Mona/Gads SH3C Binding to Hematopoietic Progenitor Kinase 1 (HPK1) Combines an Atypical SH3 Binding Motif, R/KXXX, with a Classical PXXP Motif Embedded in a Polyproline Type II (PPII) Helix*

Hematopoietic progenitor kinase 1 (HPK1) is implicated in signaling downstream of the T cell receptor. Its non-catalytic, C-terminal half contains several proline-rich motifs, which have been shown to interact with different SH3 domain-containing adaptor proteins in vitro. One of these, Mona/Gads, was also shown to bind HPK1 in mouse T cells in vivo. The region of HPK1 that binds to the Mona/Gads C-terminal SH3 domain has been mapped and shows only very limited similarity to a recently identified high affinity binding motif in SLP-76, another T-cadaptor. Using isothermal titration calorimetry and x-ray crystallography, the binding of the HPK1 motif to Mona/Gads SH3C has now been characterized in molecular detail. The results indicate that although charge interactions through an RXXK motif are essential for complex formation, a PXXP motif in HPK1 strongly complements binding. This unexpected binding mode therefore differs considerably from the previously described interaction of Mona/Gads SH3C with SLP-76. The crystal structure of the complex highlights the great versatility of SH3 domains, which allows interactions with very different proteins. This currently limits our ability to categorize SH3 binding properties by simple rules.

Hematopoietic progenitor kinase 1 (HPK1); Human Genome Organization gene symbol MAP4K1; a member of the germinal center kinase (GCK) family within the large superfamily of STE20 and p21-activated kinases (Refs. 1 and 2). As its name indicates, HPK1 expression is prominent in hematopoietic cells. Initial cloning reports implicated HPK1 in the activation of stress kinases (SAPKs/JNKs) (3, 4). Since then, HPK1 activity has been associated with lymphocyte antigen receptor signaling (BCR, TCR; Refs. 5–9) and in signaling induced by transforming growth factor-β (10, 11). It has also been shown that physiological concentrations of prostaglandin E2 raise HPK1 kinase activity in T cells and myeloid cells by a yet undefined mechanism, possibly leading to the suppression of fos gene transcription (12). In addition, a single report suggests a role for HPK1 in erythropoietin-induced signaling of two cell lines (13), but this finding remains unconfirmed for primary erythroid progenitor cells.

HPK1 is presumed to act as a MAP4K (MAP kinase kinase kinase). Its potential substrates include the mixed lineage kinase MLK3, the mitogenic kinase MEKK1, the transforming growth factor-β activated kinase TAK1 (8), and also c-Jun. Furthermore, HPK1 is activated by caspase cleavage (14, 15) and functions as a regulator of NFκB signaling (15, 16). Despite these reports, the in vivo functions and signaling pathways of HPK1 remain only marginally defined. HPK1−/− mice have been generated by targeted gene disruption but exhibit no obvious phenotype. This may not be entirely surprising, given that HPK1 is just one member of a family of similar kinases with hematopoietic expression (1, 2). Close relatives with overlapping expression patterns include GCK, kinase homologous to SPS1/STE20 (syn. GCKR), HPK1/GCK-like kinase (named NIK in mice) and germinal center-like kinase. The targeted disruption of multiple GCK family kinase genes in mice may eventually shed more light on HPK1 functions.

Physical interactions of HPK1 with other intracellular proteins in vitro and in vivo have been reported in numerous studies. The T-cell adaptor SLP-76 and its B-cell relative BLNK, also known as SLP-65 or BASH, are functionally important interaction partners of HPK1 and crucial in the signal transmission of TCR and BCR, respectively (5, 7, 17, 18). After receptor activation, SLP-76 and BLNK bind via their SH2 domains to the phosphorylated tyrosine 397 of human HPK1. This finding is supported by the demonstration of HPK1 kinase activity in T cells and myeloid cells by a yet undefined mechanism, possibly leading to the suppression of fos gene transcription (12). In addition, a single report suggests a role for HPK1 in erythropoietin-induced signaling of two cell lines (13), but this finding remains unconfirmed for primary erythroid progenitor cells.

