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Eyes Absent Proteins: Characterization of Substrate Specificity and Phosphatase Activity of Mutants Associated with Branchial, Otic and Renal Anomalies

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*The eyes absent (*Eya*) genes encode a family of proteins that combine the functions of transcriptional cofactors, signal transducers and enzymes, namely protein tyrosine phosphatases. The latter activity resides in the highly conserved C-terminal Eya domain (ED). Here, we investigated the substrate specificity of the* Arabidopsis thaliana *homologue (AtEya) by using low-molecular-weight compounds and synthetic phosphotyrosine (pY)-containing peptides that correspond either to phosphorylation sites in proteins or to peptides that were selected through the screening of a combinatorial peptide library. AtEya displayed modest peptide substrate specificity and was sensitive to charges adjacent to pY. In general, the presence of acidic residues on the N-terminal side of the phosphorylation site was critical for catalysis, whereas basic amino acids seemed to be preferred with respect to high-affinity binding. We also detected significant acyl phosphatase activity of AtEya; this suggests that Eya proteins might have further substrates in vivo. In addition, we analysed the phosphatase activity of a number of variants of the mouse Eya1 protein that harbours single point mutations that were associated with branchio–oto–renal syndrome (BOR), branchio–oto syndrome (BO) and ocular defects, respectively, in humans. While BOR mutations led to a significantly reduced phosphatase activity, BO mutants as well as those that are associated with ocular defects only displayed activity that was similar to wild-type levels.*

Introduction

The reversible tyrosine phosphorylation of proteins represents one of the key events that cells use to regulate signal transduction, gene expression and other cellular processes.[1] Protein tyrosine phosphatases (PTPs), which exclusively remove phosphate from tyrosine residues in proteins are critical components in maintaining the resting level of tyrosine phosphorylation.[2,3] All classical PTPs include the signature motif (H/V)-**C**XAGXG**R**(S/T), with the cysteine residue being crucial for the catalytic activity. This conserved cysteine is required for the nucleophilic attack on the substrate phosphate to produce a thiol intermediate, which is subsequently hydrolysed by water.[4,5] Recently, an additional class of tyrosine-specific phosphatases, the eyes absent (Eya) proteins, has been described.[6,7] Eya proteins employ an aspartate as the nucleophile in a metal-dependent reaction, and thus clearly differ from classical PTPs in their mode of action.[8] Furthermore, based on sequence homology the Eya proteins have been suggested to belong to the haloacid dehalogenase (HAD) family of phosphohydrolases. These enzymes are characterised by several conserved "core residues" within three motifs (I–III) that form the catalytic scaffold of the active site. This HAD-specific catalytic fold is required for the transfer of the phosphoryl group from a specific phosphate ester to the active site Asp, and then to a water molecule.[9,10]

Eya genes are found in invertebrates (one gene in *Drosophila*) and vertebrates (four genes in mammals) as well as in higher plants (one gene in *A. thaliana*).[11,12] In humans, the four *EYA* genes are expressed in distinct but overlapping patterns. Eya1–3 primarily occur in organs such as branchial arches, kidney, eye and central nervous system (CNS), whereas Eya4 is found in the lung and CNS.[13] Eya has been shown to act as a transcriptional cofactor by interacting with Pax6 (homologue of both *Drosophila* twin of eyeless and eyeless), Six (sine oculis) and Dach (dachshund) proteins that cooperatively regulate the formation of the respective tissues and organs.[13,14] Mutations in the human *EYA1* gene were found to be responsible for the branchio–oto–renal (BOR) syndrome that is characterised by branchial arch abnormalities, hearing loss and kidney defects.[15,16,18] In contrast, the branchio–oto (BO) syndrome is ascribed to a similar combination of branchial and otic anomalies, without the association of renal defects,[16] whereas other *EYA1* mutations are associated with congenital cataracts and ocular defects (OD) only.[17]

Human Eya proteins consist of an N-terminal region of 200–300 amino acids and a conserved C-terminal domain (Eya domain, ED) of 271–274 residues, which exhibits the phosphatase activity. Both animal and plant Eya proteins share a high sequence similarity in their ED domain. In contrast, the N-terminal region of the protein from *A. thaliana* (AtEya) is shorter and comprises only 15–23 residues compared to the animal

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proteins.[6–8] Although the main focus of most previous studies on Eya proteins has been on their activity as a transcriptional coactivator, it is generally acknowledged that the phosphatase activity is likely to be equally important. In this respect, it is interesting to note that most missense mutations in *EYA1* lie in the conserved Eya domain (ED), which mediates protein–protein interactions as well as harbours the phosphatase activity.[15,16,18] It is therefore compelling to understand how modulation of Eya1 phosphatase activity contributes to the BOR syndrome phenotypes. Moreover, until now tyrosine phosphorylation was largely neglected in plants due to a lack of evidence for a typical tyrosine phosphatase. Interestingly, recent studies demonstrated that tyrosine phosphorylation and dephosphorylation might serve important functions in plant biology, for instance in the regulation of stomatal movement.[19] It is far from clear, however, how the phosphatase activity of Eya proteins is regulated and what physiological substrates they act on. To understand the specific functions of these proteins and their roles in cellular processes more knowledge about their substrate specificity and mode of action is required.

Different approaches to define the substrate specificity and subsequently to identify potential substrates of tyrosine-specific phosphatases have been described thus far, for example, the use of synthetic peptides that correspond to known phosphorylation sites in proteins or combinatorial peptide library approaches.[20–23] The challenge of combinatorial approaches generally has been to find a reliable method to distinguish the dephosphorylated reaction product (Y-peptide) from a complex mixture of phosphotyrosine (pY) substrates.[23] Methods such as ECLIPSE (*e*nzyme-*c*atalysed *l*oss of *i*sotope *p*eak *s*ignal *e*nhancement) were developed and successfully applied to elucidate the optimal substrates of classical PTPs such as SHP-1. This approach, however, requires both a sophisticated mass spectrometry technique and mass spectra analysis.[23] In other attempts, peptide libraries that contain nonhydrolysable pY analogues have been screened against PTP1B to select substrates on the basis of high-affinity binding.[21,22] All together, these studies revealed that classical PTPs such as SHP-1 and PTP1B typically recognise specific residues N-terminal to pY. In general, contacts of the protein active site with 3–5 residues on either side of pY are considered to be important for substrate recognition.[20–23]

