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Intracellular Receptor for Human Host Defense Peptide LL-37 in Monocytes[1]

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The human cationic host defense peptide LL-37 has a broad range of immunomodulatory, anti-infective functions. A synthetic innate defense regulator peptide, innate defense regulator 1 (IDR-1), based conceptually on LL-37, was recently shown to selectively modulate innate immunity to protect against a wide range of bacterial infections. Using advanced proteomic techniques, ELISA, and Western blotting procedures, GAPDH was identified as a direct binding partner for LL-37 in monocytes. Enzyme kinetics and mobility shift studies also indicated LL-37 and IDR-1 binding to GAPDH. The functional relevance of GAPDH in peptide-induced responses was demonstrated by using gene silencing of GAPDH with small interfering RNA (siRNA). Previous studies have established that the induction of chemokines and the anti-inflammatory cytokine IL-10 are critical immunomodulatory functions in the anti-infective properties of LL-37 and IDR-1, and these functions are modulated by the MAPK p38 pathway. Consistent with that, this study demonstrated the importance of the GAPDH interactions with these peptides since gene silencing of GAPDH resulted in impaired p38 MAPK signaling, downstream chemokine and cytokine transcriptional responses induced by LL-37 and IDR-1, and LL-37-induced cytokine production. Bioinformatic analysis, using InnateDB, of the major interacting partners of GAPDH indicated the likelihood that this protein can impact on innate immune pathways including p38 MAPK. Thus, this study has demonstrated a novel function for GAPDH as a mononuclear cell receptor for human cathelicidin LL-37 and immunomodulatory IDR-1 and conclusively demonstrated its relevance in the functioning of cationic host defense peptides. ***The Journal of Immunology*****, 2009, 183: 2688–2696.**

Cationic host defense peptides are evolutionarily conserved critical elements of innate immunity. They are widely distributed in nature, existing in organisms from insects, plants, and crustaceans to mammals, indicating their importance in defense against pathogenic challenge and innate immune responses in virtually all organisms. There are two major groups of cationic host defense peptides in mammals based on their gene structures and pre-pro sequences; the defensins, and cathelicidins. The sole human cathelicidin is LL-37 (LLGDFFRKS KEKIGKEFKRIVQRI-KDFLRNLVPRTES), an amphipathic α helical peptide obtained by proteolytic cleavage from the C-terminal end of the human protein CAP18 (1). LL-37 is widely expressed in a variety of body fluids and tissues, including key immune cell types such as monocytes, neutrophils, epithelial cells, and lymphocytes (2, 3). It displays significant anti-infective and anti-inflammatory properties in vivo (4) and is up-regulated during inflammatory conditions and infections (5–7). Conversely, deficiencies in LL-37 expression lead to increased susceptibility to infections in a variety of diseases including morbus Kostmann, chronic ulcers, and atopic dermatitis (8–10).

The anti-infective properties of LL-37 can be largely attributed to its ability to mediate a diverse array of immunomodulatory activities, since its modest direct microbicidal activity has been shown to be antagonized by physiological ion concentrations (4, 11). The diverse immunomodulatory functions of LL-37 include proinflammatory activities such as direct chemoattraction of mononuclear cells, neutrophils, eosinophils, T cells, and mast cells (12–14) and the induction of the expression of chemokines required for cell recruitment (4, 15, 16), as well as anti-inflammatory functions including potent neutralization of endotoxin (17–20). LL-37 also promotes angiogenesis (21) and wound healing (9), induces degranulation of mast cells (22), and influences dendritic cell differentiation and the polarization of T cells (23). Overall, LL-37 plays a critical role in selectively balancing responses to inflammatory stimuli, such as bacterial endotoxin, in human immune cells.

Macrophages and monocytes play a key role in the clearance of pathogens. They respond to LL-37 stimulation through changes in gene expression and subsequent release of chemokines (19, 24). Although certain functions of LL-37 were previously demonstrated to involve a variety of putative cell surface receptors, e.g., formyl peptide receptor-like 1 (FPRL-1)[4] for chemoattraction and

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[4] Abbreviations used in this paper: FPRL-1, formyl peptide receptor-like 1; IDR-1, innate defense regulator 1;SILAC, stable isotope labeling of amino acids in cell culture; siRNA, small interfering RNA; MS, mass spectrometry; qRT-PCR, quantitative real-time PCR; WT, wild type; KD, knockdown; Ct, cycle threshold; MT, mock transfected; LTA, lipoteichoic acid.

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the transactivator P2X7 for IL-1β processing, respectively (12, 25), the receptor(s) through which LL-37 exerts its effects on macrophages remains unidentified. Cellular uptake of LL-37 into epithelial cells, mediated by atypical endocytic processes (26), is required for induction of chemokine expression which is further mediated through the activation of MAPKs and other pathways, as well as several transcription factors.

Based on the precedents provided by LL-37 and other cationic host defense peptides, we designed a novel innate defense regulator peptide (IDR-1; KSRIVPAIPVSLL-NH_2) that was protective, by both local and systemic administration, in murine models of infection with Gram-positive and Gram-negative pathogens, despite its complete lack of antimicrobial activity (17). Monocytes/macrophages, but not neutrophils or lymphocytes, were shown to be key to protection and, in these cells IDR-1, enhanced the levels of chemokines while reducing proinflammatory cytokine responses, as also demonstrated in a murine infection model. Thus, IDR-1 was defined as the first member of a class of innate defense regulators which counter infection by selective modulation of innate immunity.

In this study, we used stable isotope labeling of amino acids in cell culture (SILAC), followed by affinity tag pull-down experiments and quantitative mass spectrometry (MS) (27, 28) to identify macrophage GAPDH as a cellular interacting protein for LL-37, as subsequently confirmed with a variety of additional biochemical methods. A variety of LL-37-mediated transcriptional responses as well as those induced by another functionally analogous synthetic anti-infective, innate defense regulator peptide, IDR-1 (17), was significantly suppressed in the presence of GAPDH small interfering RNA (siRNA), as was LL-37-induced MAPK p38 signaling and LL-37-induced cytokine production. This is the first study to demonstrate a novel function for intracellular GAPDH in innate immunity as a functional cellular receptor for human cathelicidin LL-37.

Materials and Methods

Cell culture and isolation

Human PBMC were isolated from healthy volunteers (19) under the approval and ethics guidelines of the University of British Columbia. $CD14^+$ human monocytes were isolated from PBMC using a Dynal-negative isolation kit according to the manufacturer's instructions (Invitrogen). Murine RAW 264.7 (ATCC TIB-71) were cultured and differentially labeled with heavy amino acids using a SILAC D-MEM Flex-media kit (Invitrogen) as described below. Human THP-1 (ATCC TIB-202) were differentiated with PMA (Sigma-Aldrich) as previously described (19) to induce plastic-adherent macrophage-like cells and maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen). Cultures were maintained at 37°C in a humidified 5% (v/v) CO_2 incubator.