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binding to HPK1 was clearly demonstrated in mouse T cells by co-immunoprecipitation of the endogenous proteins (5). The same study implicated the fourth proline-rich motif in the non-catalytic part of HPK1 as the binding region for Mona/Gads but did not provide details regarding the HPK1 residues crucial for high affinity binding. We and others have recently shown that the SH3C domain of Mona/Gads can interact with nanomolar affinity with a SLP-76 motif, which lacks a typical PXXP motif (23, 24).

In this study, the interaction of HPK1 with Mona/Gads was initially analyzed by isothermal titration calorimetry (ITC). Subsequently, a suitable fragment of HPK1 in complex with Mona/Gads SH3C was crystallized and studied by x-ray crystallography. The structure of the Mona/Gads SH3C-HPK1 peptide complex was solved to 1.5-Å resolution. The data show that, different from the interaction of Mona/Gads SH3C with SLP-76, a PXXP motif, which is part of a polyproline type II helix, is essential for complex formation of murine Mona/Gads SH3C and HPK1. Alanine scanning and crystal contacts define the residues PXVPXRXXK as key sites for complex formation.

**EXPERIMENTAL PROCEDURES**

Peptide synthesis as well as expression and purification of murine Mona/Gads SH3C were done as described previously (24). The pGEX-2T expression vector for GST-Grb2 SH3N (human amino acids 3–57) is a gift of O. Janssen. Expression and glutathione-Sepharose purification of this fusion protein was done under the same conditions as for Mona/Gads SH3C. The functionality of the construct had been previously confirmed in precipitation experiments using Sos-containing cell lysate.

ITC was done essentially as described previously (24). Briefly, ITC measurements were performed on a VP-ITC MicroCalorimeter (MicroCal, Northhampton, MA). 1.43 ml of 0.05 mM affinity-purified GST-SH3 domain in ITC buffer (25 mM HEPES-KOH, pH 7.5, 100 mM potassium acetate, 5 mM magnesium acetate) was clarified for 10 min at 20,800 g and degassed for 10 min with Thermovac (MicroCal) before being transferred into the sample chamber. Synthetic peptides were diluted to 0.5 mM in ITC buffer, clarified, and degassed, and ~300 µl was loaded into the syringe. During the measurements, 5- and 10-µl aliquots of peptide solution were titrated once and 28 times, respectively, into the sample chamber at an equilibrium temperature of 25 °C. The heat of dilution was negligible. Total heat generated and $K_v$ values were calculated in ORIGIN (V5.0).

For crystallization, the Mona/Gads SH3C construct at 15 mg/ml was mixed with the 16-residue HPK1 peptide P5 at a 1:3 molar ratio. Crystals were obtained with the sitting drop vapor diffusion method at 20 °C overnight under equilibration conditions of 0.1M HEPES, pH 7.5, 2 M (NH4)2SO4 (Index Screen, Hampton Research). These were cryoproected through transfer into mother liquor containing 20% glycerol and vitrified. Single wavelength data were collected on beamline BM14 at the European Synchrotron Radiation Facility, Grenoble, France (see also Table II). The structure was solved by molecular replacement using AMoRe (25) with 1OEB.pdb as the search model. The atomic model was built subsequently defined the motif PXXRXXK, which is part of a polyproline type II helix, is essential for complex formation of murine Mona/Gads SH3C and HPK1. Alanine scanning and crystal contacts define the residues PXVPXRXXK as key sites for complex formation.

**RESULTS AND DISCUSSION**

Characterization of the Sequence Motif in HPK1 Required for Mona/Gads SH3C Binding—Until now, the SH3C domain of Mona/Gads is the only domain of this adaptor known to mediate protein complex formation independent of tyrosine phosphorylation. Previously, we have shown that the Mona/Gads SH3C domain binds with very high affinity to a PXDRXXKP motif from SLP-76 and that all of the specified residues contribute considerably to complex formation (24). However, HPK1, which also binds to Mona/Gads independently of tyrosine phosphorylation, lacks such a PXDRXXKP motif (Fig. 1A). Instead, the implicated region in HPK1 contains only an RXXK motif in the mouse protein and a KXXK motif in the human protein. Additionally, HPK1 proteins have a cluster of proline residues N-terminal to the R/KXXK motif, which is not present in SLP-76, and could possibly contribute to Mona/Gads SH3C binding. To test this hypothesis, peptides corresponding to 26 amino acids of the relevant region in mouse and human HPK1 (Table I, P1 and P2) were analyzed for binding by ITC. Both peptides bound to Mona/Gads SH3C with affinities of 4 and 21 μM, respectively. These are typical affinities for known SH3 domain-peptide interactions.

