Structure

Structural Basis for the Subversion of MAP Kinase Signaling by an Intrinsically Disordered Parasite Secreted Agonist

Graphical Abstract

Highlights

- Toxoplasmosis controls its host immune response via a protein effector, GRA24

- A recombinant complex of GRA24 and MAPK p38α demonstrates how the protein works

- An adapted KIM domain ensures activation and a sustained inflammatory response

- The recombinant complex is useful in the evaluation of p38 inhibitors

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In Brief

Pellegrini et al. show how a protein, GRA24, secreted by the causative agent of toxoplasmosis, directly activates a host MAP kinase signaling cascade (p38α). The structure of the complex provides a mechanistic view of activation and defines GRA24 as the only component required to activate p38α.

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Structural Basis for the Subversion of MAP Kinase Signaling by an Intrinsically Disordered Parasite Secreted Agonist

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SUMMARY

The causative agent of toxoplasmosis, the intracellular parasite Toxoplasma gondii, delivers a protein, GRA24, into the cells it infects that interacts with the mitogen-activated protein (MAP) kinase p38α (MAPK14), leading to activation and nuclear translocation of the host kinase and a subsequent inflammatory response that controls the progress of the parasite. The purification of a recombinant complex of GRA24 and human p38α has allowed the molecular basis of this activation to be determined. GRA24 is shown to be intrinsically disordered, binding two kinases that act independently, and is the only factor required to bypass the canonical mitogen-activated protein kinase activation pathway. An adapted kinase interaction motif (KIM) forms a highly stable complex that competes with cytoplasmic regulatory partners. In addition, the recombinant complex forms a powerful in vitro tool to evaluate the specificity and effectiveness of p38α inhibitors that have advanced to clinical trials, as it provides a hitherto unavailable stable and highly active form of p38α.

INTRODUCTION

A critical issue for the immune system in mammals is to achieve a balance between protection and immune pathology. Much research has focused on the regulatory mechanisms of this balance and their dysfunction in a number of disease settings. In the specific setting of host infection and intracellular pathogens, it has been found that a wide range of pathogens directly hijack the immunoregulatory network during the course of infection. In the case of the Apicomplexa protozoan Toxoplasma gondii, the parasite has evolved methods to trigger a transient innate proinflammatory response that guarantees both control of the parasite population and survival of the host, promoting the establishment of a persistent cryptic parasite population (i.e., parasite survival) (Hunter and Sibley, 2012). Like most pathogens, it has evolved numerous methods of hijacking cell-signaling pathways in order to survive in the host cell. As an obligate intracellular protozoan, it delivers effector proteins into its host cell (Hakimi and Bougdour, 2015; Melo et al., 2011) to control mitogen-activated protein kinase (MAPK) signaling in macrophages, which in turn dictate parasite burden and disease (Braun et al., 2013; Kim et al., 2005). MAPK signaling is characterized by a cascade of multiple kinases, through which the signal is transduced in the form of phosphorylation events from an upstream kinase to a downstream one (Chen et al., 2001; Johnson and Lapadat, 2002). The final phosphorylation event is a dual phosphorylation, which occurs on a conserved Thr-X-Tyr motif in the activation loop (AL) of the MAPK (Cobb and Goldsmith, 1995; Davis, 1995). The phospho-residues are then able to bind basic patches, changing the relative orientation from open in the inactive state to closed in the active state allowing nucleotide binding in the active site (Jura et al., 2011; Kornev and Taylor, 2010).

One of the effector proteins secreted by T. gondii, GRA24, has been shown to interact with the MAP kinase p38α, promoting its activation and nuclear translocation (Braun et al., 2013). GRA24 encodes a 542 amino acid protein with an internal putative bipartite nuclear localization signal, and two repeats, R1 and R2, at the C terminus (Figure 1A). The mechanism by which GRA24 activates p38α, either directly or by modulating the existing activation pathway, is unknown. Previous experiments that mapped the region of the interactions between the two proteins in infected cells show that only one of the repeats is essential for the activation of p38α. Through a docking model, it was proposed that binding of p38α by GRA24 is driven by the kinase interaction motif (KIM), embedded in each of the two repeat regions (Figure 1A). KIMs are linear motifs used by kinases, phosphatases, substrates, and scaffold proteins to bind MAPKs at the KIM or D-motif binding site and regulate their activity (Enslen et al., 2000; Kallunki et al., 1996; Zuniga et al., 1999). KIMs ensure specificity between partners but also have allosteric effects on the proteins (De Nicola et al., 2013; Tokunaga et al., 2014; Zhou et al., 2006).
Here, we have produced a recombinant complex of an N-terminal truncation of GRA24 (GRA24ΔN, Figure 1A) and p38α. Using a combinatorial approach of various structural techniques, the macrostructure of the recombinant complex between GRA24 and p38α and the crystal structure of the core interaction between the proteins is described. The results provide a molecular view of activation and define GRA24 as the only component required to activate p38α. The recombinant complex of GRA24 and p38α also provides a highly specific tool to determine the activity of p38α inhibitors that have advanced to clinical trials.

