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

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## 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 evolutionary 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-I kDa fragments of human placental type IV collagen are derived from the pro$\alpha$I(IV) chain, whereas the 70-II kDa fragment is derived from the pro$\alpha$2(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 hydrolyzation 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 sequences of identified peptides by online nanoLC-Orbitrap mass spectrometry (Hu et al., 2005; Perry et al., 2008). Interestingly, five of the 38 identified peptides contained the integrin-binding peptide motif RGD/E (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1987; Hynes, 1992). Custom-synthesized peptides including the RGD/E motif stimulated both cellular and systemic immune responses *in vivo* and at least the induction of lysozyme expression was MEK/ERK and p38 kinase dependent.

## 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 using the IT for analysis (Figure 1). Moreover, *de novo* peptide sequencing validated obtained results (Figure 1). For this purpose, we combined a high-resolution survey scan with high-resolution MS/MS measurements. In the case of the FTICR, measurements typically consisted of two MS/MS scans of the most intense precursor ions with collision-induced dissociation (CID). Furthermore, we validated identified peptides by the use of a FT orbital trapping mass spectrometer. Therefore, the two most intense precursor ions were measured with both CID and higher energy collision induced dissociation (HCD) as a fragmentation technique.

Overall, 38 different peptides were identified (Table 1). Four peptides with monoisotopic masses in positive mode ($[M+H]^+$) of 755.39 Da, 839.45 Da, 1034.53 Da, and 1105.57 Da, respectively, were accompanied by another peptide with a mass shift of +16 Da implicating the presence of an additional hydroxylation. Further, over 60% of all proline residues were hydroxyproline. Examination of the peptide sequences revealed that 13 of the identified peptides derived from the $\alpha$1 collagen chain and 21 from the $\alpha$2 collagen chain. Three peptides with monoisotopic masses in positive mode ($[M+H]^+$) of 506.26 Da, 634.32 Da, and 637.30 Da, respectively, were not clearly identified by the SEQUEST database; hence, we additionally performed high-resolution MS/MS analysis for *de novo* peptide sequencing (composition based sequencing, CBS) (Spengler, 2004, 2007) which resulted in the final identification of these peptides (Table 1). It is noteworthy to mention that most identified peptides still contained one or more intact cleavage sites for thermolysin, which cleaves before amino acids with hydrophobic or bulky side chains (http://merops.sanger.ac.uk). This observation suggests restricted access of thermolysin to at least some cleavage sites under used conditions. 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 positive control for further experiments. The peptide VDGKSAPNV unrelated to integrin-binding sequence was used as negative control.

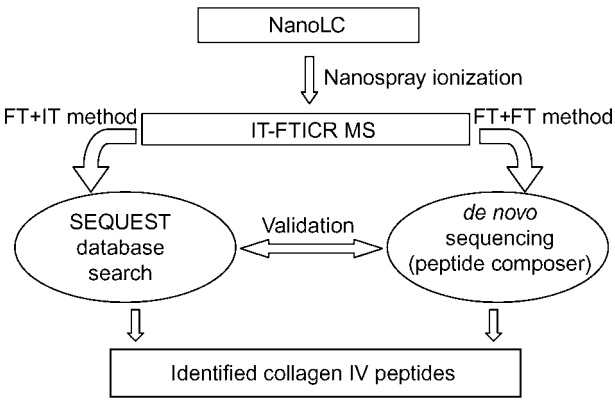


**Figure 1** Workflow for the identification of specific collagen IV derived peptides.
The schematically drawn workflow demonstrates the order of used methods for the identification of specific collagen peptides.