Stable isotope labeling with amino acids in cell culture (SILAC)

Murine RAW 264.7 (ATCC TIB-71) were cultured and differentially labeled with heavy amino acids (L-[$^{13}C_6$]lysine and L-[$^{13}C_6$]arginine) using a SILAC D-MEM Flex-media kit (Invitrogen) as per the manufacturer's instructions. Briefly, one cell population was cultured in medium with light (normal) amino acids and the second cell population was grown in parallel in medium supplemented with heavy (L-[$^{13}C_6$]lysine and L-[$^{13}C_6$]arginine) amino acids. All of the medium components including dialyzed FBS, medium supplements, and amino acids were obtained from Invitrogen. The cells were maintained at 37°C in a humidified 5% (v/v) CO_2 incubator and grown for 10 doublings. Once incorporated into cellular protein, the mass differences of heavy amino acids, which differed from unlabeled amino acids by 6 Da, were used to quantitatively compare samples.

Isolation of interacting proteins by peptide affinity chromatography

To permit its use as a bait, LL-37 was biotinylated with a carboxyl-terminal biotin tag as described previously (26). LL-37 without biotin was used as a control. RAW 264.7 cells (1×10^8 for each treatment) were washed with cold PBS and lysed on ice for 30 min in TNE lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM EDTA) containing a protease inhibitor mixture (Sigma-Aldrich) and 1% Nonidet P-40. The RAW cell lysates from the cells labeled with heavy amino acids were treated with LL-37B (50 μg/ml) and the lysates from unlabeled cells were either untreated or treated with LL-37 (50 μg/ml), to be used as paired controls, for 30 min at 37°C. After incubation, peptide-protein cross-linking was accomplished using 1% paraformaldehyde in PBS containing a protease inhibitor mixture for 15 min at 37°C, followed by quenching with 2 M glycine for 5 min at 23ºC. The samples were clarified by centrifugation at 10,000 rpm for 10 min, supernatants were passed over columns of Ultralink Immobilized NeutrAvidin resin (Pierce), and the bound proteins were eluted using 6 M guanidium hydrochloride. Similarly, $CD14^+$ monocytes were lysed in TNE lysis buffer containing a protease inhibitor mixture (Sigma-Aldrich) and 1% Nonidet P-40, were treated with either LL-37B or LL-37, any interacting cellular proteins were subsequently cross-linked, and were eluted following affinity tag pull-downs using NeutrAvidin resin as described above.

MS analysis

For MS analysis, equal quantities of protein from unlabeled (control) and labeled (test) samples were combined. Protein cross-linking was reversed by heating the samples to 100°C for 5 min and then protein samples (100 μg of total protein) were reduced with 5 mM DTT for 20 min at 37°C. Cysteine alkylation was performed by treating the samples with 20 mM iodoacetamide for 20 min at room temperature in the dark. The alkylation reaction was quenched by the addition of DTT to a final concentration of 100 mM. The samples were diluted to a concentration of 1 M guanidium hydrochloride by the addition of 20 mM ammonium bicarbonate before digestion. The reduced and alkylated samples were digested for 16 h with 2 μg of modified porcine trypsin (Promega) at 37°C. Aliquots of the peptide mixtures (5 μg of total peptide) were subsequently acidified by dilution with an equal volume of 5% acetic acid. The samples were then passed over a STop And Go Extraction stage tip (29) and loaded onto tips in the acidified digest buffer, washed with 50 μl of 0.5% acetic acid, and eluted with 20 μl of 80% acetonitrile in 0.5% acetic acid. Samples were lyophilized in a centrifugal vacuum concentrator for 20 min to evaporate the acetonitrile and resuspended to a volume of 10 μl with 0.5% acetic acid. Samples were analyzed via nanoflow liquid chromatography coupled to tandem MS. The analysis was accomplished using an LTQ Orbitrap (Thermo Fisher Scientific) coupled to an Agilent 1100 Series nanoflow HPLC via a nanospray ionization source (Proxeon Biosystems). A reverse-phase column consisting of ReproSil-Pur C_{18} resin packed into a fused silica emitter (15 cm $\times$ 75 μm internal diameter) was used for sample separation and was developed with a gradient of 0.5% acetic acid (buffer A) and 80% acetonitrile in 0.5% acetic acid (buffer B). The gradient was run from 6% B to 30% B over 60 min, followed by 30–80% B over 10 min, 80% B for 5 min, and finally from 80% B back to 6% B for 15 min to recondition the column. Full-range scans were collected from 350 to 1500 Thomson at 60,000 resolutions and the five most intense ions in each cycle were selected for fragmentation in a data-dependent manner.

For data analysis, fragmentation spectra were centroided and peak lists were assembled in Mascot generic format using the Extract_MSN.exe program supplied with the mass spectrometer control software. DTA Supercharge, a part of the MSQuant software package (http://ms-quant.sourceforge.net), was used to correct the predicted parent ion charge states. The Mascot (version 2.1; Matrix Science) search engine was used to query the MS data against a mouse-specific protein database (International Protein Index, European Bioinformatics Institute) to determine peptide sequences for protein identification. The database search criteria chosen were trypsin cleavage, allowing one missed cleavage, carbamidomethyl cysteine-fixed modification, L-[$^{13}C_6$]lysine and L-[$^{13}C_6$]arginine as variable modifications, parent ion mass tolerance of 5 ppm, MS/MS mass tolerance of 0.6 Da, and the electrospray ionization-ion trap scoring scheme. MSQuant was used for extracting quantitative information from the raw data for all of the proteins identified during the Mascot search.

Immunoblots

The samples eluted from peptide affinity purification were precipitated overnight at −20°C in 50% (v/v) acetone/methanol solution. The pellets were washed with ice-cold 50% (v/v) acetone/methanol solution and equilibrated in nonreducing sample buffer (2% SDS, 10% glycerol, 0.125 M Tris (pH 7.5), and 20% bromphenol blue) for 30 min at 23ºC. Before immunoblotting, the samples were not heated to protect the protein-peptide cross-linking for detection in subsequent probing with specific Abs. Samples were electrophoretically resolved on a 7.5% SDS-polyacrylamide gel

(SDS-PAGE), followed by transfer to polyvinylidene difluoride Immobilon-P membranes (Millipore). The polyvinylidene difluoride membranes were subsequently probed with either rabbit anti-LL-37 polyclonal Ab (a gift from Dr. T. Pearson, University of Victoria, Victoria, British Columbia, Canada), purified rabbit anti-GAPDH (Cedarlane Laboratories), or HRP-conjugated anti-biotin Ab (Cell Signaling Technology) in TBST (20 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20) containing 5% skimmed milk powder. Goat anti-rabbit HRP-conjugated Abs were used for detection as required. The membranes were developed with chemiluminescence peroxidase substrate (Sigma-Aldrich) according to the manufacturer's instructions.

