Poster # 209

# Sequestosome-1/p62 is the key intracellular target of innate defense regulator peptide

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## Background

Inimex' Innate Defense Regulators (IDRs) are synthetic peptides with no antimicrobial activity that enhance infection control while suppressing inflammation. Treatment of mice with IDRs at the time of infectious challenge provides protection from otherwise lethal bacterial infection, and modulates cytokine and chemokine expression downstream of TLR stimulation. Previously, the effects of IDR-1 were postulated to impact several signaling pathways, including MAPK p38 and C/EBP, but the preceding molecular events remained unknown.

In this study, the cytoplasmic protein p62 has been identified as a molecular target of Inimex' IDRs. p62 is a multi-domain scaffold (adaptor) protein, with many known interacting partners, including PKCζ, p38, RIP1, and TRAF6. p62 comprises an N-terminal PB1 domain that is primarily important for aPKC binding, a ZZ domain which interacts with RIP1, and a TBS sequence domain recognized by TRAF6. Additionally, a C-terminal UBA domain binds to polyubiquitin – a function considered to be the basis of the association between p62 and protein trafficking to the proteasome. Variation in p62 expression levels has been implicated in various disease states but its function in antimicrobial immunity has not yet been investigated.

p62 has recently been recognized as a nodal point in cellular signaling pathways, in particular implicated in regulation of NF-κB. In addition, recent studies demonstrate that p62 expression contributes to regulating macrophage mediated and cancer-associated inflammation, raising the question as to whether IDRs might affect inflammatory responses in the absence of pathogen stimulation.

## Results

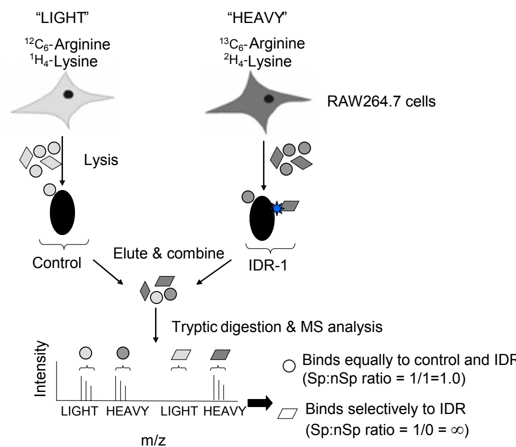


**Figure 1.** Schematic representation of the SILAC-proteomics approach. Streptavidin beads coated with desthiobiotinylated IDR-1 or desthiobiotin alone (negative control) were incubated with lysates prepared from SILAC-labeled and unlabelled RAW264.7 cells, followed by LC-MS/MS analysis of the combined bead eluent. Spectra derived from SILAC-labeled proteins and unlabelled proteins are presented as "specific (Sp)" and "non-specific (nSp)". IDR binding partners are identified by having a Sp:nSp ratio greater then 1. Proteins bound to the desthiobiotin only control will produce Sp:nSp ratio close to or less than 1, and constitute background binders.

## IDR-1 binds to p62

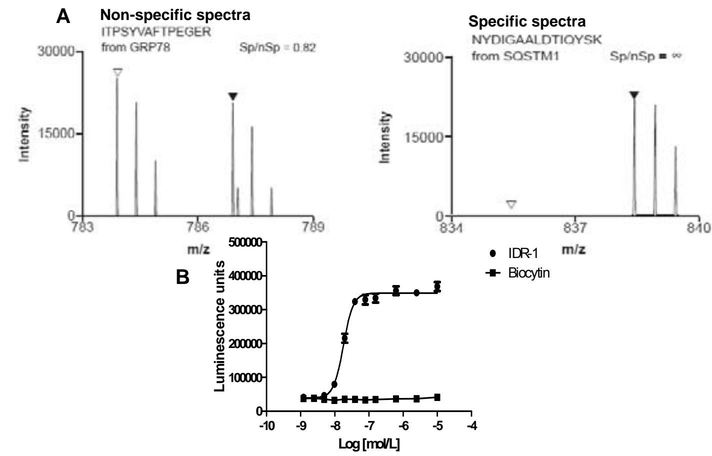


**Figure 2.** **A** SILAC; desthiobiotin-IDR-1 pull-down. Mass spectra from representative tryptic peptides of p62 are observed only in the labeled condition (▼, right) indicating specific binding of p62 to IDR-1 **B** IDR-1 binds to recombinant p62 *in vitro*. Similar results were obtained with other IDRs in Inimex' portfolio.