The essential Mona/Gads SH3C binding region in mouse HPK1 was further mapped by analyzing shorter overlapping HPK1 fragments (Table I, P3–P8). A representative example of an ITC measurement is shown in Fig. 1B. P7, a 14-amino-acid motif, was the shortest sequence with an affinity significantly below 10 μM. Further truncation by 3 amino acids (P8) resulted in a 4–5-fold decreased affinity. An alanine scan through P8 subsequently defined the motif PXVPXRXXK as essential for mouse HPK1 binding to Mona/Gads SH3C (Table I, P9–P19).
As discussed above, HPK1 has several proline-rich stretches in the non-catalytic part of the protein. In one study, using human HPK1, a region different from the site implicated in the mouse HPK1 has been suggested as the preferential binding site for Mona/Gads SH3C (6). A corresponding peptide (P20) was therefore also tested for binding to Mona/Gads SH3C by ITC. However, the affinity of this peptide was considerably lower than that of P1, and no further studies were performed with it.

The Mona/Gads SH3C binding motif identified in mouse HPK1 clearly overlaps with the PXXPxR core consensus motif for binding of the N-terminal SH3 domain of the Mona/Gads relative Grb2 as defined previously in several studies (Ref. 28 and references therein). Grb2 appears to be ubiquitously expressed and might therefore compete with Mona/Gads for some binding partners. Consequently, the affinity of this peptide was considerably lower than that of P1, and no further studies were performed with it.

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**Table I**

| Peptide | Sequence | Kd (μM) | SD |
|---------|----------|---------|----|
| P1      | WNPAAEPGQPLLVPFPKEMKRMGK | HPK1 (mouse, 26aa, aa457-482) | 4.3 | 2.0 |
| P2      | WNPSSREDKPPLLVPFPKEMKRMGK | HPK1 (human, 26aa, aa459-484) | 20.8 | 1.2 |
| P3      | GQPPLLVPFPKEMKRMGK | HPK1 (mouse, 18aa) | 2.4 | 0.4 |
| P4      | WNPAAEPGQPLLVPFPK | HPK1 (mouse, 18aa) | nbd |
| P5      | GQPPLLVPFPKEMKRMGK | HPK1 (mouse, 16aa) | 2.4 | 0.0 |
| P6      | PPLLVPFPKEMKRMGK | HPK1 (mouse, 16aa) | 4.9 | 1.0 |
| P7      | PPLLVPFPKEMKRMGK | HPK1 (mouse, 14aa) | 3.0 | 0.7 |
| P8      | PPLLVPFPKEM | HPK1 (mouse, 11aa) | 14.4 | 1.1 |
| P9      | APLLVPFPKEM | HPK1 (mouse, 11aa) P1→A | 12.8 | 2.4 |
| P10     | PPLLVPFPKEM | HPK1 (mouse, 11aa) P2→A | tlq |
| P11     | PPLLVPFPKEM | HPK1 (mouse, 11aa) L→A | 9.2 | 1.0 |
| P12     | PPLLVPFPKEM | HPK1 (mouse, 11aa) V→A | tlq |
| P13     | PPLLVPFPKEM | HPK1 (mouse, 11aa) P3→A | tlq |
| P14     | PPLLVPFPKEM | HPK1 (mouse, 11aa) P4→A | 35.7 | 3.3 |
| P15     | PPLLVPFPKEM | HPK1 (mouse, 11aa) R→A | nbd |
| P16     | PPLLVPFPKEM | HPK1 (mouse, 11aa) K1→A | 9.7 | 1.3 |
| P17     | PPLLVPFPKEM | HPK1 (mouse, 11aa) E→A | 10.3 | 0.5 |
| P18     | PPLLVPFPKEM | HPK1 (mouse, 11aa) K2→A | nbd |
| P19     | PPLLVPFPKEM | HPK1 (mouse, 11aa) M→A | 12.9 | 2.4 |
| P20     | VDIPTPAEDTPPLPPKFKFRSPSDE | HPK1 (human, 26aa, aa384-409) | 66.9 | 10.2 |