RESULTS

Structure of the GRA24KIM1-p38α Complex

To define the molecular basis of the interaction between p38α and GRA24, we determined the crystal structure of p38α bound to the GRA24 KIM1 peptide (residues 440–455) at 2.8 Å (Table 1). The complex crystallized in the triclinic space group P1 with two molecules in the asymmetric unit. The electron density for the GRA24KIM1-p38α complex was excellent and the entire peptide sequence could be traced (Figure S1A). As the density is slightly better in the second molecule (chain B), only this chain is used in subsequent descriptions. KIMs have a general consensus sequence of X-fU-X2-(q(Arg/Lys))1-2-(X)2-6-fL-(X)1-2-fA-X-fB (where fA, fB, fL, and fU are hydrophobic residues [usually Leu, Ile, or Val] and q is a basic residue [Arg or Lys]) (Gavin and Nebreda, 1999; Smith et al., 1999), shorter motifs can lack the first four residues (Sharrocks et al., 2000; Zeke et al., 2015). The KIM or D-motif binding site is formed by an acidic patch known as the common docking (CD) domain (Tanoue et al., 2001) and a hydrophobic docking groove (Chang et al., 2002; Gum and Young, 1999) (Figure 1B). The CD domain accommodates the fU and basic residues, while the hydrophobic docking groove binds fA, fB, and fL (Akella et al., 2010; Heo et al., 2004; Lee et al., 2006). The GRA24 peptide binds with the hydrophobic residues (fA-fB) in the docking groove formed by α7 and α8 and the reverse turn between β7 and β8 in the C-terminal lobe of p38α (Figure 1B). Moreover, GRA24 utilizes all interacting motifs identified so far (Peti and Page, 2013; Zeke et al., 2015), including the additional hydrophobic residues, fI and fU, and the ionic contacts with the p38α CD domain, where the KIM1 peptide forms a short α helix involving residues RRELLG (Figure 1B). An additional hydrophobic interaction, not observed in other KIMs, is made with V447. Binding of GRA24KIM1 causes significant conformational change and disorder in the AL with a rotation of the N-terminal domain of 10° toward the C-terminal domain caused by tightening between the areas linked by the peptide (Figure 1C). This movement results in the alignment of the catalytic spine (C-spine) toward the active state, one of the essential events in kinase activation (McClendon et al., 2014), the residues belonging to the C-spine and the hinge region are in a similar position to the fully activated ATP bound p38γ (Bellon et al., 1999) (Figure 1C).
Table 1. Data Processing and Refinement Statistics for the GRA24KIM1-p38α and MKK6KIM-p38αK53R Structures

| Structure                  | GRA24KIM-p38α (SETA) | MKK6KIM-p38αK53R (SETF) |
|----------------------------|----------------------|-------------------------|
| Data Collection            |                      |                         |
| Space group                | P1                   | P3,21                   |
| Unit cell dimensions       |                      |                         |
| a, b, c (Å)                | 52.0, 61.9, 75.7     | 82.4, 82.4, 123.3       |
| α, β, γ (°)                | 105.6, 96.3, 114.7   | 90.0, 90.0, 120.0       |
| Resolution range (Å)       | 45.79 (2.95–2.8)     | 46.65 (2.49–2.4)        |
| Rmeas                      | 0.09 (0.27)          | 0.108 (0.690)           |
| <(I/σ(I)>                  | 9.9 (3.0)            | 13.3 (2.1)              |
| Redundancy                 | 1.9 (1.9)            | 3.9 (3.9)               |
| Completeness (%)           | 96.5 (97.2)          | 90.3 (59.0)             |
| Refinement                 |                      |                         |
| Resolution range (Å)       | 20.0 (2.95–2.8)      | 20.0 (2.49–2.4)         |
| No. of reflections         | 36,372               | 32,069                  |
| Rwork/Rfree                | 22.3/28.3            | 16.29/23.1              |
| No. of atoms               |                      |                         |
| Protein                    | 2,715 (A), 2,705 (B) | 2,725                   |
| GRA24KIM1                  | 127 (C), 127 (D)     | 92 (MKK6KIM)            |
| Water                      | 29                   | 223                     |
| B factors (Å²)             | 63.69 (A), 59.98 (B) | 32.25                   |
| Ligand                     | 73.97 (C), 83.91 (D) | 42.33                   |
| Water                      | 46.87                | 33.48                   |
| RMSD                       |                      |                         |
| Bond lengths (Å)           | 0.006                | 0.0078                  |
| Bond angles (°)            | 1.113                | 0.931                   |
| Ramachandran favored (%)   | 95.8                 | 97.7                    |
| Ramachandran outliers (%)  | 0.4                  | 0                       |
| Rotamer outliers (%)       | 2.2                  | 1.3                     |
| Clash score                | 9.68                 | 4.82                    |
| Overall score              | 2.07                 | 1.41                    |

Values in parentheses are for the outer resolution shell. RMSD, root-mean-square deviation.

rearrangement also leads to a rotation of methionine 109 in the hinge region that prevents nucleotide binding in the active site of the inactive protein (Figure 1C). These conformational changes would allow the entry of ATP into the active site and make the AL accessible for phosphorylation between activated p38α molecules.

GRA24 Has High Affinity for p38α by Combining the Attributes of Different KIM Domains

To further characterize the interaction between GRA24 and p38α we compared the binding of GRA24 KIM1 with other KIM peptides described in the literature (Table S1) using isothermal calorimetry (ITC). The GRA24 KIM1 peptide was found to bind p38α with an equilibrium dissociation constant (K_D) of 1.6 μM (Table S1 and Figure S2), ~2-fold tighter than KIMs found in phosphatas, which have the highest measured affinity of cytosolic regulatory partners involved in classical MAPK regulation (Francis et al., 2011). While the full-length proteins may show different affinities and binding modes, these results show that the GRA24 KIM has a higher affinity than either upstream kinases or downstream phosphatase KIMs.

