Identification of collagen IV derived danger/alarm signals in insect immunity by nanoLC-FTICR MS

Boran Altincicek¹, Arton Berisha², Krishnendu Mukherjee¹, Bernhard Spengler², Andreas Römpp² and Andreas Vilcinskas¹,*

¹ Institute of Phytopathology and Applied Zoology, Justus Liebig University of Giessen, D-35392 Giessen, Germany
² Institute of Inorganic and Analytical Chemistry, Justus Liebig University of Giessen, D-35392 Giessen, Germany

* Corresponding author
e-mail: Andreas.Vilcinskas@agrar.uni-giessen.de

Abstract

The immune system can be stimulated by microbial molecules as well as by endogenously derived danger/ alarm signals of host origin. Using the lepidopteran model insect Galleria mellonella, we recently discovered that fragments of collagen IV, resulting from hydrolysis by microbial metalloproteinases, represent danger/alarm signals in insects. Here, we characterized immune-stimulatory peptides generated by thermolysin-mediated degradation of collagen IV using nanospray ionization Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) after separation by nanoscale liquid chromatography (nanoLC). The combination of FTICR MS analysis and de novo peptide sequencing resulted in the identification of 38 specific collagen IV fragments of which several peptides included the integrin-binding motif RGD/E known from numerous mammalian immune-related proteins. Custom-synthesized peptides corresponding either to the presently identified collagen peptide GIRGEHyp or to a well-known integrin-binding RGD peptide (GRGDS) were injected into G. mellonella to determine their immune-stimulatory activities in vivo. Both peptides stimulated immune cells and systemically the expression of lysozyme and a specific inhibitor of microbial metalloproteinases. Further examination using specific MAP kinase inhibitors indicated that MEK/ERK and p38 are involved in RGD/E-mediated immune-signaling pathways, whereas JNK seems to play only a minor role.

Keywords: collagen; danger signals; Galleria mellonella; innate immunity; insect model organism; integrin.

Introduction

A prerequisite for the evolution of Metazoa ranging from insects to humans has been the ability to recognize and to encounter microbial invasion (Boman, 2003; Beutler, 2004; Akira et al., 2006; Beutler et al., 2006). The highly specific recognition of a wide range of antigens by T cell receptors and B cell-derived antibodies, followed by the immunological memory of infection, are features of the adaptive or acquired immunity that arose during vertebrate evolution. Insects lacking this type of adaptive immunity rely solely on the evolutionarily conserved innate immunity and are, therefore, valuable model organisms to elucidate its underlying molecular mechanisms. The insect immune system includes Toll, immune deficiency Imd, and the Janus-kinase/signal transducers and activators of transcription JAK/STAT signaling pathways. These pathways induce multiple defense reactions such as phagocytosis, nodulation and/or encapsulation of microbes and parasites, phenoloxidase activation, hemolymph coagulation, and production of antimicrobial effectors such as reactive oxygen species or antimicrobial peptides (e.g., cecropins and defensins) (Cherry and Silverman, 2006; Jiravanichpaisal et al., 2006; Lemaitre and Hoffmann, 2007).

In mammals, Toll-like receptors, for example, recognize both pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide or flagellin as well as damage-associated molecular patterns such as extracellular uric acid, nucleic acids or HMGB1 (Matzinger, 2002; Karin et al., 2006). Using the lepidopteran model insect Galleria mellonella, we recently provided evidence that endogenously derived danger molecules also exist in insects (Griesch et al., 2000; Altincicek and Vilcinskas, 2006, 2008; Altincicek et al., 2007, 2008). G. mellonella has been the focus of increasing attention as a source of novel antimicrobial peptides with therapeutic potential (Langen et al., 2006; Wedde et al., 2007), and as a surrogate model host to investigate pathogenesis of human pathogens. It provides the advantage that it can be reared at mammalian physiological temperatures (around 37°C) to which human pathogens are adapted and which are essential for synthesis of many microbial virulence/pathogenicity factors (Mylonakis et al., 2007).