**Table 1** Identification of 38 specific collagen IV peptides by nanoLC-FTICR MS and *de novo* peptide sequencing.

| $[M+H]^+$ (Da) | Δm/m (ppm) | *z* | Sequence | Probability factor |
|---|---|---|---|---|
| 506.26129 | 0.75 | 1 | **FGIP*G** | 8.90E-02 |
| 634.31987 | 0.60 | 1 | **LQGFP*G** | 2.60E-02 |
| 637.30195 | 0.87 | 1 | **IMGFP*G** | 8.70E-02 |
| 685.38917 | -1.83 | 1 | K.**IAVQPGT**.V | 3.16E-05 |
| 724.31594 | -1.56 | 1 | D.**FGDIGDT**.I | 1.66E-04 |
| 754.37848 | -1.93 | 1 | G.**ITGFP*GF**.I | 1.86E-05 |
| 755.39483 | -1.89 | 1 | F.**IGDP*GIPA**.L | 7.12E-05 |
| 771.38970 | -1.79 | 1 | F.**IGDP*GIP*A**.L | 4.01E-05 |
| 775.44667 | -0.73 | 2 | G.**LRGIP*GF**.A | 6.51E-04 |
| 837.44766 | -1.39 | 1 | G.**LQGPVGPP*G**.F | 7.69E-06 |
| 839.44543 | -1.24 | 2 | E.**ILGHVP*GM**.L | 9.13E-04 |
| 852.37413 | -0.88 | 1 | E.**IGATGDFGD**.I | 1.50E-05 |
| 855.44020 | -1.04 | 2 | E.**ILGHVP*GM*.**L | 9.58E-05 |
| 926.45845 | -0.70 | 2 | G.**VSGPP*GVP*GQ**.A | 1.75E-05 |
| 958.44779 | -0.18 | 2 | G.**LTGPP*GSQGE**.L | 1.70E-07 |
| 1034.52770 | -1.12 | 2 | S.**IGVP*GVPGEHG**.A | 5.11E-05 |
| 1050.52243 | -0.92 | 2 | S.**IGVP*GVP*GEHG**.A | 5.36E-05 |
| 1068.53284 | -0.76 | 2 | G.**VAGVP*GPQGTP*G**.L | 2.43E-04 |
| 1080.53522 | -2.96 | 1 | F.**LGPP*GPAGTP*GQ**.I | 1.74E-04 |
| 1105.56697 | -3.00 | 1 | S.**IGVP*GVPGEHGA**.I | 4.11E-09 |
| 1121.55987 | -1.16 | 2 | S.**IGVP*GVP*GEHGA**.I | 1.10E-05 |
| 1121.62049 | -0.33 | 2 | G.**LVGIP*GPP*GIP*G**.F | 1.99E-04 |
| 1125.55505 | -1.39 | 1 | G.**VSGPP*GVP*GQAQ**.V | 8.45E-08 |
| 1181.61787 | -1.51 | 2 | G.**VAGVP*GPQGTP*GL**.I | 4.18E-04 |
| 1225.65482 | -1.05 | 2 | D.**IGDTINLPGRP*G**.L | 4.73E-04 |
| 1238.55656 | -2.44 | 1 | E.**IGATGDFGDIGDT**.I | 5.38E-04 |
| 1281.64560 | -1.74 | 2 | G.**IRGEP*GPP*GLP*GS**.V | 2.47E-04 |
| 1305.69155 | -0.43 | 3 | G.**LSGIP*GLP*GRP*GH**.I | 2.11E-04 |
| 1343.61452 | -2.32 | 2 | G.**LDGFP*GLP*GPP*GDG**.I | 9.34E-05 |
| 1366.65110 | -0.93 | 3 | G.**FRGDEGPIGHQGP**.I | 1.15E-09 |
| 1385.68040 | -2.6 | 2 | S.**IGDGDQRRGLP*GE**.M | 1.39E-04 |
| 1516.75362 | -2.19 | 2 | G.**IGP*PGARGPP*GGQGPP*G**.L | 1.61E-05 |
| 1559.78263 | -0.88 | 3 | G.**VFGLPGEK*GP*RGEQG**.F | 9.97E-05 |
| 1862.85202 | -1.61 | 2 | G.**FMGPP*GPQGQPGLP*GSP*GH**.A | 1.83E-08 |
| 1963.97374 | -0.89 | 2 | G.**IRGEP*GPP*GLP*GSVGSP*GVP*G**.I | 4.45E-08 |
| 1980.84503 | -1.65 | 2 | G.**AQGEP*GSQGEP*GDP*GLP*GPP*G**.L | 1.13E-04 |
| 2088.00931 | 0.56 | 4 | Y.**ALPKEERDRYRGEP*GEP*G**.L | 2.84E-04 |
| 2516.16372 | -2.16 | 3 | T.**VGPQGRRGPP*GAP*GEM*GPQGPP*GEP*G**.F | 4.92E-05 |

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 VDGKSAPNV 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 galiomycin 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

