Degeneracy and function of the ubiquitous RVXF-motif that mediates binding to protein phosphatase-1

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Summary

Most interactors of protein phosphatase-1 (PP1) contain a variant of a so-called ‘RVXF’-sequence that binds to a hydrophobic groove of the catalytic subunit. A combination of sequence alignments and site-directed mutagenesis has enabled us to further define the consensus sequence for this degenerate motif as [RK]-X_{0-1}-[VI]-{P}-[FW], where X denotes any residue and {P} any residue except Pro. Naturally occurring RVXF-sequences differ in their affinity for PP1 and we show by swapping experiments that this binding affinity is an important determinant of the inhibitory potency of the regulators NIPP1 and Inhibitor-1. Also, inhibition by NIPP1-(143-224) was retained when the RVXF-motif (plus the preceding Ser) was swapped for either of two unrelated PP1-binding sequences from human Inhibitor-2, i.e. KGILK or RKLHY. Conversely, the KGILK-motif of Inhibitor-2 could be functionally replaced by the RVXF-motif of NIPP1. Our data provide additional evidence for the view that the RVXF and KGILK-motifs function as anchors for PP1 and thereby promote the interaction of secondary binding sites that determine the activity and substrate specificity of the enzyme.

INTRODUCTION

The ubiquitous protein serine/threonine phosphatases of type-1 (PP1) and type-2A (PP2A) interact with dozens of different polypeptides that function as substrates, inhibitors, chaperones, anchoring/scaffolding proteins or substrate-specifiers, and are often multifunctional (1-3). For example, the glycogen-associated G-subunits not only target PP1 to the glycogen particles but also increase the specific activity of PP1 towards the substrate glycogen synthase. Similarly, protein kinase Nek2 is a substrate for associated PP1 and targets the centrosomal protein C-Nap1 for dephosphorylation by PP1. In addition, Nek2 mediates cell-cycle dependent control of centrosomal PP1. The promiscuity of PP1 and PP2A in their interaction with other polypeptides accounts for the presence of these enzymes in a large variety of different
holoenzymes. The sharing of catalytic subunits between holoenzymes also explains why higher eukaryotes can manage with 15 times fewer protein serine/threonine phosphatases than protein serine/threonine kinases (4).

Mammalian genomes contain three genes that encode isoforms of PP1 (1, 3). These isoforms (35-38 kDa) are about 90% identical and the differences are mainly concentrated in the extremities. Although some PP1 interactors, such as the Neurabins (5, 6), interact with PP1 in an isoform-specific manner, most interactors do not discriminate between PP1 isoforms, implying that the major interactor binding sites reside in the catalytic core, the central three-quarters of the protein. The surface of the catalytic core is too small to harbor specific binding sites for each of the 65 known mammalian interactors. The available evidence rather suggests that PP1 interactors compete for a limited number of common or overlapping binding sites [discussed in (1)]. The binding to these sites is mainly mediated by short (4-6 residues), degenerate motifs, and this accounts for the lack of structural similarity between PP1 interactors. Combined with observations that most PP1 interactors have multiple PP1 binding sites, this inspired us to suggest that PP1 is subject to a combinatorial control (1). According to this model, the activity and substrate specificity of PP1 is (partially) determined by the interactors that occupy different combinations of the available binding sites. Even with a limited number of interactor binding sites, the interactors could thus ‘combine’ with PP1 in many different ways. It is not yet understood exactly how protein interactors of PP1 affect the activity and substrate specificity of the enzyme, but it can be envisaged that the binding of the interactors induces conformational changes or has steric consequences that affect the accessibility of the catalytic site. An additional control by the interactors is usually conferred by targeting domains and binding sites for specific substrates.