Thermolysin-like metalloproteinases (M4-type) encompass prominent microbial virulence factors and toxins such as aureolysin, bacillolysin, and pseudolysin which reportedly cause pathologic symptoms such as increased vascular permeability, hemorrhagic edema, sepsis, and necrotic tissue destruction in mammals (Adekoya and Sylte, 2009). A recent study has revealed that injection of thermolysin into G. mellonella hemocoel elicits immune responses that are qualitatively (spectrum of induced peptides and proteins) and quantitatively (levels of induced antimicrobial activity in the hemolymph) comparable with those that are observed in response to injected bacteria (Altincicek et al., 2007). Particularly, thermolysin-mediated degradation of the evolutionarily conserved extracellular matrix component collagen IV...
resulted in peptidic fragments with potent immune-stimulatory activity in *G. mellonella* larvae (Altincicek and Vilcinskas, 2006).

Type IV collagen associated with laminin, entactin, and heparan sulfate proteoglycans forms the sheet-like basement membranes that separate epithelium from connective tissue and is conserved in animals (Lunstrum et al., 1988; Knorr et al., 2009). Recent studies provide evidence that the 140-, 100-, and 70-kDa fragments of human placental type IV collagen are derived from the proα1(IV) chain, whereas the 70-kDa fragment is derived from the proα2(IV) chain (Sage et al., 1979; Crouch et al., 1980). In insects, collagen IV plays a specific role in innate immune defense reactions in Lepidoptera (Adachi et al., 2005). Recently, we have shown that purified peptides with molecular masses below 3 kDa resulting from thermolysin-mediated hydrolysis of collagen IV exhibit significant immune-stimulatory activity (Altincicek and Vilcinskas, 2006). In this follow-up study, we identified amino acid sequence of the immune-stimulatory collagen IV peptides by an online combination of nanoscale liquid chromatography (nanoLC) and Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) interfaced by a nanospray ionization source. Additionally, we validated the sequences using online peptide sequencing. The peptide hydrolysate (Altincicek and Vilcinskas, 2006). Hence, we wanted to identify single peptides responsible for the immune stimulatory activity of the collagen IV hydrolysate (Altincicek and Vilcinskas, 2006). Hence, we examined obtained peptide sequences for known immune-related motifs and found that several peptides included the integrin-binding motif RGD/E (Table 1). To investigate the possible role of integrin-binding peptides in insect immune activation, we synthetically produced the peptide GIRGEHyp (coll-peptide) corresponding to the sequences identified in the present study along with a well-known integrin-binding RGD peptide GRGDS as a positive control for further experiments. The peptide VDGKSAPNV unrelated to integrin-binding sequence was used as negative control.

**Results**

We recently determined that degradation of collagen IV by thermolysin, which cleaves before amino acids with hydrophobic or bulky side chains, results in particular peptides, which represent potent elicitors of innate immune responses when injected into *G. mellonella* larvae (Altincicek and Vilcinskas, 2006). The number of peptides that were determined by matrix-assisted laser desorption ionization-time of flight mass spectrometric (MALDI-TOF MS) analysis was significantly lower than that predicted from *in silico* thermolysin hydrolysis (Altincicek et al., 2007). This manageable number of immune-stimulatory collagen IV peptides stimulated us to identify their exact sequences using nanoLC coupled to FTICR MS analysis.

A typical experiment using a hybrid ion trap (IT) ion cyclotron resonance (ICR) mass spectrometer included measurements in a survey MS scan in the ICR cell with high mass resolving power ($R = 100,000$) in parallel to MS/MS experiments of the five most intense ions of the survey scan. The peak list was used for the FTICR MS experiments. We validated the sequences identified in the present study along with a well-known integrin-binding RGD peptide GRGDS as a positive control for further experiments. The peptide VDGKSAPNV unrelated to integrin-binding sequence was used as a negative control.

**Figure 1** Workflow for the identification of specific collagen IV derived peptides.

The schematically drawn workflow demonstrates the order of used methods for the identification of specific collagen peptides.
Table 1  Identification of 38 specific collagen IV peptides by nanoLC-FTICR MS and de novo peptide sequencing.