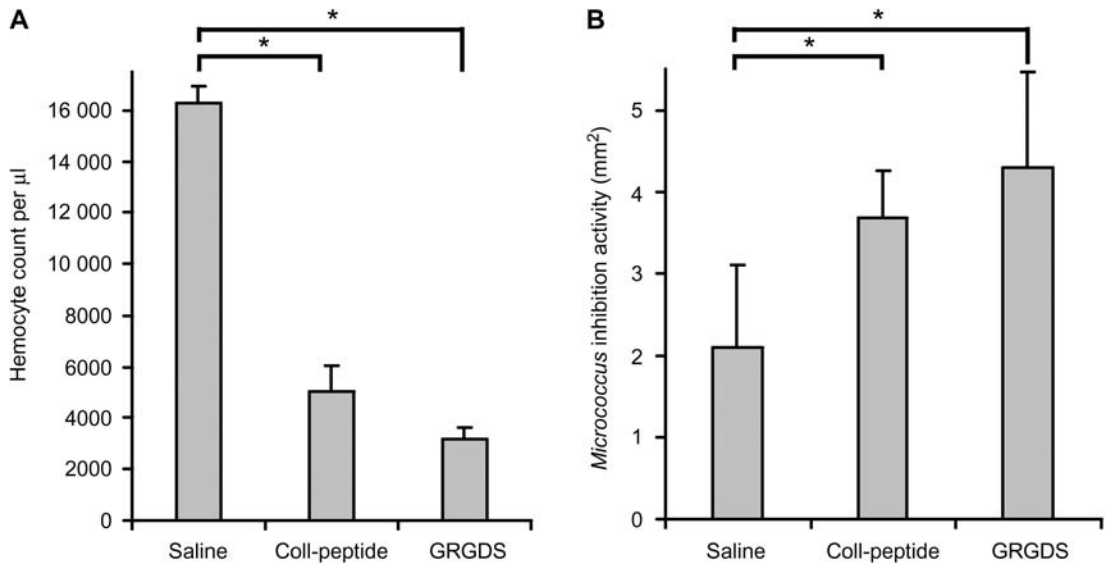


**Figure 2** Injection of coll-peptide as well as GRGDS induced similar immune responses.
Larvae injected with saline, coll-peptide, and GRGDS, respectively, were investigated in immune response assays. (A) Cellular immune stimulation and (B) humoral immune stimulation were significantly induced upon 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.

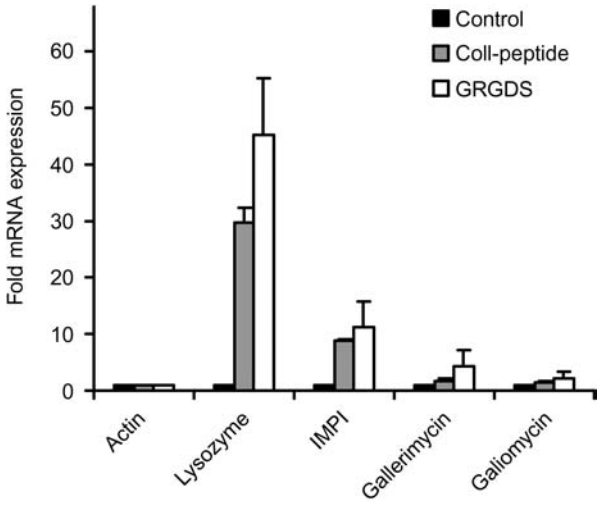


**Figure 3** Quantitative real-time RT-PCR analysis of mRNA levels of immune genes.
Levels of mRNA of lysozyme as well as IMPI were found to be significantly elevated on injection of both coll-peptide and GRGDS in comparison to control injection ($p<0.05$, Student's *t*-test). In contrast, mRNA levels were of gallerimycin, and galiomycin was not significantly induced by coll-peptide or GRGDS peptide. Results represent mean values of at least three independent determinations±SD.

a JNK inhibitor (SP600125). Injection of SB203580 or PD98059 along with coll-peptide into the hemocoel of larvae significantly reduced coll-peptide inducible lysozyme activity (Figure 4). In contrast, co-injection of coll-peptide along with SP600125 did not result in dampened lysozyme levels (Figure 4).