In vitro ELISA for peptide binding

In vitro interaction of LL-37 peptide with GAPDH was evaluated using a modified ELISA. Polystyrene microtiter ELISA plates were coated with 2 μg/ml each of either purified human GAPDH (HyTest), milk powder, or BSA in PBS overnight at 4°C. The plates were blocked with 3% (w/v) milk powder in PBS for 1 h at 37°C, followed by incubation with either LL-37 or LL-37B in PBS containing 1% (w/v) skim milk powder and 0.05% Tween 20 at 37°C. The plates were washed with PBS plus 0.1% Tween 20. Binding of the peptide was further evaluated using rabbit anti-LL-37 polyclonal, anti-biotin Ab, or goat anti-rabbit HRP-conjugated Abs. LL-37-protein interaction was detected using ImmunoPure tetramethylbenzidine substrate (Pierce) by monitoring absorbance at 450 nm.

GAPDH activity in the presence of host defense peptides

The effects of LL-37 and IDR-1 on GAPDH enzymatic activity were determined at 25°C by monitoring NADH generation spectorophotometrically at 340 nm (30). The reaction mixture contained 50 mM Na_2HPO_4 (pH 8.6), 2.5 mM EDTA, and 1 mM NAD^+. For kinetic studies, the initial velocities of the enzymatic reactions were assessed by varying the concentration of the substrate glyceraldehyde from 0.32 to 6.4 mM. Values for K_m, V_{max}, and K_i were obtained by mathematical calculation according to standard Michaelis-Menten kinetics.

Gene silencing using GAPDH siRNA

Human monocytic THP-1 cells were treated with 1 μM siRNA for human GAPDH (Dharmacon and Fisher) according to the manufacturers' instructions (GAPDH knockdown (KD)) for 96 h. In parallel, cells were also transfected with negative nontargeting Accell siRNA, D-001910–0X (Dharmacon) (mock-transfected (MT) cells). After 96 h of incubation, the cells were PMA differentiated to get plastic-adherent THP-1 cells. PMA-differentiated plastic-adherent THP-1 cells in the presence and absence of GAPDH siRNA, or the negative nontargeting siRNA, were stimulated with either LL-37 (20 μg/ml), IDR-1 (200 μg/ml), LPS (10 or 100 ng/ml), lipoteichoic acid (LTA; 2 μg/ml) from *Staphylococcus aureus* (2 μg/ml), flagellin (500 ng/ml), or IL-1β (10 ng/ml) for the indicated times.

Quantitative real-time PCR (qRT-PCR)

Wild-type (WT) and GAPDH KD THP-1 cells were stimulated with either LL-37 (20 or 50 μg/ml) or, as a paired control, bacterial LPS (100 ng/ml), for 4 h. RNA was isolated and analyzed for gene expression by qRT-PCR using a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen) according to the manufacturer's instructions in the Applied Biosystems PRISM 7000 sequence detection system. Fold changes were calculated using the comparative cycle threshold (Ct) method (31), after normalization with 18S rRNA primers (Ambion). The list of primers used is shown in supplemental Table I.[5]

Evaluation of cytokine production

Cells transfected with negative nontargeting siRNA, i.e., MT and GAPDH KD cells, were stimulated with either LL-37 (20 μg/ml) or, as paired controls, bacterial LPS (10 ng/ml), LTA (2 μg/ml), flagellin (500 ng/ml), or IL-1β (10 ng/ml) for 24 h. Cytokine production was monitored in the tissue culture supernatants with the multiplex bead immunoassays using Luminex 100 StarStation software (Applied Cytometry Systems) as per the manufacturer's instructions.

Evaluation of MAPK p38 phosphorylation using flow cytometry

WT and GAPDH KD THP-1 cells were stimulated for 30 min with either LL-37 (50 μg/ml) or, as paired controls, the TLR ligands LPS (100 ng/ml) or LTA (2 μg/ml). The cells were detached using trypsin and stained for p38 activation using an anti-phospho-p38 (Thr^{180}/Tyr^{182}) 3D7 rabbit mAb from Cell Signaling Technology according to the manufacturer's protocol. Briefly, the cells were fixed in 2% (w/v) paraformaldehyde for 15 min at 23ºC, permeabilized in ice-cold 90% (v/v) methanol for 20 min, washed twice in 0.5% BSA in PBS (w/v), and stained for 1 h at room temperature with the anti-phospho-p38 3D7 rabbit mAb diluted 1/100 in 0.5% BSA in PBS. The cells were further stained with goat anti-rabbit IgG AF647 (Invitrogen and Molecular Probes) at 2 μg/ml for 30 min at room temperature. The data were collected and analyzed using a BD FACSCalibur System and BD CellQuest Pro software.

Results

Identification of macrophage GAPDH as a direct interacting protein partner for LL-37

FPRL-1 is known to be a receptor for LL-37, mediating direct leukocyte chemotaxis (12, 14). However, pretreatment of human PBMC with the specific FPRL-1 antagonist WRW4 did not significantly suppress MCP-1 production in the presence of LL-37 (supplemental Fig. 1*A*). It was previously shown that the nucleotide scavenging receptor P2X7, possibly through transactivation, is involved in LL-37-mediated IL-1β processing and release from monocytes, as well as inhibition of apoptosis of neutrophils (25, 32). In contrast, in this study, we demonstrated that LL-37-induced MCP-1 production in PBMC was not suppressed by the P2X7 inhibitor KN-62 (supplemental Fig. 1*B*). On the other hand, using C-terminally biotinylated LL-37 (LL-37B) as previously described (26), the uptake of LL-37 into macrophages was confirmed (supplemental Fig. 2). Inhibitors of cellular uptake/endocytic mobilization of LL-37 led to the significant ($p < 0.05$) inhibition of downstream chemokine MCP-1 induction by LL-37 (supplemental Fig. 3), with the actin inhibitor cytochalasin D causing almost complete inhibition and the tubulin inhibitor nocodazole causing 45% inhibition. Taken together, these results indicated that there was an alternative interacting protein partner involved in the functioning of LL-37-mediated responses in mononuclear cells and that this receptor might be intracellular.