**Table B**

| Peptide | Sequence | Affinity |
|---------|----------|----------|
| P5      | GQPPLLVPFPKEMKRMGK | HPK1 (mouse, 16aa) nbd |
| P8      | PPLLVPFPKEM | HPK1 (mouse, 11aa) tlq |
| P21     | PPPPLLPPRRR | HAGBP (11aa) 5.4 | 0.4 |

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the HPK1 peptide (P5), the first 3 residues do not contribute to binding, although Pro-3* engages in a very weak hydrophobic interaction with Tyr-8. Pro-4* appears to be the first residue vital for peptide docking (Fig. 3C). The pyrrolidine ring of this residue rests in a hydrophobic groove formed by Tyr-52 and Tyr-8 stacking against the phenyl ring of the former and positioned at a 90° angle to the latter. This interaction is further reinforced by a hydrogen bond from the backbone carboxyl oxygen of Pro-4* to the hydroxyl of the Tyr-52 side chain. Leu-5* exhibits only a single hydrogen bond docking the peptide main chain to Nδ2 of Asn-51.

The next hydrophobic groove along the path of the HPK1 peptide on the domain surface is formed by residues Tyr-52, Pro-59, Phe-10, and Trp-36. Tight hydrophobic interactions in this region maintain the side chains of Val-6*, and to a lesser degree Pro-7*, firmly locked in place, making their presence indispensable for tight peptide binding. The pyrrolidine ring of Pro-8* stacks against the aliphatic portion of the Glu-11* side chain. Hydrogen bonds from the backbone oxygen of Pro-8* to the nitrogen of the indole ring of Trp-36 and the backbone amine of Glu-11* dock the peptide main chain onto the SH3 surface and onto itself, stabilizing the 3_{10} helix of P5, which emerges past this point.

The charged interactions of amino acids Arg-9* and Lys-12*, which flank the 3_{10} helix, involve a tight network of hydrogen bonds with residues of the Mona/Gads SH3C. Specifically, Nε1 of Arg-9* forms hydrogen bonds to Oε1 and Oδ2 of Glu-17, and Lys-12* Nε1 forms hydrogen bonds to Glu-14 Oε1, Glu-17 Oε2, and Asp-16 Oδ1 in a manner identical to that observed in the binding of the SLP-76 peptide to the same interaction surface (24). In addition, Lys-12* further stacks against the indole ring of Trp-36. Lys-10* does not contribute to SH3 domain binding at all, whereas the aliphatic portion of the Glu-11* side chain stacks between Lys-16 and Pro-8*. All residues of the 3_{10} helix contribute hydrogen bonds toward the formation of the secondary structure element itself. The 4 C-terminal residues of P5 do not participate in protein docking and have no direct interaction with any of the Mona/Gads residues in the vicinity. The observed intra-peptide interactions therein only provide structure to this end of the peptide.