Previous investigations regarding the phosphatase activity of Eya proteins focused on the effects of buffer composition, pH and metal ions on catalytic activity and on a first assessment of the phosphate-group-carrying target. It is clear from these recent compilations that peptides that contain pY indeed seem to be potential substrates of eyes absent proteins,[6–8] however, to what extent this activity is influenced by the amino acid sequence surrounding pY and the size of the peptides has not been described yet. In this report, we investigated the substrate specificity of Eya proteins by using different methods. We used the plant homologue AtEya to investigate the structural requirements of ED substrates. In a first attempt, a combinatorial peptide library that contains phosphonomethylphenylalanine (Pmp) as pY mimetic was prepared and screened for binding to the protein. Because high-affinity peptides might or might not be good substrates of AtEya, we carried out a kinetic analysis of the peptide dephosphorylations. This analysis was extended to further peptides derived from EDAEpYAARG (RR-Src), which was previously suggested to be a good model substrate for AtEya.[8] Thereby, the Src peptide served as a template for truncation and amino acid replacement studies. The contribution of individual residues on both sides of pY to binding and catalysis was assessed by a spectrophotometric assay. Our results indicate that the conversion of pY peptides containing acidic amino acids N-terminal to pY is preferred, but basic residues within the peptide sequences had a positive effect on binding. Further, the catalytic activity of AtEya toward phosphopeptide substrates was relatively low compared to classical phosphatases, hence dephosphorylation of low-molecular-weight and protein substrates has been evaluated, too. Thereby new substrates of Eya proteins have been detected such as acetyl phosphate, benzoyl phosphate and carbamoyl phosphate as well as β-casein.

In addition, to elucidate whether loss of Eya1 enzymatic activity is the reason for the various syndromes that are caused by *EYA1* mutations, we investigated the phosphatase activity of various disease-associated Eya1 mutants. Based on the high sequence identity between the human and the mouse Eya1 (mEya1) proteins we generated mEya1 constructs that harbour most of the C-terminally located, BOR-, BO- and OD-associated missense mutations that have been reported so far. We demonstrate that BOR-associated mutations in *EYA1* strongly reduced phosphatase activity, whereas mutations that are associated with BO or ocular defects did not show significantly changed phosphatase activity compared to the wild-type enzyme.

Results

AtEya phosphatase activity in comparison to SHP-1 PTP domain

Several reports indicated that the Eya domain is able to dephosphorylate pY peptidic and low-molecular-weight substrates (*p*NPP) like classical PTPs, for example, PTP1B and SHP-1.[6–8] In an experiment with *p*NPP as a substrate it was shown that AtEya was 10% as active as PTP1B and 1.3-times as active as full-length SHP-1,[6] but the catalytic activity of full-length SHP-1 is rather low in the native state because of the autoinhibitory function of its N-terminal SH2 domain.[23,32] We therefore used the phosphatase domain (PTP domain, residues 271–514) of SHP-1 to assess which concentration of AtEya is appropriate for the library screening and the phosphatase assays.

To test this, we monitored phosphopeptide dephosphorylations by HPLC. The Src-derived peptide EDAEpYAARG and the $EGFR_{988–998}$ sequence DADEpYLIPQ were previously suggested to be good model substrates of AtEya,[8] and were thus used for the HPLC analysis first. The GST fusion protein of the SHP-1 catalytic domain (GST–SHP-1(PTP)) was applied in a concentration (5 ng/mL) that has been suitable in our previous studies on SHP-1 substrate specificity;[33] however, at the same concentration no dephosphorylation occurred for AtEya. With a ten-

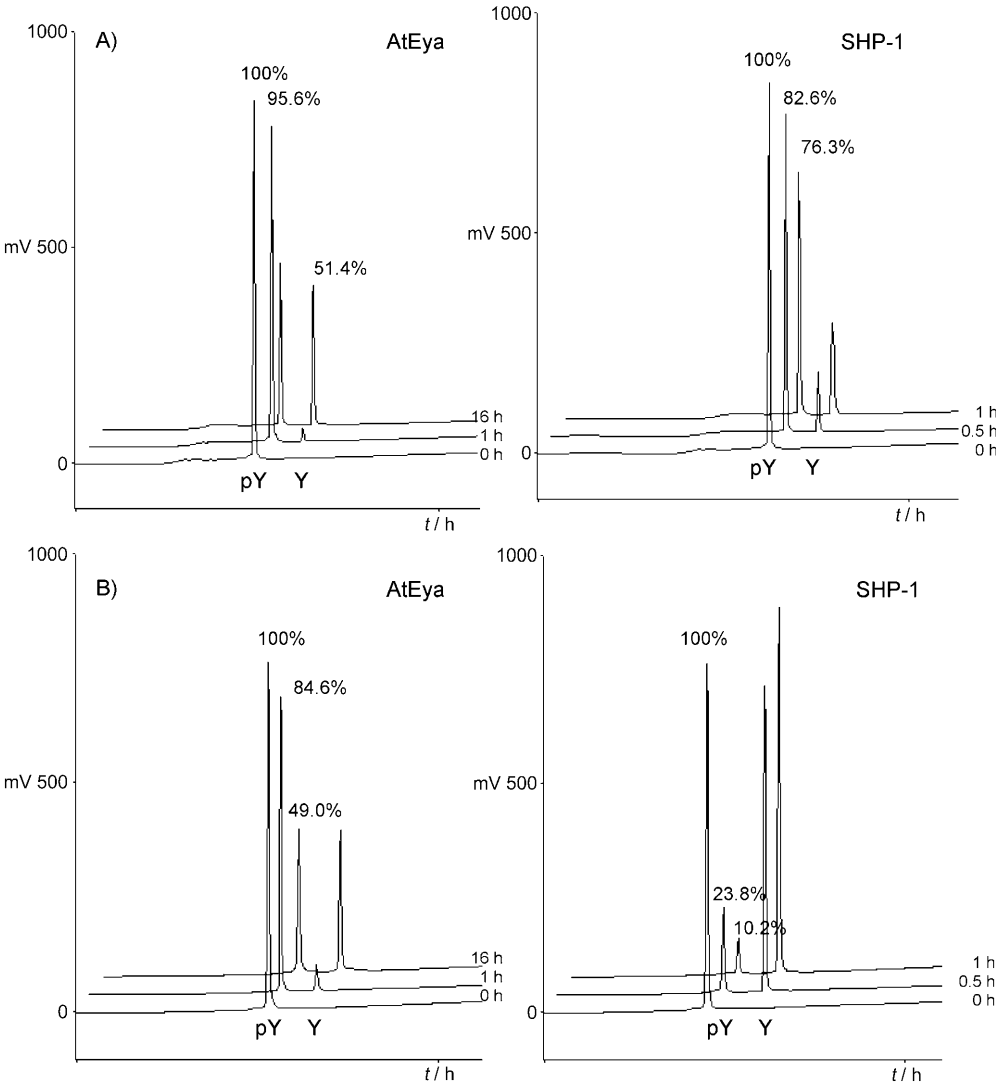

Figure 1. HPLC elution profiles of the dephosphorylation reaction of A) EDAEpYAARG-NH_2 and B) DADEpYLIPQ-NH_2. The experiments with GST–AtEya were conducted in MES buffer (20 mM) that contained $MgCl_2$ (2 mM) at pH 5.5, and in Tris (50 mM, pH 7.4)/NaCl (150 mM)/EDTA (1 mM) in the case of GST–SHP-1(PTP). The conditions for HPLC analysis were: 0 to 40% eluent B in 40 min (A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile), flow rate at 1 mL min^{-1}; UV detection at 220 nm.

fold excess of GST–AtEya compared to GST–SHP-1(PTP), we found that the peptide substrates were converted by both enzymes, albeit to a different extent (Figure 1). Both enzymes seem to prefer the sequence DADEpYLIPQ over EDAEpYAARG, though the difference is marginal in the case of AtEya. In addition, whereas DADEpYLIPQ was almost completely dephosphorylated by the catalytic domain of SHP-1 after 60 min, only about 15% of this peptide was converted by AtEya in the same time period. AtEya was significantly less potent than SHP-1(PTP) toward other peptides, too (data not shown), while SHP-1(PTP) was found to be of similar activity as PTP1B toward *p*NPP as previously described.[33] Incubation with GST alone revealed no pY peptide conversion.