Only a few interactor binding sites of PP1 have been mapped in detail. One of these is the catalytic site which binds some of the interactors (Inhibitor-1, PHI and Mypt1) in their phosphorylated form as pseudosubstrate inhibitors (7-9). Another interactor binding site is represented by the α4/α5/α6 triangle of
PP1, which mediates binding to Sds22 (10). The β12-β13 loop of PP1, which overhangs the catalytic site, is important for inhibition by both toxins and protein inhibitors (11). By far the best-characterized interactor binding site of PP1 is the hydrophobic ‘RVXF’ binding groove, which is remote from the catalytic site and is centered by the edge of one of the two β-sheets (12). Most interactors of PP1 contain an RVXF-variant that binds to this hydrophobic channel (1, 3). Surprisingly, binding of the RVXF-motif in itself has little effect on the conformation and activity of PP1 (1, 12), and yet, considerable evidence suggests that the RVXF-motif is required for interactor-specific effects on PP1. Thus, synthetic RVXF-containing peptides competitively disrupt the PP1-interactor binding or affect the enzymatic properties of various PP1 holoenzymes (12-15). Likewise, mutation of the RVXF-motif is often sufficient to prevent the high-affinity binding of an interactor to PP1. One interpretation for these findings is that the binding of the RVXF-motif to PP1 increases the local concentration of the interactor and thereby promotes the binding of secondary binding sites to PP1, which determine the activity and/or substrate specificity of the enzyme (1). In one report on the binding of the myosin targeting protein Mypt1 to PP1 as studied by Surface Plasmon Resonance Spectroscopy, it was concluded that the interaction of the RVXF-motif is a prerequisite for the (cooperative) binding of other motifs (16).

One aim of the present study was to further define the consensus RVXF-sequence, using both alignments of established RVXF-sequences and site-directed mutagenesis. This analysis revealed that the RVXF-motif is rather degenerate and that ‘X’ can be any residue except Pro. The second aim was to explore the function of the RVXF-motif by swapping studies using variant RVXF-motifs and unrelated PP1 interaction motifs. Our data suggest that the RVXF-motif has only a ‘proximity’ effect and thereby promotes the binding of secondary interaction sites to PP1.

EXPERIMENTAL PROCEDURES
Materials - The catalytic subunit of PP1 (17) and glycogen phosphorylase b (18) were purified from rabbit skeletal muscle. Phosphorylase b was phosphorylated in the presence of [γ-32P]ATP by purified phosphorylase kinase (15). Glutathione Sepharose and Blue Sepharose were obtained from Amersham Biosciences. Ni2+-pentadentatechelator-Sepharose was purchased from Affiland. Okadaic acid was obtained from Calbiochem. All peptides were synthesised by the N-(9-fluorenyl)methoxycarbonyl method on a Milligen 9050.

Preparation of recombinant proteins - Polyhistidine-tagged NIPP1-(143-224) was purified as described by Beullens et al. (15). Recombinant human Inhibitor-1 was expressed as a fusion protein with glutathione-S-transferase (GST) in BL21(DE3)pLys cells transformed with pGEX-2T-Inhibitor-1 plasmid, provided by Dr. S. Shenolikar (Duke University). The fusion protein was purified on glutathione-Sepharose as described (7). The purified protein was cleaved with thrombin and free Inhibitor-1 was purified as a heat-resistant fragment after centrifugation. Inhibitor-1 was phosphorylated with the catalytic subunit of PKA (7). Inhibitor-2 was purified by chromatography on Blue Sepharose of heat-treated lysates of BL21(DE3)pLys cells that had been transformed with the pET8d-Inhibitor-2 plasmid, donated by Dr A. DePaoli-Roach (Indiana University) (19). The pET15b plasmid encoding polyhistidine-tagged rabbit glycogen-synthase kinase-3 (gift of Dr P. Roach, Indiana University) was transformed into BL21(DE3)pLys cells and the expressed fusion protein was purified on Ni2+-Sepharose.