To determine the direct interacting partner in mononuclear cells, biotinylated LL-37B was used for SILAC and affinity tag pull-down experiments in the murine macrophage cell line RAW264.7 (ATCC TIB-71), a cell type in which LL-37 induces immunomodulatory and antiendotoxin activities (33) analogous to those observed in human monocytic cells (19). The RAW264.7 cells were differentially labeled by growing the cells either with the heavy amino acids L-[$^{13}C_6$]lysine and L-[$^{13}C_6$]arginine or corresponding unlabeled amino acids (27, 28). LL-37B peptide was used as a bait to selectively bind putative protein receptors from cell lysates labeled with heavy amino acids, while parallel lysates from unlabeled cells were either untreated or treated with LL-37 without biotin to provide paired controls. Following cross-linking of cellular proteins and bound peptides and avidin affinity purification to pull down any proteins bound to the LL-37 bait, the mixed pull-down complexes were analyzed via nanoflow liquid chromatography coupled to tandem MS. MS analysis from two independent experiments identified the 36-kDa murine GAPDH as the only protein with a heavy:light ratio of >4, indicating that GAPDH was the predominant interacting protein partner of the bait peptide (Fig. 1). All other proteins identified had measured heavy:light ratios near 1, indicating that they were nonspecifically binding to the NeutrAvidin resin.

To further validate the association of LL-37 and GAPDH, samples obtained from the SILAC-labeled RAW cells following pull-down experiments using LL-37B (from three additional independent experiments) were analyzed on immunoblots probing with either anti-LL-37, anti-GAPDH, or anti-biotin Abs. All immunoblots resulted in the detection of a product at 37 kDa that contained

[5] The online version of this article contains supplemental material.

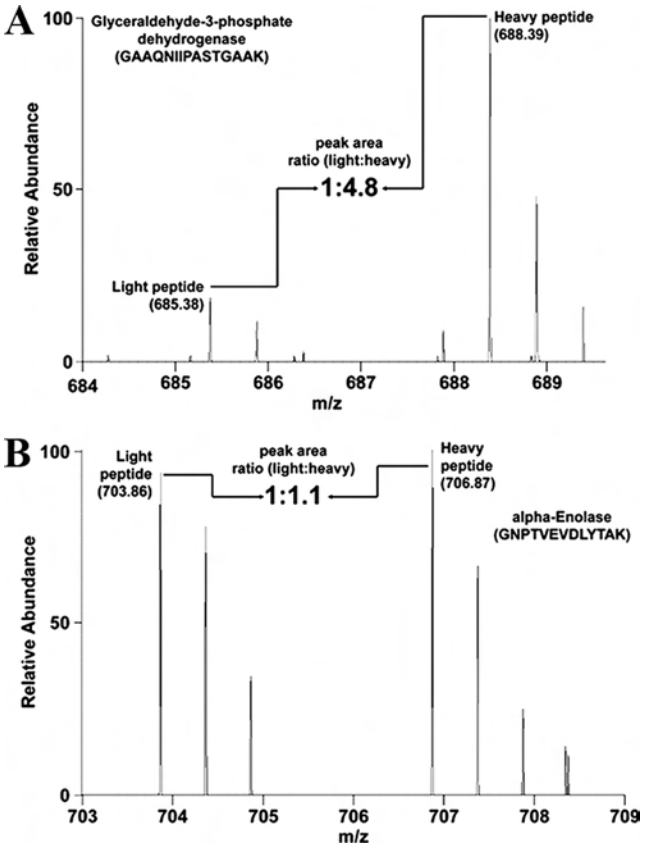

FIGURE 1. GAPDH was quantitatively enriched in samples treated with biotinylated LL-37 after avidin affinity purification. Protein extracts from unlabeled RAW cells were treated with LL-37, while extracts from cells metabolically labeled with heavy amino acids (L-[$^{13}C_6$]lysine and L-[$^{13}C_6$]arginine) were treated with LL-7B as described. After cross-linking and avidin affinity purification, equal quantities of these samples were mixed for simultaneous proteomic analysis using MS. Peptides that were identified during subsequent MS were observed in both labeled (heavy) and unlabeled (light) forms and could be differentiated on the basis of mass due to the presence or absence of heavy amino acids in the two samples. The relative quantity of each peptide in the two samples could be determined based on the size of the peaks detected. *A*, A peptide from GAPDH was detected at *m/z* ratios of 685.38 Thomson and 688.39 Thomson, representing the light and heavy forms of the peptide, respectively. The relative signal intensity of these two peaks indicated a heavy:light ratio of 4.8:1, indicating its enrichment in the LL-37B pull-down. *B*, Peptides from all other proteins detected had heavy:light ratios near 1:1 as illustrated using a peptide derived from α-enolase. Equal abundance of the light and heavy forms indicated that all other proteins were purified as a result of nonspecific interaction with the affinity column. Results were confirmed in two independent biological replicates.

both LL-37 (detected using anti-biotin and anti-LL-37 Abs; Fig. 2, *A* and *B*, respectively), as well as GAPDH (detected with anti-GAPDH Ab; Fig. 2*C*) in samples from LL-37B-treated RAW cells, but not in the paired control samples either treated with nonbiotinylated peptide or untreated. To determine whether this result was species specific and whether it also occurred in primary cells, pull-down complexes were obtained from human CD14$^+$ blood monocytes from four independent donors, revealing an analogous 37-kDa product when probed with anti-GAPDH Ab (Fig. 2*D*), when treated with LL-37B only but not with complexes obtained from CD14$^+$ monocytes that were either untreated or treated with nonbiotinylated peptide or biotin alone. These results confirmed the MS findings identifying GAPDH as a conserved, direct interacting protein partner for the human host defense peptide LL-37.

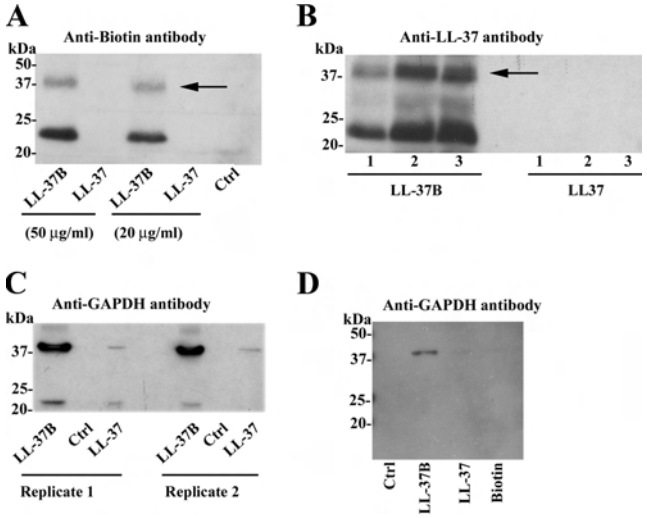

FIGURE 2. Evaluation of macrophage GAPDH as a direct interacting protein partner of cathelicidin LL-37. Immunoblots were performed using samples obtained following affinity tag pull-down experiments using either LL-37B or unlabeled LL-37 using both murine RAW macrophages and human primary CD14$^+$ monocytes. Immunoblots with samples obtained from SILAC-labeled RAW cells were probed with HRP-conjugated anti-biotin Ab (*A*), rabbit anti-LL-37 polyclonal Ab (*B*), or rabbit anti-GAPDH (*C*). Results are representative of three independent biological replicates. *D*, Immunoblots using samples obtained from pull-down affinity tag experiments from CD14$^+$ human monocytes (from four independent donors) using either LL-37B, LL-37, or biotin alone as a bait were probed with anti-GAPDH Ab. Note that the 25-kDa band that was detected in *A* and *B* contained LL-37B, but did not react with the anti-GAPDH Ab (*C*) and thus was likely a multimer or unidentified peptide-protein complex. The minor amounts of GAPDH pull-down in the nonbiotinylated LL-37 lanes in *C* were due to nonspecific interaction of complexes with the NeutrAvidin resin.