To investigate direct binding of the coll-peptide to insect hemocytes, we incubated FITC-labeled coll-peptide, integrin-binding GRGDS peptide, and control peptide with naïve hemocytes, respectively. We observed that the FITC-labeled coll-peptide bound to the cell surface of a subset of hemocytes in a specific pattern (Figure 5A, B). This pattern resembled that observed by integrin-binding FITC-labeled GRGDS peptide (Figure 5C, D). In line with our observation, a recent study showed a similar binding pattern of fluorescent RGD-peptide to mammalian cells, which indicated both binding to cell surface and subsequent internalization by endocytosis (Castel et al., 2001).

## 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

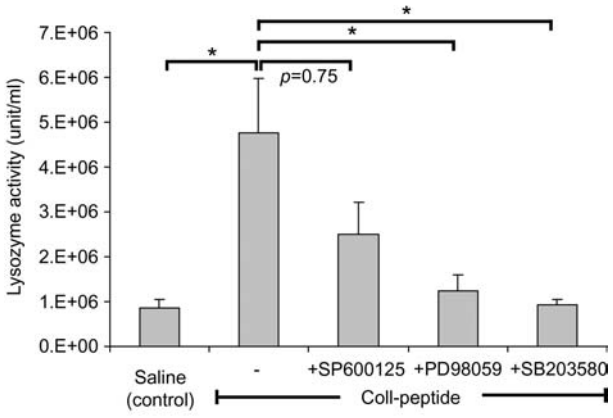


**Figure 4** Coll-peptide inducible lysozyme activity was sensitive to MAP kinase inhibitors.
Coinjection of MEK/ERK inhibitor PD98059 and p38 inhibitor SB203580, respectively, along with coll-peptide resulted in a significant decrease of inducible lysozyme activity in hemolymph 24 h post-treatment. 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.

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; Nardi et al., 2005; Zhuang et al., 2008). 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-

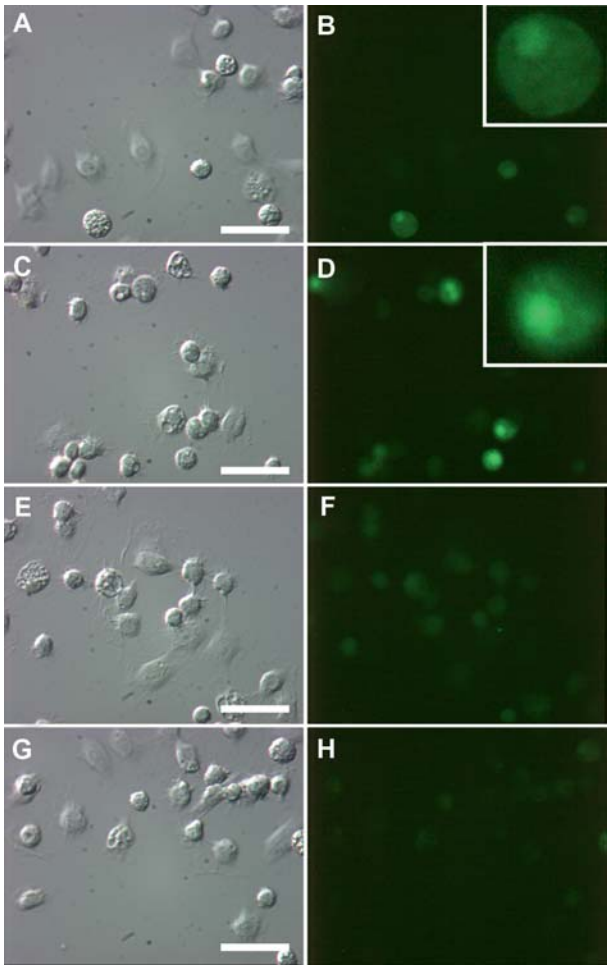


**Figure 5** FITC-labeled coll-peptide bound to the cell surface of a subset of hemocytes in a similar pattern as FITC-labeled RGD peptide.
(A, B) FITC-labeled coll-peptide bound strongly to the cell surface of a subset of live hemocytes resulting in a specific pattern of green fluorescence. Inset shows 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.

