated following addition of GBP, and afterward oenocytoids began to be gradually lysed (Fig. 1B). The lag time between stimulation and lysis of respective cells was interpreted to indicate that the hemolysis could be related to an inhibitory mechanism(s) of the GBP-dependent plasmatocyte activation.

**Discovery of GBP-binding Protein—**To examine whether oeno-
cytoids release the inhibitory factor against GBP action, we examined effects of the cell-free medium after incubation of oenocytoids on plasmatocytes by adding the medium into the plasmatocyte-spreading assay system containing GBP (Fig. 2A).

The incubation medium showed a dose-dependent capacity to decrease the GBP activity. Further, hemocyte-free plasma possesses a similar inhibitory activity against GBP (Fig. 2B), indicating that the medium as well as plasma could contain the factor...
that interacts with GBP. These results were interpreted to indicate two possibilities: the oenocytoid incubation medium and the plasma fraction might contain the factor with a specific binding capacity for GBP, or it might contain the factor with an effect against plasmatocytes to inhibit their spreading behavior.

We examined the former possibility by trying to identify the GBP binding factor(s). Because prior studies indicated that a 26-amino acid GBP containing a C-terminal biotinylated Lys26 (biotin-Lys26 GBP) retains the biological activity of a wild-type GBP (27), an affinity column was prepared using biotin-Lys26 GBP as a ligand. The fraction between 40 and 50% saturation of ammonium sulfate solution was loaded on the affinity column and a linear gradient elution of 0.1–2 M KCl in PBS was carried out after washing well with 0.1 M KCl in PBS. One major peak fraction with affinity for GBP was rechromatographed by gel permeation chromatography on a Superdex 75 column (Fig. 2C). An analysis of the active peak fraction by SDS-PAGE under reducing conditions yielded a single band indicating a molecular mass of ~49 kDa (Fig. 2C).

Based on the partially characterized primary structures of the purified GBP-binding protein, degenerate primers were synthesized. By a combination of primary PCR using these primers and following 5’- and 3’-RACE PCR, GBP-binding protein cDNA was isolated and sequenced (Fig. 3A). The deduced 430-amino acid sequence with a molecular mass of 49.5 kDa is a novel protein containing a C-terminal region displaying limited homology to several insect lipoproteins such as 30k-lipoprotein (28) and microvitellogenin (29) (Fig. 3B). Further, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and translational start codon; therefore, this protein appears not to have a signal peptide and transmembrane domain. These results are consistent with our observation that the binding protein was released through hemolysis of oenocytoids by GBP as shown in Fig. 1A.
Characterization of Binding between GBP and GBP-binding Protein—The direct and specific binding between GBP and the purified binding protein was demonstrated by real-time interaction analyses with immobilized [biotin-Lys26]GBP using surface plasmon resonance (Fig. 4A). A calculated mean dissociation constant of 74 pm (based on a 1:1 binding between the binding protein and the immobilized GBP) indicates a very stable complex. Further, the purified protein clearly decreased GBP-induced plasmatocyte spreading (Fig. 4B). The release of the binding protein from hemocytes in response to GBP and following binding between both factors in vitro was demonstrated by the evidence showing that [biotin-Lys26]GBP bound to the protein cross-reacted with anti-GBP-binding protein IgG in the medium. Further, the concentration of [biotin-Lys26]GBP bound with the binding protein was clearly increased during 30 min after addition of the biotinylated GBP (Fig. 4C). Finally, to analyze how an external stimulus affects both factors, their concentrations were measured in the hemolymph of bacteria-injected larvae. Western blots showed that both GBP and the binding protein were rapidly increased 5–15 min after the injection (Fig. 4D); at this time, a complex formation between both factors was demonstrated by the evidence that the protein immunoprecipitated by anti-GBP-binding protein IgG contains GBP (Fig. 4E). Thereafter, both factors clearly decreased from hemolymph within 30–60 min after the bacterial challenge (Fig. 4D). These results were interpreted to indicate that the GBP-binding protein could trap free GBP molecules and scavenge them to some extent.

Distribution of GBP-binding Protein and Its mRNA—To investigate the expression site of the binding protein, Northern and Western blots were carried out. The analysis of total cellular RNAs from various larval tissues showed the presence of the binding protein mRNA only in hemocytes (Fig. 5A). This result was confirmed by Western blotting using anti-GBP-binding protein antibody; the positive bands with an apparent molecular mass of 49 kDa were found in hemocytes and plasma (Fig. 5A). To confirm unambiguously which cell type(s) contains the binding protein, immunoelectron microscopy was conducted using the binding protein antibody. As we expected, oenocytoids were labeled most densely among the classes of hemocytes (data not shown). These results are consistent with our first observation that GBP-binding protein levels rose in the incubation medium during the process of oenocytoid lysis.