<table>
<thead>
<tr>
<th>[M+H]+ (Da)</th>
<th>Δm/m (ppm)</th>
<th>z</th>
<th>Sequence</th>
<th>Probability factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>506.26129</td>
<td>0.75</td>
<td>1</td>
<td>FGIP*G</td>
<td>8.90E-02</td>
</tr>
<tr>
<td>634.31987</td>
<td>0.60</td>
<td>1</td>
<td>LGQFPG</td>
<td>2.60E-02</td>
</tr>
<tr>
<td>637.30195</td>
<td>0.87</td>
<td>1</td>
<td>IMGFP*G</td>
<td>8.70E-02</td>
</tr>
<tr>
<td>685.38917</td>
<td>-1.83</td>
<td>1</td>
<td>K.IAVQPQTV</td>
<td>3.16E-05</td>
</tr>
<tr>
<td>724.31594</td>
<td>-1.56</td>
<td>1</td>
<td>D.FGIDGDTI</td>
<td>1.66E-04</td>
</tr>
<tr>
<td>754.37848</td>
<td>-1.93</td>
<td>1</td>
<td>G.ITGFPG*F</td>
<td>1.86E-05</td>
</tr>
<tr>
<td>755.39483</td>
<td>-1.89</td>
<td>1</td>
<td>F.IGDP*GIPAL</td>
<td>7.12E-05</td>
</tr>
<tr>
<td>771.38970</td>
<td>-1.79</td>
<td>1</td>
<td>F.IGDP<em>GIP</em>AL</td>
<td>4.01E-05</td>
</tr>
<tr>
<td>775.44667</td>
<td>-0.73</td>
<td>2</td>
<td>G.LRGIFPG*F</td>
<td>6.51E-04</td>
</tr>
<tr>
<td>837.44766</td>
<td>-1.39</td>
<td>1</td>
<td>G.LQGPVGP<em>G</em>F</td>
<td>7.69E-06</td>
</tr>
<tr>
<td>839.44543</td>
<td>-1.24</td>
<td>2</td>
<td>E.ILGHPV<em>G</em>M*L</td>
<td>9.13E-04</td>
</tr>
<tr>
<td>852.37413</td>
<td>-0.88</td>
<td>1</td>
<td>E.IGATGFDGDI</td>
<td>1.50E-05</td>
</tr>
<tr>
<td>855.44020</td>
<td>-1.04</td>
<td>2</td>
<td>E.ILGHPV<em>G</em>M*</td>
<td>9.58E-05</td>
</tr>
<tr>
<td>926.45845</td>
<td>-0.70</td>
<td>2</td>
<td>G.VSGQP<em>GVP</em>GQ</td>
<td>1.75E-05</td>
</tr>
<tr>
<td>958.44779</td>
<td>-0.18</td>
<td>2</td>
<td>G.LTGPP*GSOQGE</td>
<td>1.70E-07</td>
</tr>
<tr>
<td>1034.52770</td>
<td>-1.12</td>
<td>2</td>
<td>S.IGV<em>GVP</em>GHEGA</td>
<td>5.11E-05</td>
</tr>
<tr>
<td>1050.52243</td>
<td>-0.92</td>
<td>2</td>
<td>S.IGV<em>GVP</em>GHEG</td>
<td>5.36E-05</td>
</tr>
<tr>
<td>1068.53284</td>
<td>-0.76</td>
<td>2</td>
<td>G.VAGVPP<em>GPGTP</em>G</td>
<td>2.43E-04</td>
</tr>
<tr>
<td>1080.53522</td>
<td>-2.96</td>
<td>1</td>
<td>F.LQP<em>GPAGT</em>GQ</td>
<td>1.74E-04</td>
</tr>
<tr>
<td>1105.56897</td>
<td>-3.00</td>
<td>1</td>
<td>S.IGV<em>GVP</em>GHEGA</td>
<td>4.11E-09</td>
</tr>
<tr>
<td>1121.55987</td>
<td>-1.16</td>
<td>2</td>
<td>S.IGV<em>GVP</em>GHEGA</td>
<td>1.10E-05</td>
</tr>
<tr>
<td>1121.62049</td>
<td>0.53</td>
<td>1</td>
<td>G.LVGGP<em>GPP</em>G</td>
<td>1.19E-04</td>
</tr>
<tr>
<td>1125.55505</td>
<td>-1.39</td>
<td>1</td>
<td>G.VSGQP<em>GVP</em>GGAQ</td>
<td>8.45E-08</td>
</tr>
<tr>
<td>1181.61787</td>
<td>-1.51</td>
<td>2</td>
<td>G.VAGVPP<em>GPGTP</em>G</td>
<td>4.18E-04</td>
</tr>
<tr>
<td>1225.65482</td>
<td>-1.05</td>
<td>2</td>
<td>D.IGDTNLPGPR*G</td>
<td>4.73E-04</td>
</tr>
<tr>
<td>1238.55665</td>
<td>-2.44</td>
<td>1</td>
<td>E.IGATGFDGDI</td>
<td>5.38E-04</td>
</tr>
<tr>
<td>1281.64560</td>
<td>-1.74</td>
<td>2</td>
<td>G.IQGEP<em>G</em>GL<em>G</em>PSV</td>
<td>2.47E-04</td>
</tr>
<tr>
<td>1305.69155</td>
<td>-0.43</td>
<td>3</td>
<td>G.ISGP<em>GGLP</em>GPR<em>G</em>GH</td>
<td>2.11E-05</td>
</tr>
<tr>
<td>1343.61452</td>
<td>-2.32</td>
<td>2</td>
<td>G.LGQFP<em>GGLP</em>GPP*G</td>
<td>9.35E-05</td>
</tr>
<tr>
<td>1366.65110</td>
<td>-0.93</td>
<td>3</td>
<td>G.FRQDEGPIGHQGP</td>
<td>1.15E-09</td>
</tr>
<tr>
<td>1385.68040</td>
<td>-2.6</td>
<td>2</td>
<td>S.IGQDGQRRLP<em>G</em>EM</td>
<td>1.39E-04</td>
</tr>
<tr>
<td>1516.75362</td>
<td>-2.19</td>
<td>2</td>
<td>G.IGP<em>GPARGGP</em>GQQGPP*G</td>
<td>1.61E-05</td>
</tr>
<tr>
<td>1559.78263</td>
<td>-0.88</td>
<td>3</td>
<td>G.VGFLPGEP<em>K</em>GP*RQEFG</td>
<td>9.97E-05</td>
</tr>
<tr>
<td>1862.85202</td>
<td>-1.61</td>
<td>2</td>
<td>G.FMGQP<em>GPGQGQGLP</em>GSP<em>GH</em> A</td>
<td>1.83E-08</td>
</tr>
<tr>
<td>1963.97374</td>
<td>-0.89</td>
<td>2</td>
<td>G.IQGEP<em>G</em>GL<em>G</em>GQG<em>G</em>V</td>
<td>4.45E-08</td>
</tr>
<tr>
<td>1980.84503</td>
<td>-1.65</td>
<td>2</td>
<td>G.AQGEP<em>GSGQPP</em>GDP<em>GGLP</em>GPP*G</td>
<td>1.13E-04</td>
</tr>
<tr>
<td>2088.09031</td>
<td>0.56</td>
<td>4</td>
<td>Y.ALPKEEDRYBQP<em>GE</em>GPP*G</td>
<td>2.84E-04</td>
</tr>
<tr>
<td>2516.16372</td>
<td>-2.16</td>
<td>3</td>
<td>T.YGPQGOGRQPP<em>GAP</em>GEM<em>GPQGPP</em>GEP*G</td>
<td>4.92E-05</td>
</tr>
</tbody>
</table>