Peptide-binding assays demonstrating LL-37-GAPDH interaction in vitro

The LL-37-GAPDH interaction was monitored in vitro using modified ELISA-based capture assays. Purified human GAPDH was immobilized on polystyrene plates as confirmed by probing with a rabbit anti-human GAPDH (Cedarlane Laboratories). The interaction of GAPDH with a broad range of LL-37B concentrations (0–50 μg/ml) was demonstrated using HRP-conjugated anti-biotin Abs (Fig. 3*A*). In this assay, the concentration of LL-37B giving half maximal binding (K_d) was 0.4 μg/ml, i.e., 0.1 μM. To further validate the interaction of LL-37 with GAPDH in vitro, competition studies in which unlabeled LL-37 was used to inhibit the interaction of LL-37B with GAPDH were performed. Microtiter plates containing immobilized GAPDH (2 μg/ml) were preincubated with LL-37 for 30 min at 37°C, followed by the subsequent evaluation of LL-37B (2 μg/ml) interaction with GAPDH as described above. It was demonstrated that preincubation of LL-37 with GAPDH inhibited LL-37B-GAPDH interaction in a dose-dependent manner (Fig. 3*B*). Preincubation with biotin alone did not significantly inhibit the interaction of GAPDH with LL-37B in vitro. To determine the specificity of LL-37 interaction with GAPDH, a range of proteins including GAPDH, milk powder, and BSA were immobilized on polystyrene microtiter ELISA plates and then incubated with low concentrations of LL-37, followed by subsequent incubation with rabbit anti-LL-37 polyclonal and goat anti-rabbit HRP-conjugated Abs. It was demonstrated that LL-37 specifically interacted with GAPDH, since the absorbance detected

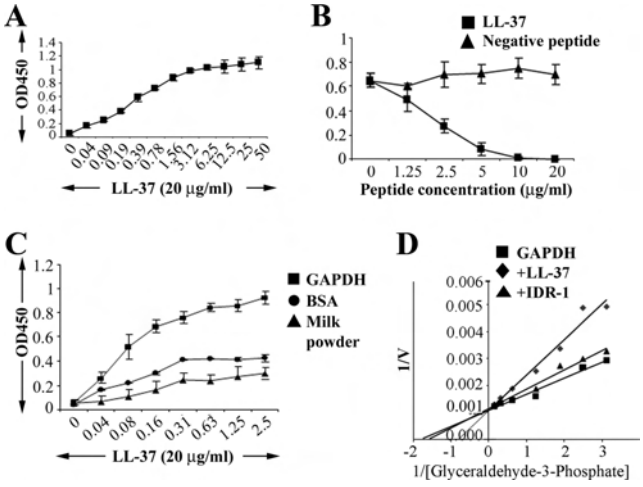

FIGURE 3. In vitro binding assays for peptide interactions. *A*, GAPDH (2 μg/ml) was immobilized on polystyrene microtiter plates followed by incubation with LL-37B (0–50 μg/ml) and the in vitro interaction of GAPDH with LL-37B was detected using HRP-conjugated anti-biotin Ab. *B*, GAPDH (2 μg/ml) immobilized on polystyrene microtiter plates was preincubated with varying concentration of either LL-37 or an unrelated negative peptide for 30 min at 37°C, followed by incubation with LL-37B (2 μg/ml) for 1 h at 37°C. LL-37B-GAPDH interaction was detected using HRP-conjugated anti-biotin Ab. *C*, Either purified human GAPDH protein, milk powder, or BSA (2 μg/ml each) was immobilized on polystyrene microtiter plates, followed by incubation with LL-37, and in vitro interaction of LL-37 with GAPDH was detected using anti-LL-37 polyclonal and goat anti-rabbit HRP-conjugated Abs. All of the microtiter plates were developed using ImmunoPure tetramethylbenzidine substrate followed by monitoring absorbance at 450 nm. Results represent an average of four independent experiments ± SD. *D*, Competitive inhibition of GAPDH dehydrogenase activity by LL-37 and IDR-1. Ten micrograms of human GAPDH (Sigma-Aldrich) was preincubated with or without peptides at 37°C and then assayed for enzymatic activity. NADH production was monitored by measuring the change in absorbance at 340 nm over time. Shown is a Lineweaver-Burke plot of GAPDH enzymatic activity.

with GAPDH was >2-fold higher when compared with the absorbance detected with either BSA or milk powder, even at a very low concentration of 0.16 μg/ml LL-37 (Fig. 3*C*). Taken together, these results demonstrated the specificity of peptide-GAPDH interaction in vitro. In addition, experiments were designed to demonstrate colocalization by immunofluorescence staining and confocal microscopy of GAPDH (using specific Ab detected with Alexa Fluor 568) and LL-37B (using streptavidin-Alexa Fluor 488); however, this was made exceptionally difficult by the high abundance of both molecules. Nevertheless, preliminary experiments demonstrated good staining of both molecules, time-dependent uptake of LL-37, and partial colocalization of LL-37 and GAPDH in human monocytic cells (supplemental Fig. 2).

To test the effect of peptide binding on GAPDH activity, enzyme inhibition studies were undertaken. It was demonstrated that both LL-37 and IDR-1 were weak enzyme inhibitors with K_I values of 61 μM and 1.8 mM, respectively (Fig. 3*D*), but although they caused a shift in the kinetics of GAPDH action on its substrate (supplemental Fig. 4), neither completely blocked the action of the enzyme at any concentration used. No inhibition was observed with a control peptide VQLHIHVAVIHA. This control peptide had the same length as IDR-1 (17) but due to the substitution of H for K residues had no immunomodulatory activity. To confirm that IDR-1 bound to GAPDH, we performed gel shifts on agarose gels. Addition of increasing amounts of IDR-1 to GAPDH led to a change in the mobility of GAPDH (supplemental Fig. 5).