Screening of a peptide library for binding to AtEya

We sought to identify the sequence preferences of AtEya by using a peptide library approach. The degenerate peptide library XXXX-Pmp-XXXX (X = all amino acids excluding Cys and Met, including Nle), which contains the nonhydrolysable phosphonomethylphenylalanine (Pmp) instead of pY was synthesised and screened against both the GST–AtEya and the GST–SHP-1(PTP) fusion proteins according to a procedure that was described earlier.[26] GST alone was used to exclude false preferences with respect to peptide binding.

We were aware of the fact that by using Pmp, we primarily screened for high-affinity binding partners rather than potential substrates. Nevertheless, such information might be of general interest because the importance of the phosphatase domain of Eya proteins has not been clarified yet. SHP-1 was introduced in this experiment to test whether there is a correlation between substrate binding and tyrosine dephosphorylation with respect to the primary sequence context, though the mechanism of catalysis is different from Eya proteins; however, the substrate specificity of SHP-1 has been well characterised in previous studies (Table 1).[23,34,35] The consensus sequences that were derived from the different approaches reflect a strong preference for acidic amino acids (D/E) at positions N-terminal to pY (−1 to −4); this is a general feature of substrate recognition by classical PTPs.

The results of the library screen that is described herein strongly suggest that the Eya domain indeed binds specific peptide sequences, however, these are different from those that are selected by the SHP-1 catalytic domain. Whereas, in accordance with the known consensus, most sequences that bind to SHP-1 contain one or more acidic residues (D/E) N-terminal to pY (63% of all sequences), the Eya domain selects for large hydrophobic and/or basic amino acids (Y/F, H/R/K) at the same positions (90% of all sequences; 57% Y/F, 71% H/R/K; Table 1). At the positions C-terminal to pY, the results are slightly different in that at pY+2, and to a lesser extent at pY+3 a wider variety of amino acids were accepted compared

Table 1. Peptide sequence selection by AtEya and SHP-1(PTP-domain)[a]

−4	−3	−2	−1	0[b]	+1	+2	+3	+4
AtEya								
H (1.8)[c]	H (1.9)	H (2.0)	H (1.2)	–	H (1.9)	X[d]	D (1.5)	V/Z (5.1)
F (1.4)	R (1.5)	R (1.6)	K (1.2)		F (1.5)	Y (1.2)	Q (1.3)	
Y (1.4)	Y (1.5)	Y (1.0)	F (1.2)				F (1.3)	
A (1.2)	A (1.2)						Y (1.3)	
	F (1.0)						H (1.1)	
SHP-1								
E (1.1)	D (1.6)	Y (2.1)	E (1.1)	–	E (1.9)	H (3.2)	V (2.3)	V/Z (5.8)
D/E (2.0)	D/E (2.1)	D/E (1.6)	D/E (1.9)		D/E (2.7)	P (1.6)	Q (1.9)	F/Y (1.7)
L (1.1)	A (1.3)	M (1.0)	V (1.0)		H/R (1.9)	Y/F (1.6)	P (1.1)	
M (1.1)	V (1.3)	M/V/L (2.1)	V/I/L(2.1)		T/S (1.9)			
L/M/V (3.1)	V/M/L (2.1)		Y/F (1.0)		M/L/V (1.9)			
Y (1.1)	H (1.0)		G (1.0)					
Y/F (1.7)								
SHP-1 consensus sequences[e]								
D/E	X	L/I/V	X	–	X	X	L/I/V	–[33]
–	–	D/E	(E/D/F/Z)	–	–	–	–	–[22]
D/E	D/E	D/E/L	X	–	X	X	F/Z	D/E[34]

[a] GST fusion proteins of AtEya and the catalytic domain of SHP-1 were screened for binding to the peptide library AAXXXXPmpXXXX-linker; M = Nle; [b] Positions are given relative to Pmp and pY, respectively (0); [c] amino acids that occurred in more than 10% of the sequences (e.g., 18% = 1.8); [d] no strong preference; [e] consensus sequences reported for the catalytic domain of SHP-1 (Z = Leu, Ile or Nle).

to the other positions. For both enzymes a strong preference for hydrophobic residues (V/I/L/M/F/Y) was detected at position +4. Whether this is an effect of the proximity to the linker and resin or not has not been clarified yet. With respect to the positions N-terminal to pY (−4 to −1), typical sequences that bound to the catalytic domain of SHP-1 were DYDY, MDDY, VGDD, YDEQ, FDEG, HHET, YDYD or DWYD; this is in good agreement with the known consensus sequences.[23,34,35] In contrast, AtEya primarily recognised sequences such as FHRR, MVRR, HQRW, YMRF, RYRK, NFHH, YHHH, HHAF, HRYT, VRAH, RAHA, or ARFF at the same positions.

Kinetic analysis of different phosphorylated substrates

To elucidate the structural determinants of AtEya substrates by a spectrophotometric assay, we chose the phosphopeptide EDAEpYAARG (RR-Src) as a template (peptide **1**, Table 2), though according to the HPLC experiment, the $EGFR_{988-998}$-derived peptide (**20**) would also have been a good candidate (Figure 1). However, only one peptide concentration was used for HPLC analysis; this is in contrast to the kinetic analysis of both peptides that was previously performed by Rayapureddi et al. who used the malachite green assay.[8] In this report, peptide **1** displayed the better substrate specificity constant ($k_{cat}/K_m = 0.96 \times 10^{-6}\,M^{-1}s^{-1}$) versus peptide **20** ($k_{cat}/K_m = 0.52 \times 10^{-6}\,M^{-1}s^{-1}$).[8] A drawback of this report is the fact that the peptide substrates that were investigated comprised very different chain lengths, which ranged from nine to thirteen amino acid residues and three to eight residues N-terminal to pY. Several studies with a variety of PTPs have shown that size and phosphotyrosine positioning in peptide substrates strongly influence substrate recognition.[36,37] We therefore used the selected peptides in the same size with the exception of the truncated versions (**7**–**14**) of template peptide **1**, which were introduced to study the size requirements for AtEya.