Cell Lysates- COS-1 cells were washed 3 times in phosphate buffered saline, resuspended in lysis buffer containing 20 mM Tris at pH 7.4, 150 mM NaCl, 0.1 % Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 mM benzamidine, 5 µg/ml leupeptin and lysed by three passages through 18-G and 22-G needles. After 10 minutes incubation on ice, the suspension was centrifuged (10 minutes at 10,000 x g) and the supernatant was used as the cell lysate.
Assays- NIPP1-(143-224), Inhibitor-1, Inhibitor-2 and their mutants were assayed as inhibitors of the phosphorylase phosphatase activity of the catalytic subunit of PP1 as described (20). The substrate was added after a preincubation of the reaction mixture for 10 min at 30°C. Phosphorylase phosphatase assays in cell lysates were performed identically but the assays were made specific for PP1 by the addition of 10 nM okadaic acid. The Inhibitor-2-mediated inactivation of PP-1 was monitored by the time-dependent decrease in trypsin-revealed phosphorylase phosphatase activity. For that purpose, the catalytic subunit was incubated at 25°C in the presence of 20 nM Inhibitor-2 in a buffer containing 20 mM imidazole at pH 7.5, 0.5 mM dithiothreitol, 5 mM β-mercaptoethanol, and 1 mg/ml bovine serum albumin. The phosphorylase phosphatase activity was then measured after prior incubation of aliquots with trypsin (0.2 mg/ml) for 5 minutes at 30°C, and the addition of trypsin inhibitor (0.4 mg/ml). After the inactivation by Inhibitor-2, PP1 was reactivated by the addition of glycogen-synthase kinase-3 (GSK-3), MgCl₂ (2 mM) and ATP (0.2 mM) for the indicated times at 30°C, and the phosphorylase phosphatase activity was again determined after a trypsin treatment.

Mutagenesis - pET16b-NIPP1-(143-224) (21), pGEX-2T-Inhibitor-1 and pET8d-Inhibitor-2 served as templates for site-directed mutagenesis using the QuickChange protocol (Stratagene). All mutations were confirmed by DNA sequencing.

RESULTS AND DISCUSSION

Degeneracy of the RVXF-motif – An alignment of established RVXF-sequences from a variety of R-subunits revealed that this motif is rather degenerate and conforms to the consensus sequence [RK]- X₀₋₁-[VI]-X-[FW] (Table 1). Thus, the RVXF-motif actually comprises either 4 or 5 residues. Although the ‘X’ at position 4 clearly represents various residues (Table 1), it cannot be deduced from the available data whether this ‘X’ can be any residue.
Moreover, it cannot be excluded that the nature of this residue is a determinant of the binding affinity of the RVXF-motif for PP1. To gain a better insight into the residues that are tolerated as ‘X’ in the RVXF-motif, we have replaced the corresponding residue (Thr202) in the central domain (residues 143-224) of the nuclear PP1 regulator NIPP1 by any of the other 19 amino acids that occur in proteins. Since the RVXF-motif is essential for inhibition of the phosphorylase phosphatase activity of PP1 by the central domain of NIPP1 (21; see also below), we have taken the inhibitory potency as a measure of the functionality of the mutant RVXF-motifs (Fig. 1). The concentration of NIPP1-(143-224) that caused half-maximal inhibition of PP1 (IC₅₀) was only drastically affected by the T202P mutation, which increased the IC₅₀ value to 76 nM as compared to 0.4 nM for the wild type protein. Thus the consensus sequence for the RVXF-motif can be more precisely defined as [RK]-X₀₋₁-[VI]-{P }-[FW]. That Pro is not tolerated at position 4 is understandable since the RVXF-motif adopts a β-strand conformation when bound to PP1 (12) and proline is well-known to prevent β-strand formation. In the muscle-type glycogen targeting subunit G₅/R₇ the ‘X’ in the RVXF-sequence is a serine and phosphorylation of this residue has been shown to prevent the interaction of this sequence with PP1 (12, 30, 31). We found that the T202E mutation did not have any effect on the IC₅₀ value of NIPP1-(143-224), while the T202D mutation resulted in a 5-fold higher IC₅₀ (Fig. 1). These data suggest that the effects of phosphorylation of the RVXF-motif can be partially mimicked by an Asp at position ‘X’. Surprisingly, the effects of phosphorylation were not mimicked by a Glu at this position suggesting than the additional hydrophobic CH₂-group in the side chain compensates for the lower affinity induced by the acidic carboxyl group. A Phe at position ‘X’ was also associated with a somewhat higher IC₅₀ (Fig. 1).