## IDR-1 binds to the ZZ domain of p62

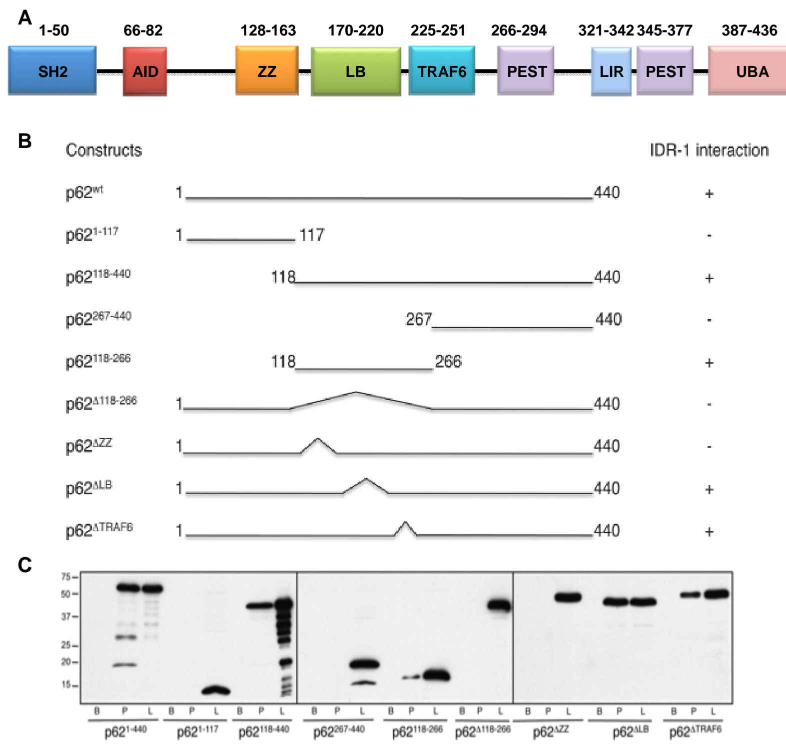


**Figure 3.** **A** Schematic representation of the p62 domains. **B** Constructs used for mapping of p62 domains that interact with IDR-1 **C** Lysates from HEK293T cells overexpressing FLAG-tagged deletion mutants of human p62 were pulled down against biocytin (B) or biotinylated IDR-1 (P). Cell lysates (L) were used as control. Proteins pulled down were immunoblotted with anti-FLAG antibody.

## IDR-1 affects intracellular p62 complexes

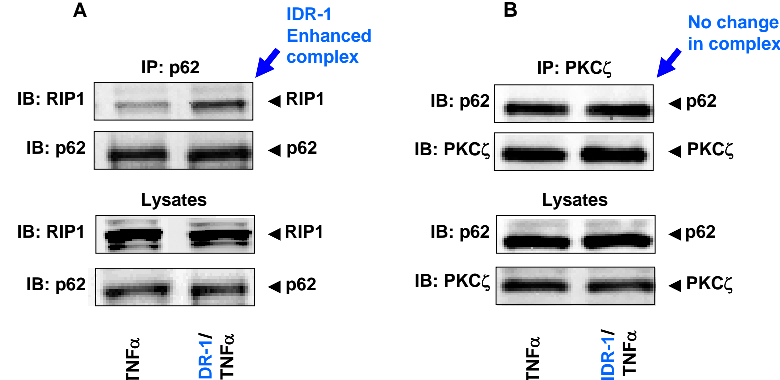


**Figure 4.** Co-immunoprecipitation analysis of p62 molecular complexes in HEK293T cells. **A** IDR-1 enhances p62-RIP complex formation in the presence of TNFα stimulation. **B** IDR-1 has no effect on p62-PKCζ complex formation in the presence of TNFα stimulation. Similar results were obtained with other IDRs in Inimex' portfolio.

## IDR induces Ubiquitylation of RIP1

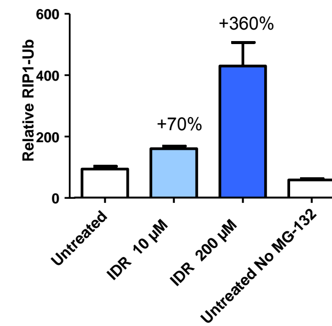


**Figure 6.** HEK293T cells were pre-treated with IDR and proteasomal inhibitor MG132, followed by 5 min TNFα stimulation. RIP1-Ub levels were determined by ELISA in cell lysates using an anti-RIP1 capture antibody and an anti-Ubiquitin detection antibody.