Table 2 summarises the Michaelis–Menten kinetic parameters of pY and the pY-containing peptides with recombinant GST–AtEya at pH 5.5 and 30 °C. The K_m and k_{cat} values that were obtained for AtEya with pY are in the range of those that were described earlier (Figure 2).[8]

Table 2. Kinetic constants for the hydrolysis of pY and phosphorylated peptides by AtEya.[a]

	Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\times 10^{-6}\,M^{-1}s^{-1}$]
	pY	2.61 ± 0.50	6693 ± 509	2.56
1	EDAE**pY**AARG-NH_2	2.01 ± 0.10	17875 ± 915	8.89
2	ADAE**pY**AARG-NH_2	1.99 ± 0.06	10143 ± 311	5.10
3	EAAE**pY**AARG-NH_2	1.95 ± 0.05	10067 ± 272	5.16
4	EDAA**pY**AARG-NH_2	2.05 ± 0.06	4995 ± 156	2.44
5	EDAE**pY**AAAG-NH_2	1.89 ± 0.19	11057 ± 1095	5.85
6	EDAE**pY**AARA-NH_2	1.85 ± 0.06	11241 ± 368	6.08
7	DAE**pY**AARG-NH_2	2.69 ± 0.10	11020 ± 356	4.10
8	AE**pY**AARG-NH_2	2.91 ± 0.28	15141 ± 1435	5.20
9	E**pY**AARG-NH_2	2.92 ± 0.37	7578 ± 971	2.59
10	**pY**AARG-NH_2	2.31 ± 0.08	2296 ± 79	0.99
11	EDAE**pY**AAR-NH_2	2.06 ± 0.08	10443 ± 401	5.07
12	EDAE**pY**AA-NH_2	2.07 ± 0.09	13838 ± 621	6.68
13	EDAE**pY**A-NH_2	1.89 ± 0.11	11169 ± 652	5.91
14	EDAE**pY**-NH_2	1.78 ± 0.06	11657 ± 423	6.55
15	EDAE**pS**AARG-NH_2	n.d.	n.d.	n.d.
16	EDAE**pT**AARG-NH_2	n.d.	n.d.	n.d.
17	ARRA**pY**VAAA-NH_2	1.37 ± 0.08	3602 ± 219	2.63
18	AAKR**pY**IRRA-NH_2	1.18 ± 0.06	4083 ± 220	3.46
19	HHRK**pY**HHFV-NH_2	1.19 ± 0.02	4986 ± 87	4.19
20	DADE**pY**LIPQ-NH_2	1.99 ± 0.13	25934 ± 1675	13.03

[a] This assay was conducted at pH 5.5 and 30 °C by using a UV/vis spectrophotometer; n.d.: not detectable.

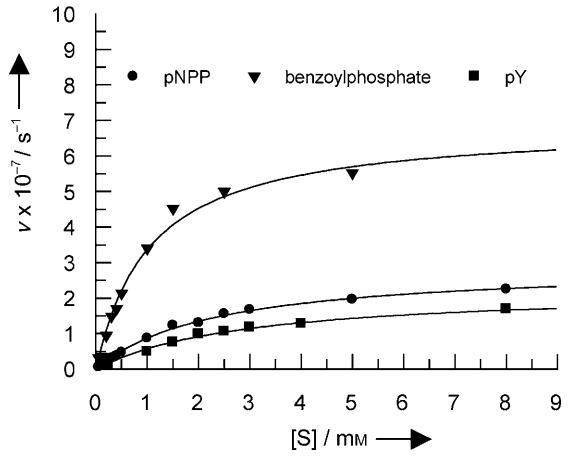

Figure 2. Conversion of phosphorylated substrates (S) *p*-nitrophenyl phosphate (*p*NPP), phosphotyrosine (pY) and benzoyl phosphate by GST–AtEya at pH 5.5 and 30 °C. The reactions follow Michaelis–Menten kinetics.

In addition, we found that reactivity toward *p*NPP and pY was comparable for GST–AtEya and AtEya; this indicates that GST had no influence on enzymatic activity. The substrate specificity constants (k_{cat}/K_m) for the phosphopeptide conversions by AtEya were not significantly different for peptides of comparable lengths. Nonetheless, some interesting features of AtEya substrate recognition were observed. In comparison to pY, with only few exceptions the K_m values slightly decreased for the peptide conversions, while k_{cat} values strongly depended on the respective substrate constitution. AtEya displayed nearly identical K_m values for peptides **1**–**6**, but reduced k_{cat} values toward peptides **2**–**6** compared to template peptide **1**. The effect is most striking for the substitution of Glu by Ala at position pY-1 in peptide **4**. The replacement of Glu and Asp at positions −4 and −3, by Ala in peptides **2** and **3**, respectively, also revealed slower k_{cat} than that of peptide **1**, however, this was less pronounced than for compound **4**. This indicates that the replacement of the acidic amino acid residues N-terminal to pY by alanine negatively influences the efficiency of peptide conversions by AtEya. The same effect as was found for peptides **2** and **3** was also observed for peptide **5**, although in this case a basic residue (R) at position +3 C-terminal to pY was replaced.

Compounds **7**–**10** represent N-terminally truncated versions of peptide **1**. We observed that the truncation had a negative effect on binding, because K_m increased for these peptides. The k_{cat} values of **7**–**10** decreased with truncation of the peptides. In contrast, the C-terminal truncation as in peptides **11**–**14** influenced the K_m values in a positive manner, albeit only to a small extent. Also, the k_{cat} for these peptides is lower than that of peptide **1**, but not as drastic as in the case of peptide **4**.

The peptides **15** and **16**, in which pY of peptide **1** was replaced by pS and pT, respectively, were introduced in the study as a control for the pY-directed activity of AtEya. There was no evidence for AtEya activity toward these peptides. This is in agreement with earlier reports of using shorter pS/pT-peptides in a different sequence context,[8] and thus indicates that the ED phosphatase activity indeed is tyrosine-specific in the case of peptidic substrates.

We included three further peptides (**17**–**19**) in our study, which primarily consisted of basic and/or hydrophobic amino acids. In **17** and **18**, pY was introduced within known sequences of typical substrates of phosphoserine/threonine phosphatases. At the same time, these peptides together with peptide **19** represented candidates containing amino acid residues that were selected by the combinatorial peptide library screening. All three peptides showed lower K_m values than the other sequences that were investigated; this indicates higher binding affinities compared to peptides that contained acidic amino acids. However, considering the k_{cat} values, these peptides are less efficient substrates for AtEya (Table 2).

Beside peptides **1**–**19**, we also investigated the conversion of the $EGFR_{988-998}$-derived peptide **20** by AtEya. The K_m value is similar to that for the Src-derived peptide **1**, the k_{cat} value for this sequence was increased compared to all other peptides. This again revealed that acidic residues are important within peptide substrates of AtEya ED, but obviously there is a preference for basic, and to a lesser extent, hydrophobic amino acids with respect to binding.