The GRA24KIM1-p38α structure shows that high-affinity and allosteric effects (disorder of the AL) is achieved by combining the sequences of both the upstream MKK KIMs and those of the downstream phosphatases (Figures 2B, 2C, and S3). To compare binding by an upstream kinase we determined a complex of a kinase dead mutant and the KIM domain from MKK6 (residues 4–18) at 2.4 Å (MKK6KIM-p38αK53R, Table 1) with no mutation in the KIM binding area, as there is in a published structure (C162S) (Garai et al., 2012). The complex crystallized in the trigonal P3,21 space group with one molecule in the asymmetric unit. Comparison of the structures shows GRA24 binding in a similar manner to the hydrophobic groove, using the same L and I residues for ϕ_A−ϕ_B but, unusually, uses the hydrophobic base of arginine in the ϕ_L pocket (Figure 2C). This is a longer linker between ϕ_A and ϕ_B pockets than predicted and is more similar to the GRA24 KIM that has a double proline spacer. There is no interaction with the CD domain or the associated secondary structural rearrangement (Figure S1B). None of the MAPK-p38α structures available show binding to the CD domain. However, GRA24KIM1 binding to the p38α CD domain is similar to that described for tyrosine phosphatases (Figure 2B), the KIMs of which comprise an RR motif usually structured in a short α helix (Zhou et al., 2009).

The only KIM with a higher affinity for p38α is from the MAPK substrate MAPKAP kinase 2 (K_D of 50 nM; Garai et al., 2012). As MAPKAP2 is only present in the nucleus (Zakowski et al., 2004) it will not compete with recruitment of p38α by GRA24 in the cytoplasm. Nevertheless, it is interesting to note that the GRA24 KIM has a very similar mode of binding to the MAPKAP2 KIM, both in the hydrophobic groove and to the CD domain, leading to very tight binding (Figure 2E). The major difference between the two KIMs is the reverse direction of the MAPKAP2 sequence (Figure S3, C- to N-terminal) and increased secondary structure elements (Figure 2E).

The Structure of the GRA24ΔN-p38α Complex Shows Two Independent Kinases Bound to an Intrinsically Disordered Protein

To define the mechanism of activation of p38α by GRA24 the complex between the proteins must be studied; we therefore attempted to produce the protein in Escherichia coli. Expression of either the wild-type (WT) or a truncation removing the N terminus to the nuclear localization sequence (GRA24ΔN, residues 337–542, Figure 1A) failed to produce detectable expression. However, when co-expressed with p38α, a stable complex between p38α and GRA24ΔN was produced (Figure S4) as the association with the kinase presumably prevents proteolysis. The complex was purified to homogeneity and analysis of staining intensity indicated a ratio of 1:2 for GRA24ΔN to p38α. We then investigated the structure of the complex using small-angle X-ray scattering (SAXS). SAXS was used to determine the average structure of the GRA24ΔN-p38α complex in solution (Table S2). The radius of gyration was determined to be 4.85 nm...
with a $D_{\text{max}}$ of 16.96 nm and ab initio modeling showing an elongated molecule (Figures 3A and 3B) best described by a disordered protein binding two globular domains. The pair distribution function (Figure 3A, inset) also suggests flexibility between the kinases (full width at half maximum of 3.8–6.2 nm about the mode of 4.85 nm) implying disorder in GRA24. The possibility that the dimer observed in the crystal structure could also be present in solution was tested by SAXS (Table S2) and found to be a crystallization artifact.

Using the crystal structure of the GRA24 KIM1-p38$\alpha$ complex and modeling GRA24 as an intrinsically disordered protein, a structure of the complex can be proposed (Figures 3B and 3C). The refined model is an excellent fit to the experimental curve ($\chi^2 = 1.08$) and provides insight into its mode of action (Figures 3A–3C). The two kinases are bound at the C terminus of GRA24, linked by ~65 residues.

Dimerization has been demonstrated to be part of an activating mechanism for p38$\alpha$ and its close homolog ERK2 (Diskin et al., 2007; Khokhlatchev et al., 1998). The presence of two KIMs in GRA24 opens the possibility that dimerization between the bound kinases could play a role in activation. The SAXS data imply movement between the kinases; however, as it only provides an average envelope of the structure the relationship between the kinases cannot be fully defined. We therefore used single-molecule approaches, negative-stain electron microscopy (EM), and atomic force microscopy (AFM), to investigate the dynamics of the complex. EM confirmed the flexibility, with class averages showing the two kinases in close proximity, but with a considerable variety of positions (Figure 3D). AFM also shows the dynamic nature of the complex at the single-molecule level (Figure 3E). In both AFM and EM data no secondary structure can be observed in GRA24 confirming its disordered nature. These data demonstrate flexibility between the kinases, eliminating the possibility of a dimer, which implies that the kinases can act independently.