Our data seem at variance with a study by Liu et al. (32) who reported that the substitution of either Ala or Val for Ser in the RVSF-sequence of the G₅/R₇ subunit abolished the binding of PP1. The presence of an Ala or Val at this position in an otherwise identical RVXF-sequence clearly did not affect the inhibitory potency of NIPP1-(143-224) (Fig. 1). Moreover, it should be pointed
out that in some regulators of PP1, for example the murine glycogen-targeting subunit R5/PTG (3) and the apoptotic regulator Bcl2 (Table 1), the penultimate position of the RVXF-motif is an Val or Ala. Since the inhibitory potency of NIPP1-(143-224) is totally dependent on a functional RVXF-motif (Figs. 2A and 4), the potent inhibition by the T202A or T202V mutants cannot be accounted for by secondary PP1 interaction sites. It is possible, however, that the corresponding mutations in the RVXF-motif of the G_{M/R_{GI}} subunit turn this sequence into a binding site for a remote regulatory element and thereby make the RVXF-motif inaccessible for binding to PP1.

Swapping of RVXF-sequences between Inhibitor-1 and NIPP1 – The RVXF-motif is essential for inhibition of PP1 by Inhibitor-1 and NIPP1, but Inhibitor-1 is a less potent inhibitor than is NIPP1 (7, 15). To study whether this difference in potency is related to their RVXF-sequence, we have replaced the RVTF-sequence in the central domain of NIPP1 with the RVXF-sequence of human Inhibitor-1 (KIQF). In Fig. 2A it is shown that this mutation turned NIPP1-(143-224) into a 3.5-fold less potent inhibitor, suggesting that the KIQF-sequence indeed represents an RVXF-motif that binds to PP1 with a relatively low affinity. In accordance with previous data (21), NIPP1-(143-224) was no longer inhibitory when the RVXF-motif was destroyed by substitution of Ala for Val and Phe. The data in Fig. 2A were corroborated by competition studies with synthetic RVXF-containing decapeptides (Fig. 2B). Indeed, the addition of 50 µM of NIPP1-(197-206), which comprises the RVTF-sequence and 3 flanking residues at each side, increased the IC_{50} of NIPP1-(143-224) about 16-fold. On the other hand, NIPP1-(197-206) with the RVXF-motif from Inhibitor-1 (KIQF) was a very poor competitor and the V201A/F203A mutant was not a competitor at all.

Since the KIQF-sequence emerged as a rather poor RVXF-motif, we wondered whether the inhibitory potency of Inhibitor-1 could be increased by replacement of the KIQF-sequence by the NIPP1-derived RVTF-sequence. In Fig. 3 it is shown that this mutation indeed turned phospho-Inhibitor-1 into a more potent inhibitor of the PP1 catalytic subunit as well as the PP1
holoenzymes that are present in cell lysates. In accordance with previous reports (33, 34), the inhibition of the holoenzymes required a higher concentration of Inhibitor-1. Non-phosphorylated Inhibitor-1 with the RVXF-sequence from NIPP1 was not inhibitory at all (not shown). This demonstrated that the inhibition of PP1 by the mutated Inhibitor-1 was still entirely phosphorylation-dependent and that the increased inhibitory potency could not be accounted for by the creation of an additional inhibitory binding site. Thus, it seems likely that the mutated Inhibitor-1 inhibited PP1 by the same mechanism as did the wild-type inhibitor, i.e. by the binding of phospho-Thr34 as a pseudosubstrate to PP1 (7). The increased inhibitory potency of the mutated Inhibitor-1 can then be explained by the higher binding affinity of the NIPP1-derived RVXF-motif for PP1, which is expected to increase the rate of association and/or decrease the rate of dissociation of phospho-Thr34.

The RVXF-motif can be functionally replaced by other PP1-binding motifs – The above data are in accordance with the view that RVXF-motifs serve to anchor R-subunits to PP1 and to promote thereby the interaction of secondary binding sites with PP1 (see Introduction). A corollary of this view is that the R-subunits could still be functional if their RVXF-motif were replaced by another PP1 anchoring motif. In order to test this experimentally, we have made use of two established PP1 binding sequences of Inhibitor-2, which were originally described as the ‘IKGI’ (35, 36) and ‘KLHY’-sequences (37). Alignments showed that part of these sequences belong to phylogenetically conserved pentapeptide motifs that conform to the consensus sequences K-[GS]-I-L-K and R-[KR]-X-H-Y (Table 2). Based on the human sequences and the nature of the conserved residues we will further refer to these sequences as the KGILK and the RKXHY-motifs, respectively.