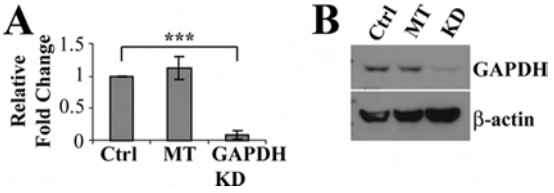

FIGURE 4. GAPDH KD in human monocytic THP-1 cells. Human monocytic THP-1 cells were treated with 1 μM siRNA for human GAPDH (Dharmacon and Fisher) according to the manufacturer's instructions (GAPDH KD) or transfected with negative nontargeting Accell siRNA (D-001910–0X; Dharmacon) (MT cells). After 96 h of incubation, the cells were PMA differentiated to obtain plastic-adherent THP-1 cells. *A*, GAPDH gene expression was evaluated by qRT-PCR. Fold changes (*y*-axis) for the GAPDH gene were normalized to the housekeeping gene for 18S RNA and further quantitated relative to gene expression in unstimulated cells was normalized to 1 using the comparative Ct method. Results shown represent the average of four independent experiments ± SD (***, $p < 0.001$). *B*, GAPDH protein was evaluated by Western blot analysis using anti-human GAPDH mAb (representative blot of four independent experiments). Ctrl, Control.

Functional validation of GAPDH in cationic peptide-induced responses

To establish the functional relevance of the identified GAPDH receptor in downstream responses induced by LL-37 and IDR-1, transcriptional responses and signaling events mediated by these peptides were evaluated in human THP-1 monocytic cells following GAPDH KD with specific siRNAs. It was previously shown that responses to LL-37 (19) and IDR-1 (17) in these cells are similar to those in primary monocytes. Therefore, WT, MT, or GAPDH KD cells were stimulated for 4 or 24 h (as indicated) with either LL-37, IDR-1, or TLR ligands such as bacterial LPS, lipoteichoic acid (sLTA), flagellin, or human recombinant cytokine IL-1β. The induced gene expression was evaluated by qRT-PCR and protein production was monitored in tissue culture supernatants by multiplex bead immunoassays. In four independent experiments, GAPDH mRNA expression was significantly ($p < 0.001$) reduced by 91 ± 5.7% (Fig. 4*A*), and GAPDH expression at the protein level, as determined by immunoblots, was inhibited by >90% (Fig. 4*B*). Both gene as well as protein expression of GAPDH was not altered in the MT cells treated with a negative nontargeting siRNA when compared with untreated control cells. In addition, the described KD treatments with GAPDH siRNA or the negative siRNA were not cytotoxic to the cells and did not alter cellular proliferation compared with the untreated control cells as evaluated using lactate dehydrogenase cytotoxicity assay, and cell proliferation reagent WST-1, respectively (data not shown).

We previously demonstrated that the induction of chemokines (e.g., CXCL-1/Gro-α), as well as anti-inflammatory responses (e.g., IL-10), are critical immunomodulatory functions in the anti-infective properties of LL-37 (19) and IDR-1 (17), and in particular chemokine responses and monocytes were demonstrated to be essential for protection of mice against *Staphylococcus aureus* infections (17). Both LL-37 and IDR-1 induced the expression of chemokines CCL-4/MIP-1β, CCL-20/MIP-3α, and CXCL-1/Gro-α and the anti-inflammatory cytokine IL-10. In each case, the silencing of GAPDH led to a significant ($p < 0.05$) inhibition of induction by 70–86% (Fig. 5, *A* and *B*). The LL-37-induced protein production of chemokine MCP-1 and cytokine MIP-1α was also suppressed >70% (Fig. 5, *D* and *E*, respectively). To eliminate the possibility of a gross defect in responsiveness of the cells as a consequence of GAPDH KD, WT and MT cells using a negative nontargeting Accell siRNA and GAPDH KD cells were also

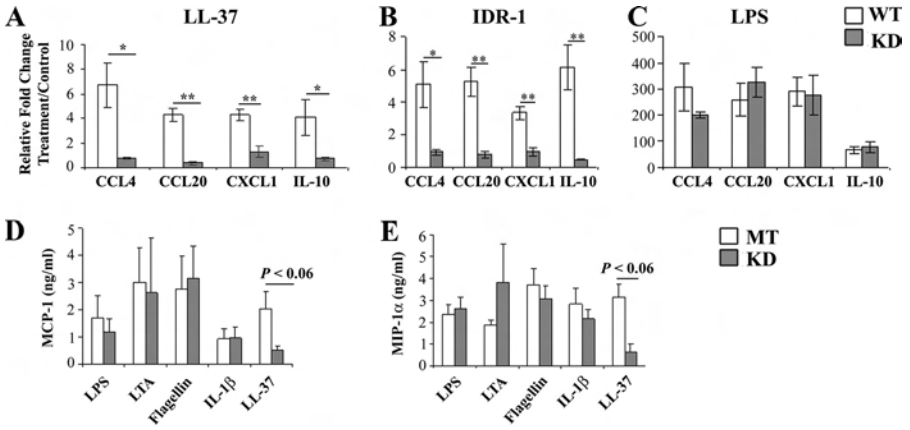

FIGURE 5. Evaluation of responses in GAPDH KD cells. WT, MT, and GAPDH KD cells were stimulated with either different TLR ligands (as indicated), host defense peptides LL-37 or IDR-1, or cytokine IL-1β. Gene expressions induced by *A*, LL-37 (20 μg/ml), *B*, IDR-1 (200 μg/ml), and *C*, LPS (100 ng/ml) were monitored by qRT-PCR after 4 h. Fold changes (*y*-axis) for each gene was normalized to 18S RNA, and is represented as a ratio of gene expression in stimulated vs gene expression in un-stimulated cells using the comparative Ct method. The tissue culture supernatants were monitored for MCP-1 (*D*) and MIP-1α protein (*E*) production after 24 h of stimulation with LPS (10 ng/ml), LTA (2 μg/ml), flagellin (500 ng/ml), IL-1β (10 ng/ml), and LL-37 (20 μg/ml). Results represent an average of at least four independent biological experiments $\pm$ SE (*, $p < 0.05$ and **, $p < 0.01$).

stimulated with either bacterial LPS (10 or 100 ng/ml), LTA (2 μg/ml), flagellin (500 ng/ml), or IL-1β (10 ng/ml) for 4 or 24 h. In contrast to peptide-induced transcriptional responses that were substantially suppressed, no significant differences in gene expression between WT and KD cells were observed in response to LPS (Fig. 5*C*) after 4 h. Similarly, protein production induced on stimulation with either TLR ligands or cytokine IL-1β was not altered in the presence of GADPH siRNA (Fig. 5, *D* and *E*). GAPDH siRNA treatment also did not alter other nonmetabolic functions associated with GAPDH, since we did not observe any difference in cycloheximide-induced apoptosis in the KD cells when compared with the WT controls or MT cells (data not shown). Taken together, these results demonstrated that GAPDH gene silencing significantly and specifically impaired downstream responses induced by these cationic peptides in human monocytic cells at both the transcriptional and protein levels.