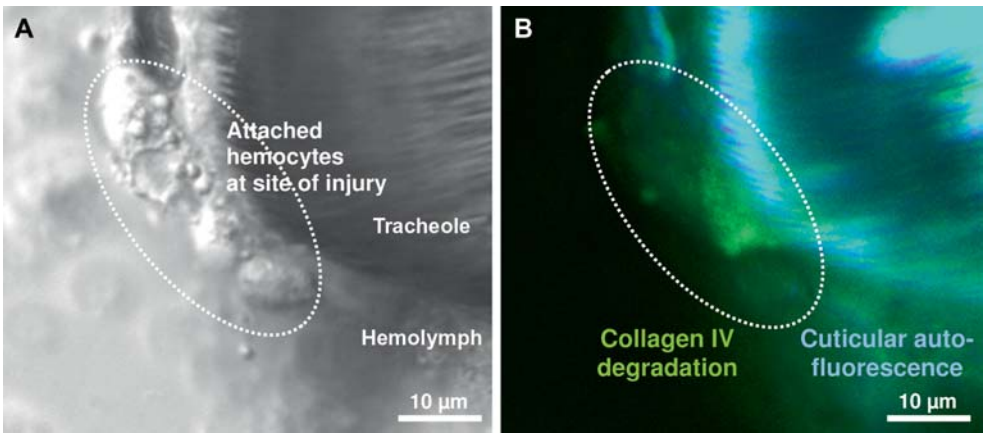


**Figure 6** Hemocytes attached to injured tracheal basement membrane exhibited significant collagen IV degrading activity. (A) On wounding of tracheal epidermis, circulating hemocytes become adhesive at the site of injury. (B) Subsequently, these hemocytes show significant collagen IV degrading activity resulting in green fluorescence with the use of soluble, fluorescent collagen IV.

sin-like metalloproteinases (Clermont et al., 2004). Hence, feedback loop regulation of microbial metalloproteinases requires danger signals resulting from their activity, which in turn elicit expression of the IMPI to neutralize the threat resulting from non-regulated digestion of cellular matrix components and other host proteins (Altincicek and Vilcinskas 2006; Wedde et al., 2007). However, we have shown that degradation of collagen IV during infection is not restricted to microbial metalloproteinases. In *G. mellonella*, we discovered a matrix metalloproteinase localized in immune-competent hemocytes, which becomes activated upon challenge with elicitors of innate immune responses and which cleaves collagen IV (Altincicek and Vilcinskas, 2008). In this study, we found that wounding alone is sufficient to force circulating hemocyte to become adhesive and to exhibit collagen IV degrading activity. Theory predicts that immediate stimulation of immune responses by danger signals at the site of injury could greatly benefit the animal host by engaging immune defense reactions, which can prevent entry and dispersal of microbes at an early time point. In addition, danger signal mediated activation of antimicrobial defense can advance the hosts ability to cope with pathogens that have evolved mechanisms to prevent host immune-recognition by PAMPs, for example, by the formation of capsules (*Pseudomonas* spp.) or by reduction of cell walls (*Mycoplasma* spp.).

Interestingly, neither coll-peptide nor GRGDS elicited expression of gallerimycin and galiomycin when injected, implicating that the spectrum of inducible immunity-related effector molecules is limited. This is in agreement with our observation that the MEK/ERK and p38 kinase dependent pathway are activated on challenge with coll-peptide but not the JNK pathway. Recent genome-wide transcriptomic profiling of insect hemocytes has provided evidence for pathogen-specific immune gene expression patterns (Baton et al., 2009). However, by identification of both particular collagen IV peptides and RGD/E-dependent pathways, this study provides novel insight into danger/alarm signal mediated activation of insect innate immune responses.

## 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 (GIRGEHyp, glycine-isoleucine-arginine-glycine-glutamate-hydroxyproline) with a purity of 94.83%, GRGDS (glycine-arginine-glycine-aspartate-serine) with a purity of 81.29%, and a control peptide unrelated to integrin-binding sequence (VDGKSAPNV, valine-aspartate-glycine-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 $Na_2HPO_4$ 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-I kDa fragments which are derived from the proαI(IV) chain (GenBank accession number: NP_001836.2), whereas an additional 70-II 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.