We therefore tested the requirement in vivo of each of the KIMs specifically, by developing 293 derived inducible stable cell lines expressing hemagglutinin (HA) Flag-tagged GRA24 where either KIM1, KIM2, or both KIMs were mutated to alanine (Figure 1A). Immunoblotting for phosphorylated p38$\alpha$ demonstrates that GRA24 induces phosphorylation with only one KIM, but at least one is required for activity as the double mutant leads to an absence of activated p38$\alpha$ (Figure 4A). This is in agreement with previous results demonstrating that a single internal repeat domain was required for activation (Braun et al., 2013). This result defines the GRA24-KIM as the essential factor in the recruitment and activation of p38$\alpha$ in vivo and that dimerization of the kinases is not required for activation.

**The Recombinant Complex Is Active and Provides a Tool to Assess p38$\alpha$ Inhibitors**

The activity of the complex was assessed by assaying its ability to phosphorylate itself (Figure 4B) and also a typical transcription
factor target of p38α, activating transcription factor 2 (ATF2) (Figure 4C). Incubation of the complex with radiolabeled ATP demonstrates the complex is active (Figure 4B) and mass spectrometry shows that phosphorylation occurs on multiple serine and threonine residues within GRA24 (Table S3, Figure 3C), including a previously identified kinase motif (Braun et al., 2013). Assaying the complex’s activity against a natural target, ATF2, shows that p38α in complex with GRA24ΔN is significantly more active against ATF2 than p38α alone (Figure 4C). As both proteins were expressed in E. coli, devoid of endogenous protein kinases, this defines GRA24ΔN as the only factor required to activate p38α and demonstrates that the complex is a powerful tool to unambiguously evaluate the pharmacological inhibition of p38α in vitro. A highly active and stable recombinant form of p38α is not currently available, hampering efforts to assay the specific effects of molecules on p38α. Using the GRA24ΔN-p38α complex, we have shown that many of the inhibitors developed against p38α, some of which are currently in phase II clinical trials, prevent ATF2 phosphorylation by the complex in vitro (Figure 4C). The ability of the compounds to impair p38α phosphorylation, in a GRA24-dependent manner, and concomitant epidermal growth factor 1 (EGR1) activation was
validated in infected macrophages (Figure 4D). Differences in the observed inhibition between in vivo and in vitro assays, as well as between different inhibitors (Figures 4C and 4D), demonstrate the importance of having a permanently active form of p38α.

**DISCUSSION**

Pathogens often evolve methods of modulating host cell-signaling pathways in order to promote their growth and persistence in the infected cell. One of the most efficient methods to interact with host signaling proteins is the use of intrinsically disordered proteins combined with short linear motifs, as the evolutionary timescales involved are shorter than those required for globular domains (Davey et al., 2012; Fuxreiter et al., 2007; Hagai et al., 2014). Short linear motifs in particular can be used to allow interactions between a pathogenic protein and that of a host in a simple manner (Elde and Malik, 2009). This study demonstrates the molecular basis by which the parasite-derived agonist GRA24 bypasses the classical MAPK phosphorylation cascade and induces p38α auto-phosphorylation, forming an active complex able to activate transcription factors, such as ATF2.

The parasite protein operates through two atypical KIMs, embedded in an intrinsically disordered protein, which combine attributes of docking domains from multiple MAPK partners to maximize binding. A recent study has analyzed all known KIMs, identified many novel sequences, and classified them by their binding modes (Zeke et al., 2015). Differences are defined in the spacing between hydrophobic residues and the linker between the CD binding region and the docking groove region. Intriguingly, the GRA24 KIM borrows attributes from all the defined classes but does not fit in a single class (Figures 2, S3,
and S5). Overall, it is closest to the HePTP class incorporating all hydrophobic residues as well as the short helix bound to the CD domain. Interestingly, its linker is the same length as the KIM from Ste7 (Figure 2F), and its binding mode for the hydrophobic groove exactly the same as that for Far1 (Figure 2D) with a larger spacer between the \( \phi_h \) and \( \psi_h \) pockets (double proline) (Figures 1, 2, S3, and S5). Ste7 and Far1 are an activator and substrate, respectively, of yeast mating decision MAPKs, and the KIMs have no known higher eukaryotic equivalent. It is interesting to note that the binding mode of the GRA24 KIM is most similar to a unicellular eukaryote interaction pathway. KIMs perform many roles in the MAP kinase cascade, being present in upstream activating kinases, scaffold proteins, and deactivating phosphatases. Each partner uses the KIM to ensure specificity and control the protein allosterically. For example, the KIM from MKK6 loosely associates the two kinases and induces disorder in the AL making it available for phosphorylation. In contrast, phosphatase KIMs bind tightly and change the conformation of the AL to present the phospho-residues for dephosphorylation (Figure 4). While two copies of the kinase are not necessary for activation, the possibility of phosphorylation between GRA24 kinase twins could enhance activity in a similar manner to scaffolding proteins (Brown and Sacks, 2009). GRA24 is itself then phosphorylated at multiple sites. Whether this has functional relevance or is just the result of an active kinase is not known, but a kinase recognition motif located between the bound kinases is highly phosphorylated, as are multiple serine and threonine residues throughout the protein (Figure 3C). Phosphorylation has been shown to play a role in protection of proteins from cellular proteases (Desagher et al., 2001; Døskeland et al., 1996) and also in nuclear translocation (Nardozzi et al., 2010), which could assist GRA24 in its role. At the N terminus, the rest of the protein is free, exposing the signal sequence to the nuclear transport machinery and allowing the complex to be imported to the nucleus where it can act on specific transcription factors (Figure 5) (Braun et al., 2013). The structural features described here show how high-affinity binding to p38\( \alpha \) by GRA24 prevents deactivation by phosphatases and promotes a sustained host kinase activation during Toxoplasma infection. The tight control of the inflammatory signaling prevents either too weak a response leading to host death or too strong a response preventing invasion.