The respective amino acid sequences of the first three identified peptides were determined by de novo peptide sequencing. Identified sequences are written in bold and are shown relative to their predicted cleavage sites, which are each indicated by a point and the respective amino acid.

*Hydroxylation; RGD/E motifs are indicated by gray shading.

Coll-peptide and GRGDS exhibited similar immune stimulation on hemocytes in vivo. The presence of coll-peptide or GRGDS in the hemolymph of G. mellonella led to a change in hemocyte behavior. Non-adherent, resting hemocytes became adherent and activated, resulting in a lesser amount of freely circulating hemocytes. We determined a >70% reduction of circulating hemocytes 4 h post-injection of the peptides (Figure 2A). Moreover, injection of coll-peptide or GRGDS significantly induced the production of antimicrobial effectors. We determined a two-fold induction of inhibitory activity against Micrococcus luteus 24 h post-injection of coll-peptide or GRGDS (Figure 2B). Consistently, we determined approximately 30-fold enhanced mRNA levels encoding for lysozyme 8 h post-injection using quantitative real-time PCR. Simultaneously, we determined an approximate 10-fold enhanced expression of the insect metalloproteinase inhibitor (IMPI; Figure 3) which specifically inhibits thermolysin-like microbial metalloproteinases (Wedde et al., 2007). The significantly induced expression of lysozyme and IMPI was not observed when the control peptide VDGKSAVPN was injected instead of coll-peptide or GRGDS (Figure 3).

However, it is noteworthy that the expression of other tested antimicrobial molecules such as gallerimycin and galloomycin were not significantly upregulated in response to challenge with coll-peptide or GRGDS (Figure 3). Hence, our results indicate a strong cellular immune activation and a selective systemic immune activation in G. mellonella.