Previous studies on the phosphatase activity of mEya3 (ED) excluded a phospholipid phosphatase activity of ED and primarily focused on aryl phosphate (*p*NPP, pY) conversion.[6] In addition, we found that riboflavin-5′-phosphate (FMN) was not dephosphorylated by AtEya (data not shown); however, other phosphate group donors might also be considered to be substrates for eyes absent proteins, which is further supported by the similarity of the Eya domain and HADs. This prompted us to test whether AtEya is able to hydrolyse other phosphorylated substrates. In cells, 1,3-bisphosphoglycerate, carbamoyl phosphate, succinyl phosphate and β-aspartyl phosphate represent substrates of acyl phosphatases.[38] For in vitro studies synthetic substrates such as acetyl phosphate and benzoyl phosphate are generally used to investigate acyl phosphatase activity.[29,30] Here, we found that AtEya efficiently hydrolysed benzoyl phosphate (Figure 2), acetyl phosphate and carbamoyl phosphate as well as a phosphoprotein, β-casein. A clear difference between the acyl phosphates benzoyl phosphate and carbamoyl phosphate and the aryl phosphates *p*NPP and pY was visible. For example, AtEya hydrolysed benzoyl phosphate faster than *p*NPP and pY and displayed a higher binding affinity (Table 3). Furthermore, benzoyl phosphate displayed the best specificity constant compared to all other substrates investigated. This suggests that the Eya domain might also recognise substrates other than tyrosine-phosphorylated proteins in vivo.

Phosphatase activity of disease-associated Eya1 mutants

Most of the disease-associated *EYA1* missense mutations are clustered in the conserved ED region which possesses an intrinsic phosphatase activity.[6,7] Interestingly, the majority of these sites are conserved (D295, S454, L472) or similar (G/A393, D/E396) in the plant Eya homologue (AtEya) that we have used for the studies described herein.

Table 3. Kinetic constants for the hydrolysis of phosphorylated substrates by AtEya.[a]

Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\times 10^{-6}\ M^{-1}s^{-1}$]
*p*NPP	0.06 ± 0.01	960 ± 42	16.0
acetyl phosphate	0.65 ± 0.20	1094 ± 106	1.68
benzoyl phosphate	0.02 ± 0.009	1668 ± 99	83.4
carbamoyl phosphate	0.15 ± 0.02	2836 ± 101	18.9
β-casein[b]	0.02 ± 0.003	798 ± 16	39.9

[a] Data were acquired by using the malachite green detection assay at pH 5.5 and 25 °C. [b] This protein represents a substrate that contains several phosphorylated residues (pS, pT, pY).[45] It was not further examined whether a single or multiple dephosphorylation reactions occurred.

We wanted to examine the effect of these mutations on Eya phosphatase function. To this end, we selected several *EYA1* mutations that are associated with BOR or BO syndrome as well as with ocular defects that lead to amino acid changes in the ED region (Figure 3 and Table 4). We introduced the mutations into the background of the Eya domain of murine Eya1. Because the mouse and human Eya1 proteins are 98.7% identical, they can be regarded as structurally and functionally equivalent. By using GST fusion proteins of full-length Eya1, ED and mutants in the ED background we performed the phosphatase assay with *p*NPP as the substrate (Figure 3). The wild-type Eya domain showed significantly higher activity than the full-length protein. This is in agreement with what had been published earlier[39] and suggests an inhibitory function of the N terminus. The mutants fell in two classes: those that retained phosphatase activity at levels that were not more than 1.7-fold different from that of the wild-type protein, and a second group that displayed loss of enzymatic activity. Within the first group, the BO-associated mutants D396G and R407Q both showed reduction of phosphatase activity whereas the mutant protein R514G, which had been identified in a patient with ocular defects only, showed increased activity compared to the wild-type protein. Activities of members of the second group were indistinguishable from those of the catalytically inactive mutant D295N or GST itself.[6,7] Interestingly, all mutants that retained phosphatase activity (D396G, R407Q and R514G) were derived from patients that displayed BO or ocular defects only, whereas those that showed reduced activity (G393S, S454P, L472R and L550P) were associated with BOR syndrome.

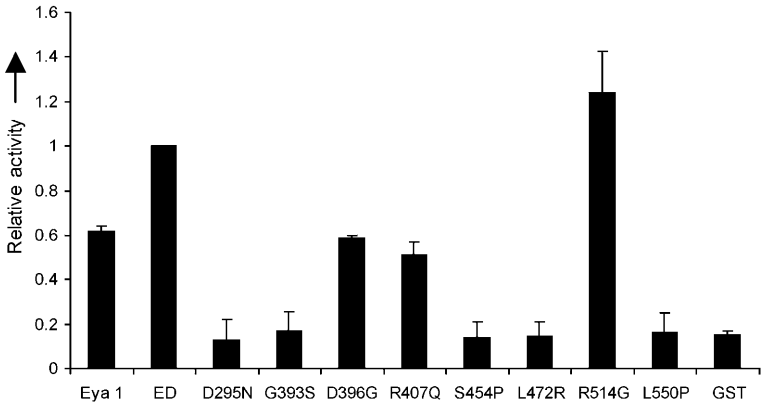

Figure 3. BOR-associated missense mutations lead to a loss of phosphatase activity. The positions of the amino acid substitutions included in the present study are based on the human Eya1 protein sequence.[18] The phosphatase activity of BOR, BO and ocular-defect-associated missense mutations on ED is depicted. Replacement of the nucleophilic Asp295 to Asn inactivates mEya1 phosphatase activity.[6,7] This mutant was employed as a negative control. ED (1.0) was used as a reference for mEya1 and the mutants. The figure represents the mean ± SD of values obtained from three independent experiments.

Table 4. Loss of phosphatase activity in BOR-associated Eya1 mutants.[a]

Protein	Phenotype	Activity relative to GST-mEya, ED (1.0)[b]
mEya1	–	0.61
D295N	–[6,7]	0.14
G393S	BOR and ocular defects[17,39]	0.16
D396G	BO[40]	0.59
R407Q	BO[41]	0.50
S454P	BOR[39,42]	0.15
L472R	BOR[39,42]	0.15
R514G	ocular defects[17,39]	1.24
L550P	BOR[43,44]	0.16

[a] MouseEya1 was used instead of the human protein based on high sequence homology. GST fusion proteins were used in this experiment. [b] Values are taken from Figure 3.