Following the replacement of the RVTF-sequence plus the preceding Ser by the KGILK and RKLHY-sequences from human Inhibitor-2, NIPP1-(143-224) was still inhibitory, with IC$_{50}$ values of 143 and 7 nM, respectively (Fig. 4). Since the replacement of the RVTF-sequence by the RATA-sequence completely abolished inhibition by NIPP1-(143-224), these data suggested that
the KGILK and RKLHY-sequences could at least in part functionally replace the RVXF-motif. There are at least two obvious and not mutually exclusive explanations for why the NIPP1-(143-224) mutants with the KGILK or RKLHY-sequences were less potent inhibitors than the wild-type protein. Firstly, it is possible that the KGILK and RKLHY-sequences bound to PP1 with less affinity than did the RVTF-sequence. This view is supported by observations that the addition of 1 mM of the competitor NIPP1-(197-206) increased the IC$_{50}$ for NIPP1-(143-224) some 200-fold, while the addition of the same concentration of the corresponding decapeptides of NIPP1-(143-224) with the RKLHY or KGILK-sequences only increased the IC$_{50}$ of the mutated NIPP1-(143-224) by 5 and 3-fold, respectively (not shown). Secondly, it is conceivable that the binding of the KGILK or RKLHY-sequences to a site of PP1 that differs from the RVXF-binding groove, hampers the optimal interaction of the secondary binding sites of NIPP1-(143-224) with PP1.

Swapping of the RKXHY and KGILK-motifs of Inhibitor-2 for an RVXF-motif – Our observation that the function of the RVXF-motif can be (partially) taken over by the KGILK or RKXHY-motifs (Fig. 4) can only be used as new evidence of the anchoring role of the RVXF-motif if these motifs do not all interact identically with PP1. While convincing evidence has been presented showing that the KGILK-motif binds to a site that differs from the RVXF-binding groove (36), some modeling data and experiments with synthetic peptides suggested that the ‘KLHY’-sequence of human Inhibitor-2 also interacts with the RVXF-binding channel of PP1 (37). However, three independent lines of evidence argue against the latter view. Firstly, while the ‘KLHY’-sequence of human Inhibitor-2 still resembles to some extent an RVXF-motif, the consensus sequences for both motifs are entirely different (Tables 1 and 2). More specifically, the Leu in position two, which was considered to be equivalent to the Val in the RVXF-motif (37), is not conserved. Actually, in some species an Ala is present at this position (Table 2) and this is known to be incompatible with a functional RVXF-motif (12). Secondly, RVXF-containing peptides were shown to antagonize inhibition by...
Inhibitor-2 (15, 37), while the deletion or mutation of the RKXHY-motif did not have major effects on the inhibitory potency of Inhibitor-2 (35, 37). This strongly indicates that the competition by these peptides cannot be accounted for by the displacement of the RKXHY-motif and thus that the RVXF and RKXHY-motifs have different binding sites on PP1. Thirdly, we found that the KLHY-sequence of human Inhibitor-2 could not be functionally replaced by the NIPP1-derived RVTF-sequence. Indeed, while the wild type and mutated Inhibitor-2 were equally potent inhibitors of the catalytic subunit of PP1 (Fig. 5A), the mutated Inhibitor-2 was a much poorer inhibitor of PP1 holoenzymes in cell lysates (Fig. 5B). However, the lesser inhibition of PP1 holoenzymes by mutated Inhibitor-2 was not affected by the simultaneous addition of 50 µM of a synthetic peptide that comprises residues 135-158 of Inhibitor-2 and that includes the KLHY-sequence (not shown). One way to account for these data is that the KLHY-sequence plays a role in the disruption of PP1 holoenzymes that accompanies their inhibition by Inhibitor-2 (38), a function that can apparently not be mimicked by the RVTF-sequence. We also found that the characteristic time-dependent and trypsin-resistant inactivation of PP1 by Inhibitor-2 (39) was faster with the mutated inhibitor (Fig. 6A) and that the MgATP-dependent reactivation of the inactive complex by glycogen-synthase kinase-3 occurred more slowly and to a lower extent with Inhibitor-2-(RVTF). (Fig. 6B). In conclusion, the KLHY → RVTF mutant of Inhibitor-2 was a poorer inhibitor of PP1 holoenzymes but promoted the conversion of the catalytic subunit into an inactive conformation.