Following pathogen infection or tissue damage, p38\( \alpha \) induces the expression of multiple genes that together regulate the inflammatory response. Therefore, interference in p38\( \alpha \) kinase activity could aid in therapy for inflammatory disorders ranging from rheumatoid arthritis to chronic obstructive pulmonary disease. Compounds targeting p38\( \alpha \) must be highly selective in order to avoid adverse side effects triggered by off-target kinase inhibition. Although in vitro p38 inhibition assays exist, they are hampered by the need to activate the MAPK either in vivo, e.g., following lipopolysaccharide treatment, or through in vitro MKK6 trans-phosphorylation (Szafranska et al., 2005) and the short lived nature of the activated enzyme. By promoting a long-lasting activation of p38\( \alpha \), GRA24 challenges the natural-negative-feedback mechanisms that prevent the MAPK activation ad infinitum and offers a powerful in vitro tool to screen for small-molecule p38\( \alpha \) inhibitors.

**EXPERIMENTAL PROCEDURES**

**Parasites and Host Cells**

Human foreskin fibroblast primary cells, and 293-TRex and J774 cell lines were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 10 mM HEPES buffer (pH 7.2), 2 mM L-glutamine, and 50 \( \mu \)g/mL penicillin and streptomycin (Invitrogen). Cells were incubated at 37°C in 5% \( \text{CO}_2 \). The Toxoplasma strains used in this study were RHku80 WT and RHku80 Δgra24 (Braun et al., 2013).

**Reagents**

Antibodies against HA (3F10; Roche), p38 MAPK (Cell Signaling Technology), Phospho-p38 MAPK (Cell Signaling Technology), Phospho-c-Fos (Ser32; Cell
Signaling Technology), H4 Acetylated (EMD Millipore), EGR1 (Cell Signaling Technology), Phospho-ATF-2 (Thr71; Cell Signaling Technology), and HAUSP (Bethyl Laboratories) were used in the immunofluorescence assay and/or in western blotting. Immunofluorescence secondary antibodies were coupled with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen). Secondary antibodies used in western blotting were conjugated to alkaline phosphatase (Promega). The p38 inhibitors were purchased from InvivoGen and Euromedex.

**Cloning, Expression, and Purification**

DNA encoding human p38α was synthesized (ShineGene Bio-Technologies) and cloned into pETBS with an N-terminal Hisα-tag with a thrombin cleavage site. The mutant p38αKSSR was obtained by PCR-based mutagenesis and verified by sequencing. Recombinant p38α and p38αKSSR were expressed and purified following the same protocol. Both proteins were expressed in E. coli BL21 Rosetta2 cells (Novagen/Merck). Cells were lysed in lysis buffer (50 mM Tris [pH 7.4], 500 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 5% glycerol, and 10 mM imidazole) by mild sonication and the lysate was loaded onto a prepacked His trap column (GE Healthcare). Protein was eluted with a linear gradient to 500 mM imidazole. Cleavage of the Hisα-tag was performed with thrombin overnight at 4°C in lysis buffer. After separation of cleaved and uncleaved protein, samples were further purified by size-exclusion chromatography using a HILoad 16/60 Superdex 200 pg column (GE Healthcare) in buffer A (50 mM Tris [pH 7.4], 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 5% glycerol). Finally, the protein was loaded onto a Mono Q 5/50 GL (GE Healthcare) with a gradient to 1 M NaCl. This final step was necessary in order to separate the mono-phosphorylated protein from unphosphorylated protein. The protein was then concentrated to 10 mg/ml and stored at -20°C.

DNA encoding *T. gondii* GRA24 N-terminal truncation (GRA24ΔN) was synthesized (ShineGene Bio-Technologies) and cloned into pETBS2a using an N-terminal Hisα-tag with a thrombin cleavage site. BL21-CondonPlus(DE3)-RIL cells transformed with pETBS p38α were then additionally transformed with pETBS2a GRA24ΔN. Co-expression of p38α and GRA24ΔN was induced overnight at 18°C with 0.2 mM isopropyl-thio-β-D-galactoside. Cells were lysed in lysis buffer (50 mM Tris [pH 8.0] and 250 mM NaCl) by mild sonication, and the lysate was loaded onto a pre-packed His trap column (GE Healthcare) in buffer A (50 mM Tris [pH 8.0], 250 mM NaCl, and 20 mM imidazole). The complex was eluted with a linear gradient to 300 mM imidazole. Pure fractions were pooled and dialyzed against buffer B (50 mM Tris [pH 7.0] and 50 mM NaCl) overnight and subsequently loaded onto a 5 mL HiTrap Q column (GE Healthcare) and eluted with a gradient to 1 M NaCl where the complex eluted as a single peak. The complex was then further purified by size-exclusion chromatography using a HILoad 16/60 Superdex 200 pg column (GE Healthcare) in buffer C (50 mM Tris [pH 7.4], 50 mM NaCl, and 5% glycerol).