DISCUSSION

The present study was focused on the postregulation of the insect cytokine GBP activity, although it is also regulated before exerting its activity. It has been reported that six members of the ENF family are produced as part of a precursor protein, with the active peptide at the C terminus of the protein (6, 12, 30–32). In all ENF peptide precursors characterized so far, either a Lys-X-Gly-Arg or an X-Lys-Gly-Arg residue precedes the putative cleavage site for activation of proENF peptide. Thus, these conserved sequences likely serve as recognition sites for processing endoproteinase(s). These sequences are similar to the consensus cleavage site (Glu | Asp-Gly-Arg) for factor Xa, a main component of the mammalian blood coagulation system. In fact, Manduca sexta PP2, one of the ENF family peptides, was produced by processing of proPP2 by bovine factor Xa (12). Further, a serine protease partially purified from the Golgi body-rich fraction of P. separata larval fat body processed proGBP (33) as well as a synthetic peptide substrate (Boc-Ile-Glu-Gly-Arg-MCA) specific for factor Xa.2 Because insects generally have a poor blood coagulation system that is mainly managed by hemocytes and because plasma gelation has been observed only in limited species such as a locust and cockroach (34–36), it is worth emphasizing that a serine protease whose substrate specificity resembles factor Xa serves as a processing enzyme of the insect cytokine precursor.

Although the protein precursor of the ENF peptide is activated in a manner analogous to those with most of the neuroactive and other biogenic peptides (37), the latter events following activation of the cytokine precursor have been poorly understood in insects as well as mammals. In the last instar larvae of Lepidoptera, the proportion of immune potent cells such as plasmatocytes and granular cells is extremely high; the two cell types comprise greater than 90% of the total circulating hemocyte population in Pseudoplasia includens (13, 38). If the hemolymph concentration of the ENF peptide continuously keeps high enough to activate these immune cells, the larvae would possibly suffer serious damages through unnecessary excessive stimulation of these cells. The present study identified a novel hemolymph protein with a strong affinity specific for GBP. The kinetic (Fig. 4A) and biological (Fig. 4, B and C) analyses for this protein indicated that it has a strong binding capacity to inhibit GBP action. Further, once GBP and the binding protein form a complex, it is likely to be promptly degraded in hemolymph (Fig. 4, D and E). Based on these facts, it is reasonable to expect that this factor would serve as a scavenger protein against GBP. In mammals, it is well known that insulin-like growth factor-binding proteins (IGFBPs) regulate the cellular actions of the IGFs because of their strong affinities (39). IGFBPs generally potentiate IGF actions by increasing their circulating half-life and by affecting their tissue distribution and localization (40). Although at least six IGFBPs have been reported, none of them shares any significant similarity with the GBP binding peptide in terms of their biological activities as well as structures. Recently, an interleukin-18-binding protein (IL-18BP) has been found in human urine. IL-18BP expressed constitutively mainly in the spleen acts as a natural inhibitor of IL-18-induced interferon-γ and suppresses the T-lymphocyte helper type 1 response (41). Although IL-18BP functions as a cytokine inhibitor just as GBP-binding protein does, the two proteins do not share any structural similarity.

Biological (Figs. 1, A and B, and 2A) and immunocytochemical (Fig. 5B) experiments demonstrated that the binding protein is released from oenocytoids through the GBP-induced hemolysis. This conclusion was supported by the fact that the amino acid sequence of the binding protein deduced from its cDNA does not contain the signal peptide region. Therefore, it is thought that GBP stimulates two different types of hemocytes to regulate cellular immunity: the defense reaction is initiated and terminated by stimulation of plasmatocytes and oenocytoids, respectively.

Given that GBP serves to stimulate plasmatocytes as a potent cytokine and that the GBP-induced oenocytoid lysis follow the plasmatocyte activation, it is reasonable to propose that the GBP-binding protein released from oenocytoids scavenges GBP in the hemocoel in order to prevent GBP from excessively stimulating the immune cells. It is also reasonable to propose that this termination system of cellular immunity is triggered through the specific hemolytic action of GBP on oenocytoids. It is generally acknowledged that, although insects lack lymphocytes to produce unique immunoglobulins, insects have a cellular immunity that reacts quickly to foreign objects. The present study demonstrated that GBP would be able to regulate the insect cellular immunoreaction through the stimulation of completely different types of the cells.

---

2 H. Matsumoto and Y. Hayakawa, unpublished data.
Acknowledgment—We thank Prof. Michael R. Strand (University of Georgia) for critical reading of this manuscript. We also thank Dr. Megumi Moriya of this laboratory for technical assistance with the electron microscopic studies.