Measurement of the samples was accomplished on a binary nanoHPLC system containing the units Switchos, Famos, and Ultimate (LCPackings/Dionex, Idstein, Germany). Solvent A was water (HPLC grade; Fluka, Neu-Ulm, Germany) containing 2% acetonitrile (v/v) (Uvasol® grade; Merck KGaA, Darmstadt, Germany) and 0.1% formic acid (v/v) (puriss p.a. for MS; Fluka). Solvent B was acetonitrile containing 20% water (v/v) and 0.08% formic acid (v/v). Separation was performed as follows: 0% to 50% B in 32 min, 50% to 100% B in 4 min, 20 min 100% B, and 5 min 0% B. The injection volume was 1.2 μl. Separation was performed on a C18 PepMap precolumn (5 mm×300 μm ID) and a fused silica C18 PepMap100 capillary column (150 mm×75 μm ID) at a flow rate of 200 nl/min. The separation was monitored by an UV detector at 214 nm. The nanoLC system was coupled to MS by a nanospray source. Pico-Tip® emitters (New Objective, Woburn, MA, USA) were used as nanospray needles. The separated peptides were measured on a linear IT Fourier transform ion cyclotron resonance hybrid mass spectrometer (Finnigan LTQFT Ultra; Thermo Scientific GmbH, Bremen, Germany). Survey MS spectra were measured on the FTICR with a high mass accuracy below 2 ppm. CID in the IT was used for fragmentation. In the next step, the five most intense ions were selected for fragmentation in the IT in order to acquire a high number of MS/MS spectra for database searches. In addition, MS/MS spectra were acquired with high mass resolving power (R=100 000 at *m/z*=400 Da) and high mass accuracy of greater than 2 ppm for analysis with the composition based sequencing approach. MS/MS data were obtained on the FTICR instrument using the wide scan range method with three microscans in order to decrease the loss of ions owing to the time-of-flight effect. In addition, coupling of nanoLC with a linear IT/Fourier transform orbital trapping (IT-FTOT MS) hybrid mass spectrometer (LTQ Orbitrap Discovery; Thermo Scientific GmbH) equipped with a nanospray ion source was used for validation of identified peptides. This mass spectrometer allowed the additional detection of low mass fragments and uses a HCD technique, providing additional information for the validation of the database searches. MS/MS spectra on the LTQ Orbitrap were acquired with a resolving power of R=30 000 and a mass accuracy of greater than 3 ppm. Calibration was performed according to the manufacturer's instructions.

The sequence of collagen type IV chains (α1 chain: NP_001836.2; α2 chain: NP_001837.2) was received from The National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Data obtained from LC-MS measurements were analyzed employing the SEQUEST database search as part of the BioWorks software version 3.3 (Thermo Scientific GmbH, Karlsruhe, Germany). The database searches were carried out using the following settings: precursor ion tolerance 3 ppm and fragment ion tolerance 1 Da. The following possible modifications were selected: phosphorylation on serine, threonine, and tyrosine, hydroxylation on proline, threonine, and methionine, and carbamidomethylation on cysteine. These modifications had to be considered by the software with an increase in mass of the concerned amino acid, for hydroxylation +15.99492 Da, for phosphorylation +79.96633 Da, and for carbamidomethylation +57.02146 Da. The amino acids alanine, isoleucine, leucine, phenylalanine, valine, and methionine were selected as cleavage sites of thermolysin in the BioWorks software. Peptides with a probability value below 0.001 and a mass accuracy of greater than 3 ppm were considered as possibly identified. To verify and supplement these results, the measured precursor ions were also analyzed manually using high-resolution MS/MS measurements on the FTICR instrument or the FT orbital trapping instrument as described previously (Römpp et al., 2007). Validation using MS/MS data with high mass accuracy is especially important in this application as identified peptides were synthesized and tested for their bioactivity. False positives would otherwise have led to unnecessary allocation of resources. Peptides that were not identified in a standard database search could be verified using the *de novo* CBS approach. This method takes advantage of the high accuracy of fragment ion masses. A combination of combinatorial and iterative steps leads to a high reliability of identification (Spengler, 2004, 2007; Thieu et al., 2006).

### 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 Vilcinskas, 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 TTC CAG TCC-3′, galiomycin-reverse: 5′-GTC GAC TTA CTC GCA CCA 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 ($NaH_2PO_4$, pH 7) including no substrate and 100 μg/ml DQ™ collagen (type IV from human placenta, fluorescein conjugate; Invitrogen, Carlsbad, CA, USA), respectively, and were monitored with an Axioplan 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 TC100 insect medium (PAA, Pasching, Austria) 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.

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