Because RGD/E peptides are well-known ligands of integrins and integrin-signaling is predominantly mediated by MAP kinases, we investigated a potential regulatory role of MAP kinases in immune responses induced by the coll-peptide. We used lysozyme activity in the hemolymph as a convenient and reliable read-out method and injected the coll-peptide with or without a p38 kinase inhibitor (SB203580), a MEK/ERK inhibitor (PD98059), or
Injection of coll-peptide as well as GRGDS induced similar immune responses. Larvae injected with saline, coll-peptide, and GRGDS, respectively, were investigated in immune response assays. (A) Cellular immune stimulation and (B) humoral immune stimulation were significantly induced upon injection of coll-peptide and GRGDS, respectively. Results represent mean values of at least three independent determinations ± SD. Statistical significances (p < 0.05) were determined by Student’s t-test and are indicated by asterisks.

Quantitative real-time RT-PCR analysis of mRNA levels of immune genes. Levels of mRNA of lysozyme as well as IMPI were found to be significantly elevated on injection of both coll-peptide and GRGDS in comparison to control injection (p < 0.05, Student’s t-test). In contrast, mRNA levels were of gallerimycin, and gallerimycin was not significantly induced by coll-peptide or GRGDS peptide. Results represent mean values of at least three independent determinations ± SD.

Discussion

The insect immune system encompasses locally restricted cellular and systemic humoral immune responses, which are tightly linked with each other because many humoral factors affect hemocyte function and hemocytes are an important source of many cytokine-like molecules and antimicrobial peptides (Strand, 2008). By microscopic examination, we observed that epidermal wounding with a sterile needle caused local changes in hemocyte behavior in Galleria larvae. Immune-competent non-adhesive hemocytes became adhesive and attached to tracheal epidermis at the wounding site where they displayed significant collagen IV degrading activities (Figure 6). We recently demonstrated that peptides derived from hydrolyzed collagen IV are able to activate both cellular and humoral immune responses in G. mellonella (Altincicek and Vilcinskas, 2006). Here, we identified particular immune-stimulatory collagen IV derived peptides containing the integrin binding motif RGD/E by nanoLC-FTICR MS and found that immune responses induced by RGD/E peptides were dependent on MEK/ERK and p38 MAP kinases.

Integrin receptors are the main mediators of cell adhesion to the extracellular matrix. They bind to extracellular matrix proteins by interacting with short amino acid...
Coll- peptide inducible lysozyme activity was sensitive to MAP kinase inhibitors. Coinjection of MEK/ERK inhibitor PD98059 and p38 inhibitor SB203580, respectively, along with coll-peptide resulted in a significant decrease of inducible lysozyme activity in hemolymph 24 h post-treatment. In contrast, JNK inhibitor SP600125 had only little influence on inducible lysozyme activity which was not statistically significant (p > 0.75, Student’s t-test). In contrast, the peptide, VDGKSAPNV, unrelated to integrin-binding sequence showed no significant inducible lysozyme activity. Results represent mean values of at least three independent determinations ± SD. Statistical significances (p < 0.05) were determined by Student’s t-test and are indicated by asterisks.

sequences, such as the RGD sequence (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987). Soluble, small RGD-based peptides have been used to block integrin-binding to ligands, thereby interfering with cell adhesion, migration, and survival, whereas substrate-immobilized RGD sequences have been used to enhance cell binding to artificial surfaces. In mammals, integrins are multifunctional, bidirectional signaling molecules known to contribute to numerous immune-related processes (Springer, 1990; Shimizu et al., 1999; Hynes, 2002; Ortega-Velázquez et al., 2003; Luo et al., 2007). Moreover, a recent study demonstrates that integrin engagement mediates the human polymorphonuclear leukocyte response to fungal β-glucan by a cross-regulatory relationship to well-known immune signaling pathways (Lavigne et al., 2007). In insects, several studies provided evidence for the involvement of integrins in hemocyte adhesion and aggregation, encapsulation of parasitoids, and phagocytosis of bacteria (Pech and Strand, 1995; Foukas et al., 1998; Lavine and Strand, 2003; Levin et al., 2005; Zhuang et al., 2005). Integrins have been shown to be involved in killing of *Onchocerca* microfilariae in a black fly, *Simulium damnosum* (Hagen and Kläger, 2001), and to interact with tetraspanin in *Manduca sexta* to enable proper cellular innate immune responses *in vivo* (Zhuang et al., 2007). Additionally, recent reports indicated that bacteria directly stimulate the integrin-mediated signaling pathway, which activates FAK/Src and MAP kinases leading to secretion of bioactive molecules in the medfly *Ceratitis capitata* (Lamprou et al., 2005, 2007; Mamali et al., 2008).