While the RKXHY-motif of Inhibitor-2 does not appear to be essential for inhibition of the catalytic subunit of PP1 (Fig. 5A; 37), the inhibitory potency of Inhibitor-2 was severely decreased by mutation of the KGILK-motif into EGGLK (Fig. 7), in accordance with data from Huang et al. (35). However, when the last four residues of KGILK-motif were replaced by RVTF, the IC_{50} for inhibition of PP1 only increased from 1 nM to 2 nM (Fig. 7). Moreover, the replacement did not significantly affect the inhibition of PP1 holoenzymes (not shown). Thus, the KGILK-motif can be functionally replaced
by an RVXF-motif, indicating that the KGILK-motif of Inhibitor-2 has a similar function as the RVXF-motif in most other PP1 interactors, although it does not bind to the RVXF-binding groove (36).

CONCLUDING REMARKS

The consensus RVXF-motif that we have delineated here as [RK]-X$_{0-1}$-[VI]-{P}-[FW] occurs in about a third of all eukaryotic proteins, and only a small fraction of these are PP1 interactors. Therefore, the presence of an RVXF-consensus sequence in itself is not sufficient to classify a protein as a putative PP1 interactor. Additional information on the functionality of an RVXF-consensus sequence could come from the use of competing RVXF-containing peptides or from RVXF-mutants. It is currently not entirely clear why the majority of the RVXF-consensus sequences do not mediate binding to PP1. However, crystal structure studies of p53BP2, which represents the C-terminal part of ASPP2, have revealed that the RVXF-motif in the unbound interactor is not very structured (40). On the other hand, a peptide comprising the RVXF-motif of the muscle-type G-subunit was shown to bind to the RVXF-binding groove as a $\beta$-strand (12). Thus, RVXF-consensus sequences may only function as PP1 interaction sites when they are present in a flexible and exposed loop that can be modelled into a $\beta$-strand.

Although our alignment and swapping studies strongly suggest that the RVXF-motif contains 4 or 5 residues, this does of course not rule out the possibility that flanking residues can affect the affinity of this motif for PP1. By the panning of a random peptide display library, Zhao and Lee (41) identified a number of peptides that bind to PP1. The consensus PP1-binding peptide sequence that they delineated, i.e. [RK]-[RK]-X$_{0-2}$-V-[RH]-[FW]-X-[DE], is surprisingly similar to the consensus RVXF-motif that we have proposed. The main difference is that the consensus sequence proposed by Zhao and Lee contains an additional N-terminal basic residue and a C-terminal acidic residue. Interestingly, the established RVXF-motifs are also often
preceded by one or more basic residues and followed by one or more C-terminal acidic residues (Table 1), which is in accordance with the view that these flanking residues affect the binding affinity of the RVXF-motif.

Our swapping experiments suggest that both the ubiquitous RVXF-motif and the Inhibitor-2-specific KGILK-motif have no other function than to mediate binding to PP1. By increasing the local concentration of the interactor this initial binding promotes the interaction with PP1 of secondary, lower-affinity binding sites, which have interactor-specific effects on the activity and substrate specificity of PP1. In agreement with this view, PP1 interactors with a mutated RVXF-motif still have the same effects on PP1, but these effects are only seen at much higher concentrations. For example, both NIPP1 and Inhibitor-1 are still inhibitory after mutation of their RVXF-motif, and their inhibitory potency is still controlled by phosphorylation, but the inhibitory concentrations are much higher. Our view of RVXF-motifs as ‘anchors’ for the binding to PP1 is consistent with the function deduced for the RVXF-motif based on Surface Plasmon Resonance Spectroscopy (16) and peptide competition studies (12-15). While the KGILK-sequence may act as an anchor for the binding of Inhibitor-2 to PP1, the RKXHY-motif does not appear to have this function since its mutation or deletion had no major effects on the inhibition of the catalytic subunit of PP1 by Inhibitor-2 (35,37) and since the RKXHY-motif could not be functionally replaced by the RVXF-motif (Figs. 5B and 6).