To determine kinetic parameters for the dephosphorylation of different substrates by mEya (ED) and several mutants (G393S, D396G and R514G), we additionally performed a phosphatase assay with the soluble proteins. The K_m and k_{cat} values that were obtained for the dephosphorylation of *p*NPP and carbamoyl phosphate are shown in Table 5. For both substrates the BOR-associated mutant G393S showed a significant decrease in catalytic activity, whereas the activity of the two other mutants was similar to wild-type levels. A multiple phosphorylated protein, β-casein, was also subjected to dephosphorylation by mEya (ED) and the mutant proteins. Indeed, β-casein was dephosphorylated by the proteins, and the ratio between mEya (ED) and the mutants was comparable to the other substrates (data not shown). Due to aggregation and protein precipitations at concentrations higher than 1 mM kinetic parameters could not be determined. Because β-casein has been shown to contain multiple phosphorylated serine residues, but also phosphothreonine and phosphotyrosine,[45] the origin of the released phosphate was not determined; however, considering our data that were acquired for monophosphorylated peptide substrates (Table 2), the dephosphorylation most likely occurred at phosphotyrosine residues.

Discussion

Previous studies revealed that Eya proteins belong to the HAD superfamily of aspartate-dependent hydrolases, though sequence homology is restricted to the three motifs (I–III) that form the catalytic fold.[39] These motifs are located in the

Table 5. Kinetic constants for the hydrolysis of phosphorylated substrates by mEya (ED) and several mutant mEya1 proteins.[a]

Substrate	*p*NPP			Carbamoyl phosphate		
Protein	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\times 10^{-6}$ $M^{-1}s^{-1}$]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$\times 10^{-6}$ $M^{-1}s^{-1}$]
$mEya1_{(ED)}$	0.01 ± 0.01	112 ± 9	11.2	0.14 ± 0.02	114 ± 15	0.8
G393S	0.01 ± 0.01	23 ± 2	2.3	0.06 ± 0.06	15 ± 4	0.2
D396G	0.06 ± 0.03	102 ± 10	1.7	0.1 ± 0.01	89 ± 11	0.9
R514G	0.04 ± 0.001	129 ± 1	3.2	0.08 ± 0.005	84 ± 2	1.0

[a] Data were acquired by using the malachite green detection assay at pH 5.5 and 25 °C.

highly conserved C-terminal Eya domain (ED) that exhibits a phosphotyrosine-specific phosphatase activity. In spite of the unknown physiological relevance of Eya phosphatase activity, a critical question in understanding Eya function is how these proteins recognise their substrates. In this study, we investigated the substrate specificity of the plant eyes absent homologue AtEya, which shares a 39% sequence similarity with the Eya domain of the animal proteins.

According to our data, there is a difference between peptide sequences that efficiently associated with AtEya, and compounds that represented good substrates. Whereas peptides that are rich in hydrophobic (Y, F, V/L/I/Nle, A) and basic (H, R, K) amino acids on either side of pY/Pmp are the preferred interaction partners as was determined by the peptide library screening, acidic residues (D, E) on the N-terminal side of pY seem to be the positive determinants for pY-substrate recognition by AtEya. Our results indicate however, that the degree of specificity is not as high as for other protein tyrosine phosphatases. AtEya can dephosphorylate both acidic and basic substrates, though to a different extent. Of the phosphopeptides that were examined, $EGFR_{988-998}$ (DADEpYLIPQ) was the best pY-containing substrate, followed by other peptides with a net negative charge. Herein, we focussed on monophosphorylated peptides first. Whether multiple phosphorylation or substitution of multiple Asp and/or Glu residues produce an additive effect on the reactivity of the peptides with AtEya as was observed for the Cys-dependent protein tyrosine phosphatases is currently under investigation.

In general, AtEya was specific for aryl monophosphate esters (*p*NPP, pY) and showed no activity toward aliphatic phosphate esters (pS, pT, FMN). Due to the similarity between the Eya domain and the catalytic fold of HAD enzymes, we examined whether AtEya can hydrolyse other phosphate group donors too. We found that compared to *p*NPP and pY-peptides the acyl phosphates acetyl phosphate, benzoyl phosphate and carbamoyl phosphate were also efficiently converted. Among those, benzoyl phosphate was the best substrate, and, in agreement with the observations that were discussed above, aromatic compounds displayed a higher affinity for AtEya than aliphatic-substituted phosphates. Thus, our data suggest that in addition to pY-containing proteins small physiologically relevant molecules like carbamoyl phosphate might also be substrates of the Eya proteins. Differences in substrate specificity between Eya proteins and other PTPs might yet prove to be of physiological significance.

Currently, in the absence of a crystal structure analysis of Eya proteins it is difficult to discuss stabilisation effects for a specific substrate or of amino acids in the vicinity of the phosphorylation site within pY peptide substrates. Nonetheless, the central motif II (hhhT, h = hydrophobic) and the N-terminal region of the C-terminal motif III ($Kx_{(n)}$hhhh) that were suggested to be responsible for substrate binding[8] provide both interaction of a negatively charged substrate with a positively charged environment (motif III) as well as hydrophobic interactions with active-site residues of motifs II and III. On the other hand, one may speculate that the library-derived sequences that efficiently associated with the plant Eya protein might represent high-affinity interaction partners independently of a phosphorylated tyrosine. Also, regions other than the active-site residues within ED might be involved in these interactions. Interestingly, amino acid sequences that are highly similar to the peptides that were identified through our library screening can be found within well-established binding partners of the Eya domain. It has been demonstrated that the interaction between Eya and Six proteins (So in *Drosophila*) is mediated by the highly conserved Six domain.[46,47] For example, regions Six_{63-70} (FRELYKIL), Six_{88-93} (LKAHYI), $Six_{105-113}$ (AVGKYRVRR) and $Six_{139-147}$ (LREWYAHNP) of human Six1 closely resemble library-derived peptides. We therefore speculate that although there is no evidence for a Six homologue in *Arabidopsis*, the binding preferences for all Eya family members share the same characteristics.

After having characterised the phosphatase activity of the prototypic Eya protein from *Arabidopsis* we also wanted to examine whether disruption of Eya's phosphatase activity might contribute to the different diseases that are associated with *EYA1* mutation in humans. Our data show that whereas BOR-related mutations lead to a loss of enzymatic activity compared to the wild-type protein, BO and ocular-defect-associated mutations do not influence phosphatase activity significantly. A similar analysis has been done before, albeit not with the same set of mutants. Rayapureddi et al. have introduced several mutants into the background of the Eya domain of murine Eya1 and/or Eya3.[39] With the exception of one mutant, the authors reach similar conclusions to ours, that is, mutants from BOR patients showed a lack of phosphatase activity, whereas mutants that are associated with ocular defects still displayed measurable activity. The exception was mutant G393S, which was derived from a patient with BOR and ocular defects. In our experiments, this mutant displayed significantly reduced activity. This was also the case when Rayapureddi et al. introduced the mutation into Eya3, but not when it was inserted into the background of Eya1.[39] It is unlikely that this is because we have used GST-tagged proteins, because in our experiments

there was no difference between the tagged and untagged proteins. Mutsuddi et al. have recently introduced various mutations from *Drosophila* and humans into GST–mouse Eya3 fusion proteins and have determined dephosphorylation of the peptide IpYGEF.[44] Whereas most of the BOR mutants lacked enzymatic activity, no clear correlation between BOR and OD-associated mutations on the one hand, and the absence or presence of phosphatase activity on the other hand was obtained. An explanation for the discrepancy between their and our data might be the difference in background in which the mutants were incorporated.