Supporting these results, a p38 MAP kinase has been determined to regulate the expression of a defensin gene in *Aedes aegypti* cells (Chen-Chih Wu et al., 2007). Hence, several lines of evidence indicate the importance of integrins in cellular as well as in systemic immune reactions in insects.

Our results from *G. mellonella* implicate that integrin-mediated signaling can be activated by particular collagen IV peptides as well as by typical RGD-peptide stimulating immune responses. We are aware that collagen-derived peptides are surely not the sole danger/alarm molecules in *Galleria* and peptides derived from other extracellular as well as intracellular proteins might also stimulate immune responses. In conclusion, collagen IV could be a substrate for which degradation by microbial metalloproteinases can indicate the presence of harmful pathogens, which release toxins. This assumption is supported by the observation that challenge with coll-peptide induces expression of both lysozyme and the IMPI that represents the only peptide reported from animals capable of specifically inhibiting thermoly-

![Figure 4](image_url) **Figure 4** Coll- peptide inducible lysozyme activity was sensitive to MAP kinase inhibitors. Coinjection of MEK/ERK inhibitor PD98059 and p38 inhibitor SB203580, respectively, along with coll-peptide resulted in a significant decrease of inducible lysozyme activity in hemolymph 24 h post-treatment. In contrast, JNK inhibitor SP600125 had only little influence on inducible lysozyme activity which was not statistically significant (p > 0.75, Student’s t-test). In contrast, the peptide, VDGKSAPNV, unrelated to integrin-binding sequence showed no significant inducible lysozyme activity. Results represent mean values of at least three independent determinations ± SD. Statistical significances (p < 0.05) were determined by Student’s t-test and are indicated by asterisks.

![Figure 5](image_url) **Figure 5** FITC-labeled coll-peptide bound to the cell surface of a subset of hemocytes in a similar pattern as FITC-labeled RGD peptide. (A, B) FITC-labeled coll-peptide bound strongly to the cell surface of a subset of live hemocytes resulting in a specific pattern of green fluorescence. Inset shows a magnification of a single cell. (C, D) We observed a similar pattern of binding of FITC-labeled GRGDS peptide to a subset of hemocytes. Insets show magnification of single cells. (C, D) No significant binding of FITC-labeled control peptide was detected or (E, F) FITC-Tris that used as control. (A, C, E) Differential interference contrast (Nomarski) and (B, D, F) fluorescence imaging. Scale bars: 100 μm.
Figure 6  Hemocytes attached to injured tracheal basement membrane exhibited significant collagen IV degrading activity. (A) On wounding of tracheal epidermis, circulating hemocytes become adhesive at the site of injury. (B) Subsequently, these hemocytes show significant collagen IV degrading activity resulting in green fluorescence with the use of soluble, fluorescent collagen IV.

Materials and methods

Insect rearing and manipulation

Galleria mellonella larvae were reared on an artificial diet (22% maize meal, 22% wheat germ, 11% dry yeast, 17.5% bee wax, 11% honey, and 11% glycerin) at 32°C in darkness. Last instar larvae, each weighing between 250 and 350 mg, were used for injection experiments. A total of 10 μl of sample volume per larva was injected dorsolaterally into the hemocoel using 1-ml disposable syringes and 0.4×20 mm needles mounted on a microapplicator. Peptides coll-peptide (GIRESVHyp, glycine-isoleucine-arginine-glycine-glutamate-hydroxyproline) with a purity of 94.83%, GRGDS (glycine-arginine-glycine-aspartate-serine) with a purity of 92.31%, and a control peptide unrelated to integrin-binding sequence (VDGKSAPNV, valine-aspartate-lysine-serine-alanine-proline-asparagine-valine) with a purity of over 92.31% were custom-synthesized by GL Biochem (Shanghai, China) and were dissolved in sterile saline (20 mM Na2HPO4 buffer, 100 mM NaCl, pH 7) prior to application. To investigate a possible regulatory role of MAP kinases, we coinjected SP600125 (Calbiochem, Darmstadt, Germany) to inhibit JNK signaling, PD98059 (Calbiochem) to inhibit MEK/ERK signaling, and SB203580 (Calbiochem) to inhibit p38 signaling. In general, peptides and inhibitors were injected in a volume of 10 μl with a concentration of 20 μM resulting in a final concentration of approximately 1 μM in the larva.