REFERENCES
1. Strand, M. R., Hayakawa, Y. & Clark, K. D. (2000) J. Insect Physiol. 46, 817–824
2. Hayakawa, Y. (1990) J. Biol. Chem. 265, 10813–10816
3. Hayakawa, Y. (1991) J. Biol. Chem. 266, 7982–7984
4. Hayakawa, Y. (1992) Biochem. Biophys. Res. Commun. 185, 1141–1147
5. Hayakawa, Y. (1995) J. Insect Physiol. 41, 1–6
6. Hayakawa, Y. & Ohnishi, A. (1996) Biochem. Biophys. Res. Commun. 250, 194–199
7. Skinner, W. S., Dennis, P. A., Li, J. P., Summerfelt, R. M., Carney, R. L. & Quistad, G. B. (1991) J. Insect Physiol. 37, 1445–1450
8. Clark, K. D., Pech, L. L. & Strand, M. R. (1997) J. Biol. Chem. 272, 23440–23447
9. Aizawa, T., Hayakawa, Y., Ohnishi, A., Fujitani, N., Clark, K. D., Strand, M. R., Miura, K., Koganesawa, N., Kumaki, Y., Demura, M., Nitta, K. & Kawano, K. (2001) J. Biol. Chem. 276, 31813–31818
10. Rowly, A. F. & Ratcliffe, N. A. (1980) Immunology 40, 483–492
11. Strand, M. R. & Pech, L. L. (1995) Annu. Rev. Entomol. 40, 31–56
12. Wang, Y., Jiang, H. & Kanost, M. R. (1999) J. Insect Physiol. 45, 1075–1086
13. Strand, M. R. & Clark, K. C. (1999) Arch. Insect Biochem. Physiol. 40, 213–223
14. Larvins, M. D. & Strand, M. R. (2002) Arch. Insect Biochem. Physiol. 51, 1237–1242
15. Pech, L. L., Trudeau, D. & Strand, M. R. (1994) Cell Tissue Res. 277, 159–167
16. Clark, K. D., Volkman, B. F., Thoetkiattikul, H., Hayakawa, Y. & Strand, M. R. (2001) J. Biol. Chem. 276, 37431–37435
17. Rizki, R. M. & Rizki, T. M. (1990) J. Insect Physiol. 36, 523–529
18. Laemmli, V. K. (1970) Nature 277, 680–685
19. Cleaveland, D. W., Fisher, S. G., Kirschner, M. W. & Laemmli, V. K. (1977) J. Biol. Chem. 252, 1102–1106
20. Burnette, W. N. (1981) Anal. Biochem. 112, 195–203
21. Chomczynski, P. & Sacchi, N. (1987) Anal. Biochem. 162, 156–159
22. Hayakawa, Y. & Noguchi, H. (1988) Eur. J. Biochem. 185, 810–816
23. Hiraoka, T., Hayakawa, Y. & Downer, R. G. H. (1995) Cell Tissue Res. 279, 465–468
24. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
25. Reynolds, K. S. (1963), J. Cell Biol. 17, 208–212
26. Ashida, M., Chiba, T., Nishi, Y. & Niki, T. (1988) Tissue Cell 20, 599–610
27. Ohnishi, A., Oda, Y. & Hayakawa, Y. (2001) J. Biol. Chem. 276, 37974–37979
28. Mori, S., Izumi, S. & Tomino, S. (1991) Biochem. Biophys. Acta 1090, 129–132
29. Wang, X. Y., Cole, K. D. & Law, J. H. (1988) J. Biol. Chem. 263, 8851–8855
30. Hayakawa, Y., Ohnishi, A., Yamanaka, A., Izumi, S. & Tomino, S. (1995) FEBS Lett. 376, 185–189
31. Clark, K. D., Withereill, A. & Strand, M. R. (1998) Biochem. Biophys. Res. Commun. 250, 479–485
32. Kaminuma, M., Nakahara, Y., Kanamori, Y., Tsuzuki, S., Hayakawa, Y. & Kiuchi, M. (2001) Biochem. Biophys. Res. Commun. 286, 67–73
33. Hayakawa, Y., Ohnishi, A. & Endo, Y. (1998) J. Insect Physiol. 44, 859–866
34. Frank, H. (1960) Zool. Jahrb. Abt. Allg. Zool. Physiol. Tierle 88, 499–518
35. Frank, H. (1960) Zool. Jahrb. Abt. Allg. Zool. Physiol. Tierle 89, 131–132
36. Brehelin, M. M. (1972) Acta Zool. 53, 165–175
37. Crine, P. & Boileau, G. (1987) in Immunobiology of Mammalian Tissue Transplants, (Boulton, A. A., Biber, G. B. & Pittman, Q. G., eds) pp. 1–44, Humana Press, Clifton, NJ
38. Strand, M. R., Clark, K. C. & Gardner, E. M. M. (1999) Arch. Insect Biochem. Physiol. 40, 41–52
39. Rajaram, S., Boylins, D. J. & Mohan, S. (1997) Endocrin. Rev. 18, 801–883
40. Martin, J. L. & Baxter, R. C. (1999) in The RGF System (Rosenfeld, R. G. & Roberts, C. T., Jr., eds) pp. 227–255, Humana Press, Clifton, NJ
41. Novick, D., Kim, S.-H., Fantuzzi, G., Reznikov, L., Dinarello, C. A. & Rubinstein, M. (1999) Immunity 10, 127–136