The mutations in GRA24 were introduced in plasmids pcDNA-GRA24 WT-HA-Flag (HF) with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene). The following oligonucleotides for deletion of KIM sequence were prepared in the backbone vector pcDNA-GRA24 WT-HF vector (Braun et al., 2013) using Microcal Origin software. A 70 m

**Isothermal Titration Calorimetry**

ITC was performed at 25°C using an ITC200 System (MicroCal, GE Healthcare). WT p38α or mutant proteins were dialyzed against the titration buffer (50 mM Tris [pH 7.0], 50 mM NaCl, and 1 mM DTT). MKK6 and GRA24 KIM peptides were also prepared in the same buffer. A 70 μM protein solution was titrated using 26 stepwise injections of 1.5 μL with each of the peptide solutions placed in the syringe. The GRA24 KIM peptide was used at 0.5 mM and the MKK6 peptide at 10 mM, the latter due to significantly lower affinity. The heat generated after each ligand injection was measured on the integral of the calorimetric signal. The resulting binding isotherms were analyzed by a nonlinear least-squares fit of the experimental data to a single site model. The heat generated after each ligand injection was monitored following 18 hr of infection with RHku80 and RHIku80 grara24 strains and by using specific p38 inhibitors, control inhibitors, and DMSO vehicle.

**In Vivo Inhibition Assays**

Specific pharmacological inhibition of GRA24-dependent p38α phosphorylation in J774 MØ was monitored following 18 hr of infection with RHku80 and RHIku80 grara24 strains and by using specific p38 inhibitors, control inhibitors, and DMSO vehicle.

**293-TREX Transfection**

Twenty-four hours before transfection, cells were plated (80% confluency) in six-well tissue culture dishes. Flag-fusion protein-expressing plasmids (1 μg) and 0.1 μg puromycin selection plasmid were co-transfected into 293-TREX cells with Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Seventy-two hours later, cells were diluted in the presence of 5 μg/ml puromycin (Sigma-Aldrich) for selection. Individual drug-resistant clones were expanded and tested for tetracycline-inducible gene expression.

**Crystallization**

Crystallization conditions were established at the EMBL High Throughput Crystallization Laboratory (Grenoble, France). To obtain crystals of p38α in complex with the KIM peptide from GRA24 (GRA24KM1-p38α) protein was incubated overnight at 4°C with a 3-fold molar excess of peptide GLLERRG SELPPLYI (Eurogentec). The complex was then mixed 1:1 with 0.2 M MgCl₂, 0.1 M Tris/HCl (pH 8.5), and 25%–28% PEG 3350 and set up in sitting-drop plates. Crystals were harvested directly from the mother liquor (Pellegri et al., 2011) using a MicroMesh loop (MiTeGen), plunged into liquid nitrogen, and stored at 70 K.

For p38αKSSR in complex with the MKK6 peptide (MKK6KM1-p38αKSSR), protein was incubated overnight at 4°C with a 3-fold molar excess of peptide SKGKRNLGKLKIPKA (Eurogentec). Crystals were obtained as above but equilibrated against a buffer containing 25% (w/v) PEG 3350 and 0.1 mM Bis-Tris (pH 6.5). Crystals were transferred to a cryoprotection buffer, prepared as described above (reservoir supplemented with 20% (v/v) PEG 400) and harvested using a MicroMesh loop, plunged into liquid nitrogen, and stored at 70 K.

**Data Collection and Structure Solution**

Diffraction data were collected at beamline ID23-2 (Flot et al., 2013) at the ESRF (Grenoble, France) on a MAR225 charge-coupled device detector to between 2.4 and 2.8 Å resolution. Crystals of MKK6KM1-p38αKSSR formed long needles (with approximate dimensions of 100 x 20 x 20 μm) that were difficult to visualize. Crystals of the GRA24KM-p38α complex were 500 μm in the largest dimension and displayed considerable heterogeneity in diffraction quality (Bowler and Bowler, 2014). Crystals were centered using automated mesh scans (Bowler et al., 2010; Brockhausen et al., 2012; Gabardinho et al., 2010; Svensson et al., 2015). The MKK6KM1-p38αKSSR complex crystallized in the trigginal space group P32,-1, with one molecule in the asymmetric unit and the GRA24KM-p38α complex crystallized in the triclinic space group P1 with two molecules in the asymmetric unit (Table 1). Data were processed with XDS (Kabsch, 2010) and programs from the Collaborative Computational Project Number 4 suite (Winn et al., 2011). The structures were solved by molecular replacement using MolRep (Vagin and Teplyakov, 2010). For the p38αKSSR structure (PDB: 1WFC; Jin et al., 2012) was used as a search model.
with all water molecules removed. For the p38x and MKK6KIM-p38x/K53R structures, the p38x/K53R structure was used as a search model. Refinement was carried out alternately using Phenix (Afonine et al., 2012) and by manual rebuilding with COOT (Emsley and Cowtan, 2004). For the GRA24KIM-p38x complex, tight NCS restraints between monomers in the asymmetric unit were used at the beginning of refinement. Models were validated using MolProbity (Chen et al., 2010).