We have used here a combination of bioinformatics tools and mutagenesis studies to delineate the consensus sequence and function of three PP1-binding motifs. The same approach could also be used to explore other PP1-binding motifs, such as the recently described F-X-X-R-X-R motif, which also appears to be shared by various PP1 interactors (42).

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Table 1

*Degeneracy of RVXF-motifs*

The table only shows the sequence of established RVXF-motifs and their flanking residues, as first shown in the cited references. Only human sequences are shown, except for Reg1, which is a yeast protein. The residues of the RVXF-motif are in bold. N-terminal basic residues and C-terminal acidic residues are underlined.

| Protein     | Residues | Sequence          | Access. nr | Reference |
|-------------|----------|-------------------|------------|-----------|
| AKAP149     | 151-160  | L S S P K G V L F S S K | CAA66000   | 22        |
| AKAP220     | 1192-1201| E H S G K K V Q F A E A | AAF07045   | 23        |
| Bcl2        | 144-153  | V N W G R I V A F F E F | AAA35591   | 24        |
| GADD34      | 552-561  | P L K A R K V R F S E K | AAC25631   | 25        |
| G12/RG1     | 60-69    | S S G T R R V S F A D S | AAB94596   | 12        |
| Inhibitor-1 | 6-15     | D N S P R K I Q F T V P | AAB02402   | 14        |
| Neurabin I  | 97-106   | I P A N R K I K F S S A | BAA86536   | 26        |
| NIPP1       | 197-206  | K R K N S R V T F S E D | AAD31541   | 15        |
| Mypt1       | 32-41    | K R Q K T K V K F D D G | BAA22378   | 12        |
| ASPP2 (p53BP2) | 916-927 | I A H G M R V K F N P L | CAC83012   | 12        |
| PNUTS/p99   | 395-404  | G R K R K S V T W P E E | CAA73697   | 27        |
| Reg1        | 462-471  | P T K N R H I H F N D R | CAA87807   | 28        |
| Spinophilin | 443-452  | P A P S R K I H F S T A | CAC37685   | 29        |

**Consensus**

\[ [RK][X_{0-1}][VI]-[X][FW] \]
The KGILK motif is not present in *Arabidopsis* and the first residue of this motif is not conserved in *Saccharomyces cerevisiae*.

| Species                          | Residues | Sequence | Residues | Sequence | Accession number |
|----------------------------------|----------|----------|----------|----------|-----------------|
| Homo sapiens                     | 10-19    | P I K G I L K N K T | 142-151  | M K R K L H Y N E G | AAC51206       |
| Arabidopsis thaliana             | -        | -        | 120-129  | A H R K A H Y D E F | BAA97463       |
| Takifugu rubripes                | 6-15     | P I K G I L K N K N | 147-156  | M M R K M H Y N E G | JGI-9884       |
| Drosophila melanogaster          | 10-19    | P C K G I L K T S R | 122-131  | R R R K A H Y R E F | AAF50198       |
| Caenorhabditis elegans           | 32-51    | P K K S I L K M K Q | 155-164  | K K R R A H Y N E G | AAF60485       |
| Schizosaccharomyces pombe        | 2-11     | K V K S I L K H S R | 264-273  | E L R K K H Y F A M | CAB11509       |
| Saccharomyces cerevisiae         | 1-9      | - M G I L K N P L | 194-203  | E M R K K H Y D V R | AAA53673       |
| **Consensus**                    |          | **K-[GS]-I-L-K** |          | **R-[KR]-X-H-Y** |                 |
LEGENDS TO THE FIGURES

FIG. 1. The penultimate position of the RVXF motif of NIPP1-(143-224) can be held by any residue except Pro. The figure shows the concentration of human NIPP1-(143-224) and its Thr202 mutants that caused 50% inhibition of 0.5 nM purified PP1 (IC50), using glycogen phosphorylase as substrate. The results represent the means ± S.E. of 4 assays.