LL-37 induces the phosphorylation of the MAPK p38 in human monocytes and this is essential for LL-37-mediated chemokine production and other downstream responses (24). To further validate the functional relevance of GAPDH in LL-37-mediated responses, LL-37-induced phosphorylation of p38 MAPK was compared in WT and GAPDH KD THP-1 monocytic cells by flow cytometry to measure intracellular phosphorylation of p38 (at T180/Y182) using an anti-phospho-p38 rabbit mAb 3D7. KD of GAPDH completely abolished MAPK p38 phosphorylation in response to LL-37, while having no significant effect on responses to bacterial LPS or LTA (Fig. 6).

Identification of a potential link between GAPDH and p38 signaling

To determine possible routes through which the GAPDH:LL-37 complex might affect p38 signaling, we examined the human interactome for possible connections between GAPDH and p38 signaling pathway members using the InnateDB human and mouse biomolecular interaction database (www.innatedb.ca) (34). According to InnateDB, human GAPDH participates in 61 unique protein-protein interactions, 9 of which represent interactions involving multiprotein complexes. Through these 61 binary and complex interactions, GAPDH interacts with a total of 195 unique proteins, 74 of which are annotated by InnateDB as being potentially involved in innate immunity (supplemental Table II). The signaling pathways in which these 195 unique interactors participate were retrieved from InnateDB and submitted to the InnateDB pathway overrepresentation analysis tool, which identifies pathways that occur more frequently in a given data set than would be

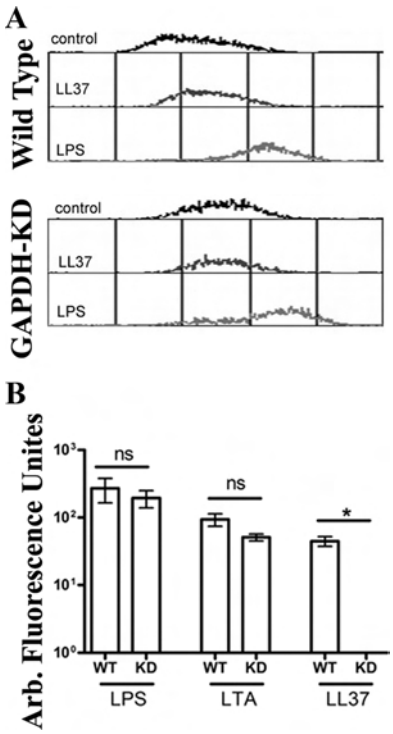

FIGURE 6. Evaluation of LL-37-induced MAPK p38 phosphorylation in GAPDH KD cells. Phosphorylation of MAPK p38 in WT and GAPDH KD cells were evaluated with either LL37 (50 μg/ml), LPS (100 ng/ml), or LTA (2 μg/ml) after 30 min. *A*, Histograms of FL4 fluorescence of WT and GAPDH KD cells stained with anti-phospho-p38 3D7 rabbit mAb (Cell Signaling Technology) and anti-rabbit AF647 secondary Ab. The data are representative of three independent experiments. *B*, Bar charts representing geometric means of the fluorescence histograms shown in *A* following background subtraction of fluorescence of the unstimulated cell controls. Error bars represent SEs of the mean and the statistical comparison using the two-tail *t* test (*, $p < 0.05$; ns, nonsignificant).

expected to occur through chance alone. The list of significantly ($p \leq 0.05$) enriched pathways included regulation of p38α and p38β ($p = 0.03481$), in which 3 of the 30 pathway members (lymphocyte-specific protein tyrosine kinase Lck, the v-yes-1 oncogene homolog Lyn, and the MAPK kinase kinase MAP3K3) are GAPDH interactors. The GAPDH-Lck interaction was reported in an experiment using SDS-PAGE and tandem MS to characterize the CD4-Lck complex (35), while GAPDH-Lyn was identified via a pull-down of proteins associated with the epidermal growth factor receptor (36). MAP3K3 was identified as an interactor of GAPDH through a study using tandem affinity purification with MAP3K3 as bait (37).

The importance of MAP3K3 (also known as MEKK3) and LCK has long been recognized in p38 activation (38), while LYN's role as both an activator and inhibitor of p38 signaling is beginning to be understood (39). We hypothesize that these three proteins, either alone or in combination represent the link between LL-37 binding to GAPDH and the downstream activation of the p38 signaling pathway. Other GAPDH interactors were also identified as regulators of p38 signaling despite not appearing in the initial search results and may also represent candidates for the GAPDH-p38 link. Stratifin has been shown to activate p38 signaling (40), while thioredoxin is a known inhibitor of the p38 activator ASK1.

Discussion

Our findings provide a new insight into the mechanism of action and the cellular protein interactor through which cationic host defense peptides mediate their diverse activities. Our data clearly support a role for GAPDH as a functional direct binding partner for both LL-37- and IDR-1-mediated immunomodulatory (chemokine) and anti-inflammatory (IL-10) responses (Fig. 5). The ability of host defense peptides to promote chemotaxis by inducing the production of chemokines appeared to be signaled through the association of the peptide with GAPDH, since the LL-37-induced production of chemokine MCP-1 was suppressed by>70% in GAPDH KD cells in the presence of the peptide (Fig. 5*D*). This is a very significant observation since LL-37 is a key natural peptide involved in the modulation of human innate immunity, while IDR-1 has been proposed as the prototype of an entirely novel class of therapeutics for infectious diseases. These two peptides have overlapping immunomodulatory and anti-inflammatory properties (17, 19) and both are taken up into cells (17, 19, 26), although they have absolutely no sequence similarity. Thus, it can be anticipated that many sequence unrelated peptides with analogous properties might also interact with GAPDH as a key binding partner. Consistent with this, we have observed that the responses of other sequence unrelated synthetic cationic amphipathic peptides are inhibited by siRNA mediated KD of GAPDH (data not shown), while ELISA and enzyme kinetic inhibition studies similar to those demonstrated in Fig. 3, *B* and *D*, have indicated that mCRAMP, the mouse homolog of LL-37, also binds to GAPDH (N. Mookherjee and R. E. W. Hancock, unpublished data). We feel that such promiscuity in terms of peptide sequences might be explained if these peptides bind to a surface on GAPDH, rather than a defined pocket. Importantly, this suggestion would help to explain the observed substantial overlap in the immunomodulatory properties of host defense peptides in the face of considerable sequence diversity. Interestingly the key responses to both peptides LL-37 and IDR-1 are remarkably analogous in both mouse and human cells, including PBMC, and in animals, indicating that any receptor would be highly conserved; consistent with this GAPDH shows ≥95% identity across mammals.