In summary, our data suggest that the loss of phosphatase activity of Eya1 contributes to the BOR phenotype of human patients, but BO syndrome and ocular defects are not caused by loss of the enzymatic activity of Eya1. This also means that Eya1 must have other functions, which, when disrupted lead to branchial and otic anomalies. Because these anomalies are also found in BOR patients, this additional function must also be hampered in BOR mutants. This activity could involve specific protein interactions of Eya1 or other functions that have not yet been identified. One additional implication of the lack of phosphatase activity specifically in BOR patients is that the phosphatase activity of Eya1 seems to be required for normal kidney development. This could be tested by the generation of appropriate mouse models. In addition, the identification of the physiological substrates of the Eya protein's phosphatase activity will be an important and revealing task for the future.

Experimental Section

General: Fmoc-protected amino acids, coupling reagents (HBTU, HOBt) and Rink-amide MBHA resin were purchased from Novabiochem (Merck Biosciences AG, Darmstadt, Germany). TentaGel S NH_2 resin and Fmoc-Pmp-OH were obtained from Activotec (Cambridge, UK). Peptide synthesis reagents and solvents were of reagent grade from Fluka (Sigma–Aldrich). Acetyl phosphate, carbamoyl phosphate and β-casein were purchased from Sigma. HPLC gradient grade acetonitrile was obtained from VWR International (Dresden, Germany) and Roth (Karlsruhe, Germany), respectively. The α-cyano-4-hydroxycinnamic acid was from Bruker (Leipzig, Germany) and trifluoroacetic acid was purchased from Solvay (Hannover, Germany).

Plasmid construction: To clone *A. thaliana* Eya (AtEya), RT-PCR was performed by using primers AtEya forward 5′-AGA GGG AAT TCA TAA TGA TAC ATC AAA AAA GCT GGG-3′ and AtEya reverse 5′-GCT TGA GCT CGA GTT ACT CTT TGC TGG AAT CAG-3′ that contained the EcoRI and SacI sites, respectively and cDNA from seedling leaves. The RT-PCR product was cloned into the TOPO vector (Invitrogen) according to the manufacturer's instruction. After identification of a positive clone and verification by sequencing, the insert was released and subcloned into the expression plasmid pGEX-KG by using EcoRI and SacI. To generate full-length mouse Eya1 and Eya1 domain (ED, amino acids 291–591) as glutathione S-transferase (GST) fusion proteins in the expression vector pGEX-KG, PCR-mediated cloning by using pHM6 *Eya1* (gift from K. Kawakami) as a template was performed. The primers that were used were HA-mEya1s 5′-ACT GGA ATT CTG GAA ATG CAG GAT CTA ACC AGC-3′and Eya1D/GST.s 5′-ACG TGA ATT CGG CTG CGT CGA GGT TCA-3′ both contained the EcoRI sites and Eya1/GST as 5′-GAT CGA GCT CTT ACA GGT ACT CTA ATT CCA AGG CAT-3′ which harboured a SacI site. The respective PCR products were then cloned into pGEM-T Easy Vector (Promega). After identification of a positive clone and confirmation by sequencing, the insert was released and subcloned into the expression plasmid pGEX-KG by using EcoRI and SacI. Missense mutations by using reverse complementary primer pairs were then introduced into ED by Quick Change™ Site Directed Mutagenesis Kit (Stratagene). All mutations were confirmed by sequencing. The following primers (only the sense strand primer is depicted) were used for mutagenesis. D295N: 5′-AGA GTG TTA CTC TGG AAC CTG GAC GAG ACC ATC-3′, G393S: 5′-GCA ACT GGT GTC CGA AGT GGT GTG GAC TGG ATG CG-3′, D396G: 5′-GTC CGA GGT GGT GTG GGC TGG ATG CGG AAA CTG-3′, R407Q: 5′-GCC TTC CGC TAC AGA CAA GTA AAA GAG ATC TAC-3′, S454P: 5′-CTG AAG GCC CTC CCC CTC ATC CAC TCC C-3′, L472R: 5′-GTA ACA ACT ACG CAG CGC AGC CCA GCA TTG GC-3′, R514G: 5′-ATC CAA AGG TTT GGA GGG AAA GTG GTA TAC CTT-3′, L550P: 5′-TCG GAC CTC ATG GCA CCG CAT CAT GCC TTG GAA-3′.

Expression and purification of proteins: AtEya, murine Eya1 (wild-type) and Eya1 ED (Eya domain) and mutant recombinant proteins were expressed as glutathione S-transferase (GST) fusion proteins. *E. coli* BL21 (DE3) that harboured the GST–AtEya, GST–ED or the mutant fusions encoding vector were grown to an OD_{600} of 0.5–0.6 and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) for 3 h at 25 °C. Cells were harvested by centrifugation and resuspended in a pH 7.6 buffer that contained Tris (50 mM), NaCl (400 mM), EDTA (1 mM), DTT (1 mM), glycerol (10%), NP-40 (0.1%), phenylmethylsulfonylfluoride (PMSF) and protease inhibitor cocktail (Roche). Lysis was achieved by sonication at 50% cycle and 50% power for 5×20 s. Following centrifugation at 10000 rpm for 30 min, clarified lysates were incubated with glutathione agarose beads at 4 °C for 1 h, after which the beads were washed extensively with PBS that contained protease inhibitor cocktail. GST–AtEya, ED and selected mutant proteins were eluted with reduced glutathione (20 mM) in Tris pH 8.8 (100 mM) and protease inhibitor cocktail. In the case of the initial phosphatase assay in which we compared murine Eya1 and its mutant forms, a similar protocol was followed except that the proteins were not eluted from the glutathione beads.

The catalytic domain of SHP-1 (residues 271–514) was expressed as GST fusion protein in BL21(DE3)pLys cells. The obtained protein was purified by affinity chromatography and gel filtration on a Sephacryl™ 16/60 S-100 high resolution column (Amersham Bioscience) by using a Pharmacia LKB GP-10 (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as previously described.[24] The purity of both proteins was ascertained by SDS-PAGE and by gel staining with Coomassie and Western blotting. The protein concentrations were determined by the Bradford method by using Roti–Nanoquant (Carl Roth GmbH, Karlsruhe, Germany).