## IDR-1 modulates p62 mediated signaling

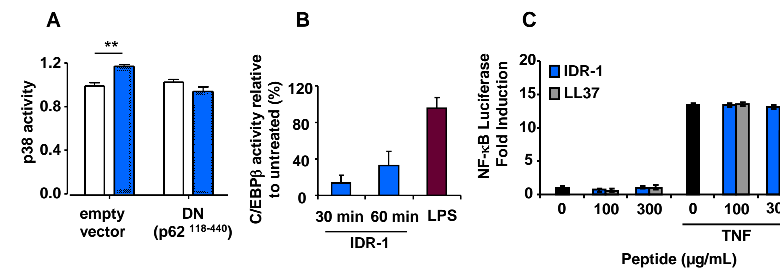


**Figure 7.** **A** IDR-1 treatment activates the p38 signaling pathway in a p62 dependent manner as determined by a p38 activity driven luciferase assay in A549 cells **B** Treatment of A549 cells with IDR-1 induces C/EBPβ activity **C** IDR-1 treatment of NF-κB-luciferase-A549 cells does not affect NF-κB activity in the presence or absence of TNFα stimulation. ** p<0.01

## Proposed IDR/p62 mechanism of action

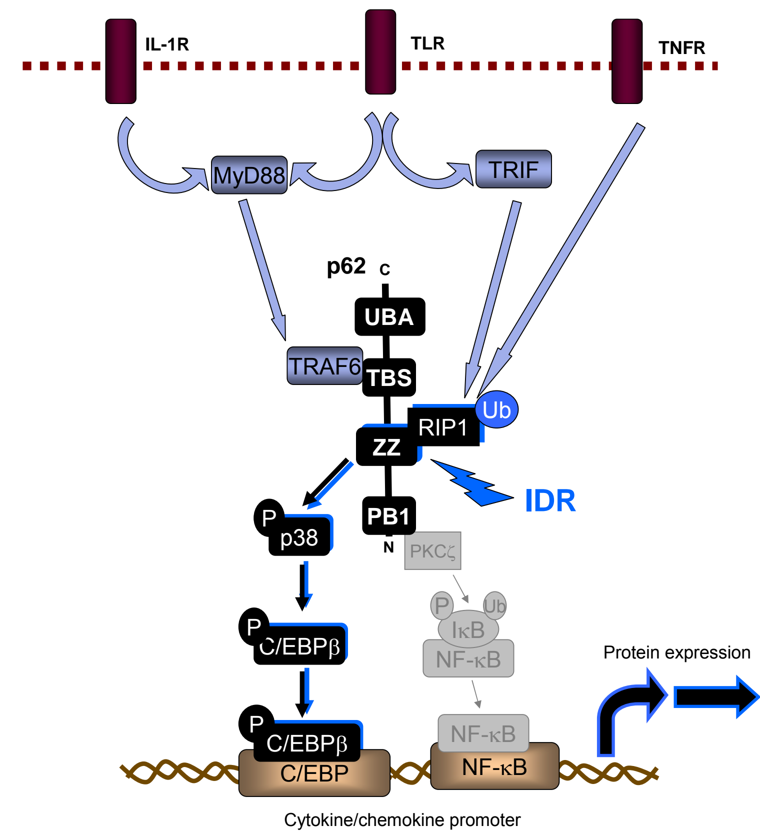


**Figure 8.** IDR binds to the ZZ domain on the p62 protein. This event results in stabilization of the intracellular complex with RIP1. Ubiquitination on RIP1 is also increased. IDR interaction with p62 then activates the p38 MAPK pathway, which results in phosphorylation and activation of the transcription factor C/EBPβ. In the presence of TNFα or TLR stimulation, the NF-κB pathway is activated, although Inimex' IDRs do not change complex formation with PKCζ nor, consequently, modulate NF-κB activity. Activation of transcriptional complexes results in modulation of cytokine/chemokine production in anti-infectious and inflammatory immune responses.

## Conclusions

- Inimex' IDRs bind to the ZZ domain of p62
- IDR binding to p62 results in selectively enhanced complex formation, thereby affecting the balance of pro-inflammatory and anti-infectious signaling
- p62 plays a key role in infection control (See Poster # 330)