Data obtained from binding experiments with SH3- and HPK1-derived Ala mutant peptides in solution (Table I, ITC data) and the contact points between SH3 and HPK1 peptide identified in the crystal structure (summarized in Fig. 1A, indicated by arrows) agree in clearly delineating the residues PXVPXRRXXX in mouse HPK1 as important for Mona/Gads SH3C binding. This motif differs considerably from the previously identified contact motif PXIDRXKKXPL derived from the SLP-76 protein (24), although the RXXK core motif is present in both mouse proteins. Nevertheless, it is evident from the human HPK1 sequence (Fig. 1A) that the arginine can be substituted with a lysine, leaving only a single lysine residue as a strictly conserved position. It is therefore not possible to effectively predict additional candidate binding partners of Mona/Gads SH3C.
FIG. 3. Divergent stereo electrostatic potential surface representations of the Mona/Gads SH3C with the docked peptide(s) in stick representation. Negative potential is drawn in red, and positive potential is drawn in blue. A, superposition of a HPK1 peptide P5 (green) and an SLP-76 peptide (yellow) on the Mona/Gads SH3C domain. The SLP-76 peptide structure is derived from 1OEB.pdb. B, secondary structure elements in the HPK1 peptide (P5) are highlighted with yellow (3_10 helix) and red (PPII helix). C, atom-specific coloring of the P5 residues.
faces are quite similar, 1048 Å^2 for the bound HPK1 peptide as compared with 1143 Å^2 for the complexed SLP-76 peptide and therefore do not provide a simple explanation for the considerable differences in binding affinity of the SLP-76 versus the HPK1 peptide (0.181 and 2.4 H9262 M, respectively; Table I, P5, and Ref. 24.

Stable conformational characteristics of the HPK1 peptide can be identified by analysis of the comparative mean square atomic displacement values (Å^2), which provide an indication of the positional stability of each atom in the crystal structure (Fig. 4A). Temperature factors (B = (u^2)8π^2/3) of P5 atoms fluctuate between 9.6 and 30.9 Å^2. This variability is introduced by the terminal residues of the peptide, which are not stabilized by binding to any docking regions on the face of the Mona/Gads domain. The core of P5, defined by the bulk of the two secondary structure arrangements present, one 3_10 and one PPII helix, is extremely stable. Residues 4* to 12*, which are directly involved with SH3 domain binding, exhibit average B values for main chain atoms of 12.6 Å^2 with the side chain atoms showing only marginally higher values averaging 13.8 Å^2. Although a direct comparison with the previous Mona/Gads SH3C complex with an SLP-76 peptide is not possible due to variability in crystallographic data quality and crystal lattice environment, we can normalize the average B values for the atoms of the peptides implicated in SH3C binding to a common mean value. Comparative values generated thus suggest that the binding stability of the core elements of the respective peptides is similar. However, whereas most residues of the SLP-76 peptide contribute to SH3C binding, terminal residues of P5, amounting to a third of the HPK1 residues present, do not participate in binding. Moreover, the relative stability of the PPII region of P5 cannot be fully accounted for by an induced fit on binding SH3C since its N-terminal part does not participate in complex formation. The PPII element appears, rather, to be inherently stable irrespective to SH3C binding.

Fig. 4B shows the large degree of overlap but also some
differences in the contacts that the Mona/Gads SH3C makes with the HPK1 and SLP-76 peptides, respectively. Fourteen amino acid residues of the SH3 domain contribute in both cases to peptide binding. However, there are also three contacts to residues within the Mona/Gads SH3C, which are unique to the interaction with the SLP-76 peptide (Leu-45, Gly-46, Thr-38). These are part of two of the characteristic SH3 domain β-strands, which contribute to the β-barrel structure. Binding of the HPK1 peptide involves only one SH3 domain residue, Ser-32 within the n-Src loop, which is not involved in the binding of the SLP-76 peptide.

SH3 domains are highly versatile protein interaction modules. Peptide binding can occur via distinct regions of their domain surfaces (discussed in Ref. 29), but a particular SH3 domain surface area can also accommodate rather distinct peptides. Due to space limitations, only two selected examples of other SH3 domain-peptide interactions are compared here with the Mona/Gads SH3C complexed with the HPK1 peptide. The first is the interaction of the SH3 domain from the endocytosis-regulating adaptor protein STAM2 with a peptide derived from the ubiquitin peptidase UBPY (also known as USP8), which binds with moderate affinity (27 μM; Ref. 30). We and others have previously shown that Mona/Gads SH3C can bind to the UBPY protein and RXXK-containing peptides derived from UBPY in vitro (23, 24), but we have failed to detect a complex of the two proteins within cells. The RXXK-containing UBPY peptide was very recently co-crystallized with the STAM2 SH3 domain. SH3 domain-based superposition of the complexed HPK1 peptide with the shorter UBPY peptide (1UJO.pdb) shows that not only do both peptides contain a 310 helix in the RXXK region but that they also occupy overall similar positions on the respective SH3 domain surfaces (Fig. 5A, SH3 domains)