SAXS Experiments
SAXS data were collected at the bioSAXS beamline BM29 (Pernot et al., 2013) at the ESRF with a PILATUS 1 M detector (Dectris) at a wavelength of 0.992 Å and a camera length of 2.87 m. Scattering curves were measured from solutions of p38x without substrates, in complex with the GRA24 peptide, and of the GRA24KIM-p38x complex. Measurements were performed at protein concentrations between 2 and 15 mg/mL to verify that any inter-particle effects that may have been present could be accounted for and rule out their influence on the analysis. To exclude the possibility of radiation damage, ten frames, each of 1 s duration, were collected while continuously exposing fresh samples to the beam, the resulting frames were then compared to ensure that no differences in the SAXS profiles were induced by exposure to X-rays. All data were processed using the ATASS program package (Petoukhov and Svergun, 2007). Radii of gyration (Rg) were evaluated from Guinier plots using PRIMUS (Konarev et al., 2003) and pair distance distribution functions, P(r), were computed with GNOM (Svergun, 1992). The model of the GRA24KIM-p38x complex was refined using CORAL (Petoukhov et al., 2012).

Electron Microscopy
The GRA24KIM-p38x complex was absorbed to the clean side of a carbon film on mica, stained with 2% sodium silicotungstate, and transferred to a 100–300 mesh copper grid. The images were taken under low-dose conditions (<10 e⁻/Å²) at a nominal magnification of 49,000 x with defocus values between 1.2 and 2.5 μm on an FEI Tecnai 12 electron microscope at 120 kV accelerating voltage using Kodak SO-163 films. Four micrographs were scanned with a Zeiss scanner at 7 m and a camera length of 2.87 m. Scattering curves were measured from solution of p38x without substrates, in complex with the GRA24 peptide, and of the GRA24KIM-p38x complex. Measurements were performed at protein concentrations between 2 and 15 mg/mL to verify that any inter-particle effects that may have been present could be accounted for and rule out their influence on the analysis. To exclude the possibility of radiation damage, ten frames, each of 1 s duration, were collected while continuously exposing fresh samples to the beam, the resulting frames were then compared to ensure that no differences in the SAXS profiles were induced by exposure to X-rays. All data were processed using the ATASS program package (Petoukhov and Svergun, 2007). Radii of gyration (Rg) were evaluated from Guinier plots using PRIMUS (Konarev et al., 2003) and pair distance distribution functions, P(r), were computed with GNOM (Svergun, 1992). The model of the GRA24KIM-p38x complex was refined using CORAL (Petoukhov et al., 2012).

Atomic Force Microscopy
Samples were adsorbed to mica plates at a concentration of 0.1 mg/mL. A Chypher AFM (Asylum Research, Oxford Instruments) was used with MSNL cantilevers (Bruker) in AFM amplitude modulation mode.

ACCESSION NUMBERS
Coordinates and structure factors have been deposited in the PDB under accession codes PDB: 5ETA and 5ETF for the GRA24KIM1-p38, and a camera length of 2.87 m. Scattering curves were measured from solutions of p38x without substrates, in complex with the GRA24 peptide, and of the GRA24KIM-p38x complex. Measurements were performed at protein concentrations between 2 and 15 mg/mL to verify that any inter-particle effects that may have been present could be accounted for and rule out their influence on the analysis. To exclude the possibility of radiation damage, ten frames, each of 1 s duration, were collected while continuously exposing fresh samples to the beam, the resulting frames were then compared to ensure that no differences in the SAXS profiles were induced by exposure to X-rays. All data were processed using the ATASS program package (Petoukhov and Svergun, 2007). Radii of gyration (Rg) were evaluated from Guinier plots using PRIMUS (Konarev et al., 2003) and pair distance distribution functions, P(r), were computed with GNOM (Svergun, 1992). The model of the GRA24KIM-p38x complex was refined using CORAL (Petoukhov et al., 2012).

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Supplemental Information

Structural Basis for the Subversion of MAP Kinase Signaling by an Intrinsically Disordered Parasite Secreted Agonist

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Supplementary Figure 1, related to Table 1 and Figure 1. Density for KIM peptides. A Simulated annealing OMIT map contoured at 3σ (green mesh) is shown for the GRA24 KIM1 peptide bound to p38α (grey surface) on Molecule 2. B. Simulated annealing OMIT map contoured at 3σ (green mesh) is shown for MKK6 KIM1 peptide bound to p38α (grey surface).
Supplementary Table 1, related to Figure 2. Thermodynamic and dissociation constants determined for complexes between p38α and KIM peptides.

| Protein complex   | $K_d$ (μm) | $\Delta H$ (kcal mol$^{-1}$) | -TΔS (kcal mol$^{-1}$) | ΔG (kcal mol$^{-1}$) |
|-------------------|------------|------------------------------|------------------------|---------------------|
| p38α MKK6KIM      | 161.5 ± 8.5| -2.6                         | -2.6                   | -5.17               |
| p38α GRA24KIM1    | 1.6 ± 0.1  | -14.6                        | 6.7                    | -7.9                |
| HePTPKIM31*       | 5.15 ± 1.29| -19.6                        | 12.4                   | -7.2                |
| FAR1*             | 7 ± 1      | ND                           | ND                     | ND                  |
| Ste7*             | 12 ± 1.5   | ND                           | ND                     | ND                  |
| MAPKAP2*          | 0.05       | ND                           | ND                     | ND                  |

*Data from:

Francis, D.M., Rozycki, B., Koveal, D., Hummer, G., Page, R., and Peti, W. (2011). Structural basis of p38alpha regulation by hematopoietic tyrosine phosphatase. Nature Chem Biol 7, 916-924.