NanoLC-FTICR MS

Human collagen type IV was purchased from Sigma (Taufkirchen, Germany). Collagen type IV is composed of 140-, 100-, and 70-kDa fragments which are derived from the proα2(IV) chain (GenBank accession number: NP_001836.2), whereas an additional 70-kDa fragment is derived from the proα2(IV) chain (GenBank accession number: NP_001837.2) (Sage et al., 1979; Crouch et al., 1980). Fragment peptides of this extracellular matrix protein were produced by hydrolyzing 500 μg of protein with 5 μg of thermolysin (Sigma) in 500 μl hydrolyzing buffer (50 mM Tris-HCl and 150 mM NaCl, pH 7.5) for 16 h at 22°C as described previously (Altincicek and Vilcinskas, 2006). The obtained low-molecular weight collagen peptides were isolated by using 3000 molecular mass cut-off microcon centrifugal filter devices (Millipore, Billerica, MA, USA) according to the manufacturer’s recommendation. The sample was stored at −20°C and used without further treatment for subsequent experiments.
Determination of humoral and cellular immune responses

Lysozyme-like activity was measured by a lytic zone assay using Micrococcus luteus cell wall components as substrate. The presence of further antimicrobial peptides was determined by using an inhibition zone assay with live M. luteus bacteria (Altincicek and Vlcnisksas, 2006; Altincicek et al., 2007). Inducible levels of lysozyme and antimicrobial peptides were determined in hemolymph samples obtained from G. mellonella larvae 24 h post-treatment. Cellular immune stimulation in vivo was indirectly determined by counting circulating hemocytes in the hemolymph from larvae 4 h post-treatment because immune-stimulation correlates with the switch from non-adherent, resting hemocytes to activated, adherent cells (Altincicek et al., 2008).

Quantitative real-time reverse transcriptase (RT) PCR

RNA from untreated larvae and larvae 8 h post-treatment was isolated using TriReagent isolation reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions and quantitative real-time RT-PCR was performed with the real-time PCR system Mx3000P (Stratagene, La Jolla, CA, USA) using the FullVelocity SYBR Green QRT-PCR Master Mix (Stratagene) as described previously (Altincicek et al., 2008). We used appropriate primers along with 10 ng of RNA per reaction to amplify the housekeeping gene actin, lysozyme, IMPI, gallerimycin, and galiomycin as described previously (Altincicek et al., 2007). In the case of galiomycin amplification, we used primers galiomycin-forward 5'-GGA TCC ATG GCG AAA AAT-3' and galiomycin-reverse: 5'-GTC GAC TTA CTC GCA ACA ATT GAC GTT-3'.

Fluorescence microscopy

Tracheal epidermis dissected from last instar larvae was injured with a sterile needle in the presence of naive hemolymph and equal volume of buffer A (NaH2PO4, pH 7) including no substrate and 100 μg/ml DNA collagen (type IV from human placenta, fluorescein conjugate; Invitrogen, Carlsbad, CA, USA), respectively, and were monitored with an Axioskop 2 microscope (Zeiss, Jena, Germany) with a fluorescence filter according to manufacturers' recommendations. Fluorescein isothiocyanate (FITC) was used to prepare FITC-labeled peptides as proposed by the manufacturers’ instructions (KMF Laborchemie Handels-GmbH, Lohmar, Germany). For binding assays, freshly isolated hemocytes were allowed to attach to microscope slides for 5 min in a humidity chamber and were subsequently incubated with phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 6.9) containing 500 μM FITC-labeled peptide for 15 min. Hemocytes were carefully washed three times with fresh buffer prior to microscopic examination.

Acknowledgments

We thank Meike Fischer for excellent technical assistance and Katja Altincicek for critical reading of the manuscript. Financial
support by the European Union (STREP project LSHG-CT-2005-518194), the Bundesministerium für Bildung und Forschung BMBF (NGFN project 0331442), and the Deutsche Forschungsgemeinschaft DFG (project SP3/14-10-1) is gratefully acknowledged. This work was supported in part by the Deutsche Forschungsgemeinschaft (DFG) with a Heisenberg fellowship (AL 902/4-1) to B.A.

References


Received July 24, 2009; accepted July 29, 2009