FIG. 2. Swapping of naturally occurring RVXF-sequences reveals differences in affinity for PP1. Panel A shows how swapping of the RVTF-sequence of human NIPP1-(143-224) for an inactive RVXF-sequence (RATA) or for the RVXF-motif of Inhibitor-1 (KIQF) affects the inhibitory potency of this NIPP1 fragment. B shows the effect of the addition of 50 µM of the synthetic decapeptide NIPP1-(197-206) and the same peptide in which the RVTF-sequence was replaced by RATA or KIQF on the inhibition of 0.5 nM PP1 by NIPP1-(143-224). The NIPP1-(197-206) peptides themselves were slightly (20-40 %) stimulatory to the phosphorylase phosphatase activity of PP1 (not shown). The results represent means ± S.E. of 4 assays.

FIG. 3. The inhibitory potency of Inhibitor-1 is increased by swapping of its KIQF-sequence for RVTF. Wild-type human Inhibitor-1 and Inhibitor-1 with the KIQF12-sequence replaced by RVTF were compared as inhibitors of the catalytic subunit (0.1 nM) of PP1 (A) and of the PP1 holoenzymes that are present in a COS-1 cell lysate (B). The phosphorylase phosphatase assay in the cell lysates was made specific for PP1 by the addition of 10 nM okadaic acid to block PP2A. The results are means ± SE of 4 assays.

FIG. 4. The RVXF-motif of NIPP1 can be functionally replaced by PP1 binding motifs from Inhibitor-2. The figure shows the effect on the inhibitory potency of replacement of the SRVT203 sequence of NIPP1-(143-224) by
SRATA, RKLHY or KGILK. The results are expressed as means ± S.E. of 4 assays.

FIG. 5. Effects of swapping of the RKXHY-motif for an RVXF-motif on the inhibitory potency of Inhibitor-2. Rabbit wild-type Inhibitor-2 and a mutant version in which \(^{145}\)KLHY\(^{148}\) was replaced by RVTF, were assayed as inhibitors of the catalytic subunit of PP1 (A) and of PP1 holoenzymes in COS-1 cell lysates (B). The phosphorylase phosphatase assay in the cell lysates was made specific for PP1 by the addition of 10 nM okadaic acid to block PP2A. The results represent means ± SE of 4 assays.

FIG. 6. Swapping of the RKXHY-motif of Inhibitor-2 for an RVXF-motif interferes with the inactivation and re-activation of PP1. Rabbit wild-type Inhibitor-2 (20 nM) or a mutant version in which \(^{145}\)KLHY\(^{148}\) was replaced by RVTF, were incubated with 1 nM PP1. At the indicated times samples were taken for the assay of trypsin-resistant phosphorylase phosphatase activity (A). Panel B shows the effect on the inactivated PP1 of the addition of MgATP in the absence (open symbols) or presence (filled symbols) of GSK-3. The results are means ± SE of 4 assays.

FIG. 7. The KGILK-motif of Inhibitor-2 can be functionally replaced by an RVXF-motif. The figure shows the effect of the substitution of EGGLK or KRVT for the \(^{12}\)KGILK\(^{16}\) sequence of rabbit Inhibitor-2 on its ability to inhibit the catalytic subunit of PP1. The results are expressed as means ± S.E. of 3 assays.
Fig. 1
A. Swapping of RVXF-sequences

B. Competition with peptides
A. Catalytic subunit

B. PP1 holoenzymes
Fig. 4

Phosphorylase phosphatase activity (% of control)

NIPP1-(143-224)(nM)
A. Catalytic subunit

![Graph showing Phosphorylase phosphatase activity (% of control) vs. Inhibitor-2 (nM) for Catalytic subunit.]

B. PP1 holoenzymes

![Graph showing Phosphorylase phosphatase activity (% of control) vs. Inhibitor-2 (nM) for PP1 holoenzymes.]

Fig. 5
A. Inactivation of PP1

![Graph showing inactivation of PP1 at 25°C.]

B. Reactivation of PP1

![Graph showing reactivation of PP1 at 30°C.]

FIG. 6
Fig. 7

Phosphorylase phosphatase activity (% of control) vs. Inhibitor-2 (nM)

Legend:
- IEGG
- RVTF
- GILK

Data points are shown with error bars.
Degeneracy and function of the ubiquitous RVXF-motif that mediates binding to protein phosphatase-1

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