Based on the studies described previously (12, 26, 41, 42) and in this article, we can reconstruct the action of LL-37 and IDR-1 on mononuclear cells as follows (Fig. 7). These peptides interact with the surface of cells either at key surface receptors, such as FPRL-1, or by inserting directly into the membrane (42). This then triggers a vesicle-mediated uptake pathway involving cellular cytoskeletal elements (26, 42) (supplemental Figs. 2 and 3). The vesicles containing these peptides will then arrive at the vicinity of GAPDH and interact with this enzyme to interfere with the association of GAPDH with one or more activators of the p38 MAPK signaling pathway, likely the kinases MAP3K3, LYN, or LCK (see supplemental Table II). The resultant activation of the p38 MAPK pathway (and possibly others) is known to be involved in the activity of LL-37 and other host defense peptides in modulation of innate immunity. It is important to note that the involvement of GAPDH in modulating innate immunity is not all obvious, as GAPDH is a key glycolytic enzyme; on the other hand, the described interactions do not prevent enzyme activity and thus will not preclude the essential growth functions of GAPDH.

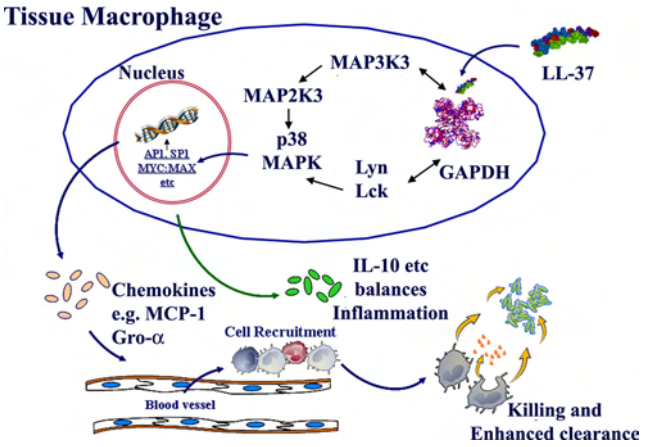

FIGURE 7. Model describing the mechanism of LL-37 immunomodulatory activity due to its interaction with GAPDH. LL-37 (or IDR-1) enters cells using pathways typical of cell-penetrating peptides involving the cell cytoskeletal machinery (26) (supplemental Figs. 1 and 3; note that this may be preceded by interaction with a surface receptor (12, 25). LL-37 then interacts with GAPDH and through its interacting partners, essentially MAP3K3, Lyn, Lck, and others such as MAP3K14, TNFR-associated factor 1, thioredoxin, and YWHAZ (supplemental Table II), to increase signaling through the p38 MAPK pathway (24) (since disruption of this interaction caused interference with p38 MAPK signaling; Fig. 6). This causes the migration of certain transcription factors into the nucleus, e.g., AP-1, SP-1- and MYC:MAX, which are all downstream of the p38 pathway and activated by LL-37 and IDR-1 (our unpublished data) and the resultant transcription of chemokine genes (4, 15, 16). The induced chemokines including MCP-1 and others lead to cell recruitment that aids resolution of the infection (17).

Despite substantial overlap (>30%) in the gene responses to LL-37 and IDR-1, both in the absence and presence of the TLR-4 agonist LPS (17, 19, 20), there are clear differences in functions mediated by these peptides. For example, LL-37 is a modulator of apoptosis in epithelial cells (43, 44) and neutrophils (32) and causes degranulation of mast cells (22), whereas IDR-1 does not (17). Other LL-37 functions such as direct chemotaxis (which tends to involve diverse receptors for different cationic host defense peptides), angiogenesis, and wound healing (9, 12, 21) have not been described for IDR-1. We anticipate that this diversity of responses reflects the possibility of multiple receptors for LL-37 as suggested previously (24, 26, 43). In this study, we have clearly shown that the known receptors and/or transactivated receptors are not required for key immunomodulatory properties (supplemental Fig. 1), but instead GAPDH is a key interactor for these conserved

functions. However, we feel that in addition to these conserved downstream responses involving GAPDH, there should be substantial diversity in accessory functions, reflecting an analogous diversity of interacting partners, and even the functions mediated through GAPDH might be further modulated by the interaction of host defense peptides with other intracellular receptors.

Although GAPDH has not been formally linked to innate immunity, our bioinformatics analyses (supplemental Table II) clearly favor such a role. GAPDH is a predominantly cytosolic protein, but has also been shown to associate with membranes and the nucleus (45–47). Although classically considered to be a glycolytic enzyme, mammalian GAPDH has been implicated in several nonmetabolic processes including membrane transport, microtubule assembly, phosphotransferase/kinase activity, and apoptosis (48). For example, GAPDH associates with the cell surface in both murine and human macrophages and is a functional transferrin receptor involved in endosomal trafficking (47). GAPDH was also shown to directly interact with the cytoplasmic domain of the macrophage scavenger receptor and therefore was hypothesized to be involved in macrophage scavenger receptor-related functions (49). Although GAPDH is an essential glycolytic enzyme, it seems that the interaction with LL-37 and indeed its many other interacting partners do not lead to complete inhibition of enzyme function (as clearly revealed by enzyme inhibition studies). Although the essentiality of GAPDH precludes conventional knockout strategies in mice, the use of siRNA KD strategies as performed here have provided compelling evidence for the functional importance of the interactions of LL-37 and IDR-1 with GAPDH (Figs. 5 and 6), and the cytokines/chemokines that were knocked down have been clearly implicated in the immunomodulatory activity of IDR-1 in mouse infection models (17).

A key feature of host defense peptides in their activity as immunomodulators is an absolute requirement for uptake into cells. It has been shown previously that LL-37 is taken up into epithelial cells (26) and dendritic cells (43), as also shown here for mononuclear cells (supplemental Fig. 2). This likely reflects the similarity of host defense peptides to at least one class of so-called cell-penetrating peptides that tend to carry strong cationic charge (42). For example, LL-37 has been demonstrated to be capable of carrying cargoes into cells (50), while analogies have been drawn between viral nuclear localization signal proteins like HIV Tat and the cationic antimicrobial peptides (a subset of host defense peptides) (51). We propose therefore that the property of cell penetration is an essential requirement for immunomodulatory cationic peptides, but may not be sufficient to enable their immunomodulatory properties, since interaction with GAPDH is also required.

Overall, this study has for the first time demonstrated the direct and functionally relevant interaction of a cellular protein, GAPDH, with diverse immunomodulatory cationic peptides. Thus, we have demonstrated a novel function for the multifunctional mammalian GAPDH protein and propose this will open new avenues for the investigation of immune and anti-infective mechanisms induced by cationic host defense peptides.

Acknowledgments

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Disclosures

The authors declare that they have competing financial interests since a patent will be filed on the use of GAPDH as a receptor for identifying and characterizing novel innate defense regulators. Furthermore, R.E.W.H. has filed patents that protect the commercial use of IDR-1 and his University, which owns these patents, has licensed this peptide to Inimex Pharmaceuticals in which he is a shareholder.

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