Fig. 5. Comparison of the Mona/Gads SH3-HPK1 peptide complex with other such structures. A, stereo view superposition of peptides from three different SH3-peptide complexes. SH3 domains are omitted for clarity. The HPK1 is colored (complex with Mona/Gads SH3C) in green, the Sos peptide (bound to Grb2 SH3N; 1GBQ.pdb) is colored in orange, and the UBPY (USP8) peptide (in complex with Stam2 SH3; 1UJ0.pdb) is colored in yellow. Peptide orientation is identical in all three cases with N termini pointing up. B, detail of the interactions between the RXXK motif within the HPK1 peptide and Mona/Gads SH3C. C, the corresponding region from the Sos peptide-Grb2 SH3N complex (1GBQ.pdb). For clarity, peptide residues are outlined by a light gray underlay.
are omitted for clarity). Why UBPY fails to bind to Mona/Gads SH3C in vivo is not self-evident from a comparison of the crystal structures and needs to be investigated through biochemical and biological studies. The third superimposed peptide is the Sos peptide structure as determined in complex with Grb2 SH3N (1GBQ.pdb). As mentioned above, the Sos peptide contains a PXXFXR motif that is crucial for Grb2 SH3N binding. Such a motif is also found in the HPK1 peptide, but its binding to Grb2 SH3N is nevertheless marginal (Table I). Again, comparison of the bound HPK1 peptide to the Sos peptide shows a high degree of similarity with respect to peptide orientation relative to the SH3 domains and also secondary structure in the proline-rich segments. However, the comparison of the structure of Grb2 SH3N in complex with a Sos peptide and the Mona/Gads SH3C-HPK1 peptide structure provides a possible explanation for the low affinity binding of RXXK motifs to the Grb2 SH3N domain. Detailed views of the relevant regions are shown in Fig. 5, B and C. In the Grb2 SH3N structure, both O_E and O_1 of Glu-16 (Glu-17Mona) form hydrogen bonds to O_3 of Thr-12 (Leu-13Mona). Therefore Grb2 Glu-16 can no longer bind to the incoming arginine of the RXXK motif in the same way as its equivalent Mona/Gads Glu-17 can. The resulting displacement of the arginine to further along the binding groove forces it to associate with the next available residue, Asp-15 (Asp-16Mona), thus rendering the latter unavailable for binding to the lysine of the RXXK motif. Therefore the affinity of this interaction is severely impaired. Furthermore, Ala-13 (Glu-14Mona) substitutes for the third hydrogen-bonded partner of the N_7 atom of the lysine within RXXK, although that should not greatly affect binding potential, as shown by the high affinity Mona/Gads SH3C-SLP-76 complex (1OEB.pdb), in which both scenarios are present (24).

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**Mona SH3C in Complex with an HPK1 Motif**

**HPK1 on LAT via two Mona/Gads molecules could have interesting implications for signal integration by molecular cross-talk. However, in-depth biochemical and biological studies are needed to clarify the actual composition of Mona/Gads-mediated signaling complexes in T-cells and other cells.**

Our results further confirm that there is not a set of simple rules that can be applied to genomics of great complexity, such as those of mammals, to reliably predict biologically relevant SH3 domain interactions. Motif scanning approaches are very unlikely to single out functionally important homologies that are based on only 2 amino acid residues such as the crucial basic R/KXXK motif shared between the Mona/Gads SH3C binding sites in the HPK1 and SLP-76 proteins. The challenge is even greater when considering subfamilies of SH3 domains with considerable homology, such as Grb2 SH3C, Mona/Gads SH3C, and Stam2 SH3 (30). Simple extrapolation of results from one species to another may also be limited at times, given existing sequence differences, for example, between the relevant regions in mouse and human HPK1 (Fig. 1A).
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