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Supplementary Figure 2, related to Figure 2. ITC titrations and fits determined for complexes between p38α and KIM peptides. The raw data of the heat produced by p38α as the peptide is titrated in is shown. The lower panel shows the thermodynamic constants contributing to dissociation constant.
Supplementary Table 2, related to Figure 3. SAXS experimental data and comparison with calculated values for crystal structures

| Protein complex                  | Rg (nm) | Dmax (nm) | Molecular weight (kDa) | Chi square of fit |
|----------------------------------|---------|-----------|------------------------|-------------------|
| p38α                             | 2.47    | 8.61      | 45.9 – 61.2            | -                 |
| p38α GRA24KIM1                   | 2.48    | 8.50      | 42.8 – 57.1            | -                 |
| p38α GRA24KIM1 TCEP              | 2.67    | 9.34      | 39.2 – 52.3            | -                 |
| p38α -GRA24ΔN                    | 4.85    | 16.96     | 103.8 – 138.3          | 1.08              |
| p38α crystal structure           | 2.88    | 7.7       | 41.5                   | 2.9               |
| p38α GRA24KIM1 crystal structure | 2.83    | 7.33      | 40.5                   | 2.9               |
| p38α GRA24KIM1 dimer crystal     | 3.73    | 9.45      | 83                     | 25.8              |
### Supplementary Figure 3, related to Figure 2 – GRA24 combines the binding motifs of different MAPK partners.

Sequence alignment between KIM domains, interaction points are identified, GRA24 uses all possible interactions.
Supplementary Figure 4, related to Figure 3 and Experimental Procedures. SDS-PAGE of fractions from size exclusion chromatography of the GRA24ΔN-p38α complex. Lane 1: Load; Lane 3: marker; Lanes 4 to 17: fractions from Superdex200 column. p38α runs just below 44 kDa and GRA24ΔN just below 29 kDa, band intensity analysis gives a ratio of 2:1 p38α : GRA24ΔN.
**Supplementary Table 3**, related to Figure 3. Phosphorylation sites identified in GRA24 ΔN by mass spectrometry. HCD and MSA were used to identify phosphorylated peptides. The higher the probability and score difference the higher the confidence. Sites are mapped to the sequence (shown below) as well as the confidence with which they were found.

| Sequence                  | Phospho (STY) | Probability* | Score difference | MS run |
|---------------------------|---------------|--------------|------------------|--------|
| HSTV*TPQ**TPPAR           | 2 but only 1  | 0.948        | 10.01            | HCD    |
| KHSTV*TPQ**TPPAR          | 3 but only 1  | 0.839        | 6.39             | HCD    |
| GSTAFSTRPP**SSR           | 1             | 0.829        | 11.27            | HCD    |
| HSTVTOPQ**TPPAR           | 1             | 0.928        | 11.13            | HCD    |
| LRPEPSL*SSLTEK            | 1             | 0.759        | 7.34             | HCD    |
| HSTV*TPQ**TPPAR           | 2             | 0.999; 0.904 | 30.23; 9.73      | MSA    |
| KHSTVTPQ**TPPAR           | 2 but only 1  | 0.984        | 16.29            | MSA    |
| LRPEPSLS*L*TEK            | 2             | 0.801; 1.000 | 6.29; 57.62      | MSA    |
| HSTV*TPQ**TPPAR           | 3             | 1.000; 1.000 | 39.94; 97.92; 112.46 | MSA    |
| KHSTV*TPQ**TPPAR          | 3 but only 2  | 0.962; 0.995 | 11.55; 21.05     | MSA    |
| GSTAFSTRPP**SSR           | 1             | 0.977        | 16.53            | MSA    |
| HSTV*TPQ**TPPAR           | 1             | 0.899        | 9.92             | MSA    |
| TRPPFNPWPSTK              | 1             | 1.000        | 93.82            | MSA    |

STY pink coloured residues – possible other sites for the remaining phosphorylation on the found peptide, but insufficient evidence to localise.

>GRA24 ΔN

MVEPAGLqTESRLRPEPSLSSLTEKGSTAFSTRPPrSSSRSALEGLTQETVEMLLDTPSYPISSVSSPPARKSSTSSSQHL

EARLSQSQRPPRTRPPFNWPSTKTGLLERGVSelpгляPRPLASGYRNPADSRKHSTVTPQTPPARKSSTSSSQHL

HLEARLSQSQRPPRTRPPFNWPSTKTGLLLERGVSelpглядVKPPTKGN

Colour key:  **RED** (found only in HCD data)

**BLUE** (found only in MSA data)

**PURPLE** (found in both)

**KIM** sequences

Text size = proportional to how many times found
Supplementary Figure 5 – Comparison of KIM peptides showing the major motifs, related to Figure 2.
Supplemental movie 1. Structure of the GRA24ΔN-p38α complex, related to Figure 3. *ab initio* model of the GRA24ΔN-p38α complex (blue mesh) with the final model (internal repeats purple, KIM yellow, p38α is shown with N terminal lobes grey and C-terminal green and Activation Loop blue).

Supplemental movie 2. Structure of the GRA24ΔN-p38α complex, related to Figure 3. Model of the GRA24ΔN-p38α complex (internal repeats purple, KIM yellow, p38α is shown with N terminal lobes grey and C-terminal green and Activation loop blue) showing phosphorylation sites (magenta) identified by MS, scaled according to the number of times found.