Synthesis of the peptide library: TentaGel S NH_2 resin (2.0 g, 0.3 mmol g^{-1}) with a diameter of 90 μm were used in solid-phase synthesis by the Fmoc/t-butyl protection strategy. Fmoc-protected amino acids, HBTU and HOBt (4 equiv each) in dimethylformamide (DMF) were coupled for 1 h at room temperature. The complete synthesis of the library was carried out according to the split-and-pool synthesis method.[25] Coupling reactions were repeated once to ensure complete reaction. According to previous reports, Ac-Gly (5%) was added to the coupling reaction of Leu and Lys, and Ac-Ala was added to the coupling of Nle to facilitate sequence determination by mass spectrometry as described.[26] Side-chain deprotection of the resin-bound library was carried out with reagent K (150 μL; reagent K contained 0.75 g phenol, 0.25 mL ethandithiol,

0.5 mL thioanisole) in 95% TFA (1 mL) per 100 mg resin at room temperature for 1 h. Then, the library was washed with TFA, CH_2Cl_2 and MeOH before drying for storage at −20 °C. The screening of the library was carried out by using biotinylated GST-tagged proteins as described.[26] Positive beads that bound the corresponding protein were selected manually under a microscope. Sequence identification of the positive beads was performed by the partial Edman degradation (PED) method followed by mass spectrometry as previously reported.[26]

Synthesis of individual peptide substrates: The phosphorylated peptides were synthesised by solid-phase peptide synthesis according to the Fmoc strategy at the 0.5 g scale by using Rink-amide MBHA resin (0.64 mmol g^{-1}). Fmoc-Tyr(PO_3H_2)-OH, Fmoc-Ser(PO(OBzl)OH)-OH, and Fmoc-Thr(PO(OBzl)OH)-OH were used for the synthesis of the phosphopeptides. For amino acid couplings, Fmoc-protected amino acids (4 equiv) were activated with HBTU/HOBt (4 equiv each) in the presence of iPr_2EtN (8 equiv) for 1 h (double couplings). The phosphorylated Fmoc-protected amino acids (2 equiv) were coupled with iPr_2EtN (6 equiv).[27] Peptides were cleaved from the resin by using a mixture of 95% TFA, 2.5% iPr_3SiH and 2.5% H_2O for 5–6 h at room temperature. After precipitation in cold Et_2O, peptides were centrifuged and washed several times with Et_2O prior to lyophilisation from H_2O and 80% *t*BuOH. Semipreparative purifications of the peptides were performed on a Shimadzu LC8A HPLC instrument that was equipped with a C18 column (Eurospher 100, Knauer, Berlin, Germany) by using a gradient of 0–50% eluent B in 120 min at a flow rate of 10 mL min^{-1} (eluent A: 0.1% TFA in H_2O, eluent B: 0.1% TFA in 90% acetonitrile/H_2O); detection was at 220 nm.

Peptide analysis: Analytical HPLC was performed on a Shimadzu LC-10AT system (Duisburg, Germany) by using a Vydac 218TP54 C18 reversed-phase column (5 μm particle size, 300 Å pore size, 4.6×25 mm) from Macherey–Nagel (Dueren, Germany). Peptides were eluted with the gradient 0–30% eluent B in 30 min at a flow rate of 1.0 mL min^{-1}, where A was 0.1% TFA in H_2O and B 0.1% TFA in acetonitrile. Detection was at 220 nm. Peptide identity was confirmed by MALDI-TOF mass spectrometry on a Laser Tec Research spectrometer (Perspective Biosystems, Weiterstadt, Germany) by using α-cyano-hydroxycinnamic acid as a matrix.

Peptide substrate preparation: To determine the concentration of the peptide solutions used for the phosphatase assays, amino acid analysis and the malachite green assay (BIOMOL Research Laboratories, Hamburg, Germany) were performed by using hydrolysed samples. The peptides were dissolved (4–5 mM) in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.5, 20 mM) that contained $MgCl_2$ (2 mM). Aliquots (100 μL) of these solutions were evaporated to dryness and hydrolysed by using 6 M HCl at 110 °C for 24 h; HCl was then removed by evaporation. The hydrolysis products were repeatedly dissolved in H_2O and evaporated. The remaining residues were dissolved in sample dilution buffer (Onken GmbH, Gruendau-Breitenborn, Germany) and provided for amino acid analysis using an LC 3000 amino acid analyzer from Eppendorf Biotronik (Hamburg, Germany). The same solutions were used to perform a malachite green assay (duplicates) to determine the content of inorganic phosphate.[28] The average of the results of both experiments was used as peptide concentration for the phosphatase assay.

Phosphatase assays: Initially we compared the efficiencies of *p*NPP conversion of GST-tagged and untagged AtEya protein (GST was removed by usage of a thrombin cleavage site). Because both proteins behaved indistinguishably, we used only the fusion proteins for subsequent experiments. Phosphatase assays were performed by employing two different techniques, namely liquid chromatography and spectrophotometry. In the first case, samples were obtained from the incubation of the corresponding enzyme (GST–AtEya, GST–SHP-1(PTP)) with the peptides (three independent experiments) and were analysed by RP-HPLC to monitor the reaction progress. The dephosphorylation was investigated at 30 °C in a total volume of 50 μL for the assay reaction. The peptides were used in a final concentration of 0.5 mM. The reaction was initiated by the addition of the corresponding enzyme (10 μL, 50 ng mL^{-1} for GST–AtEya, 5 ng mL^{-1} for GST–SHP1(PTP)). The dephosphorylation reaction was terminated by the addition of 10% TFA (50 μL) after different reaction times (e.g., 30, 60, 120 min and 16 h). Afterwards, the solutions were centrifuged, the supernatants were removed, frozen and freeze-dried. For HPLC analysis the peptides were dissolved in doubly distilled H_2O (100 μL).

The spectrophotometric phosphatase assay was performed in quartz microcuvettes with a final reaction volume of 200 μL. Due to the limitations of solubility, the peptides were used in concentrations from 0 to 4 mM. Phosphotyrosine and *p*NPP were used from 0 to 8 mM, and the other phosphorylated compounds from 0 to 5 mM. Benzoyl phosphate was prepared as described,[29] and the assay was carried out according to the literature by using this acyl phosphate.[30] The molar absorption coefficients of the peptides were determined according to Zhang et al.[31] The reaction was initiated by the addition of GST–AtEya (30 μL, 0.04–0.07 mg mL^{-1}). The reaction progress was monitored on a UV/Vis spectrophotometer (Perkin–Elmer). The initial reaction rates were calculated from the linear region of the curves. Kinetic constants were determined from a nonlinear regression hyperbolic fit to the Michaelis–Menten equation by using Grafit 3.0.

The investigation of the activity of GST–mEya (wt), GST–mEya1 (ED, 271–274 amino acids) and the mutants was performed by using two spectrophotometric assays. In the first approach, bead-bound proteins (200 μL) were centrifuged, and the supernatant was removed. The beads were then incubated with *p*NPP (200 μL, 2 mM in 20 mM MES and 2 mM $MgCl_2$) at pH 5.5 and 30 °C. After incubation, the mixture was centrifuged and the absorption of the supernatant was measured at 405 nm. An aliquot of the bead-bound recombinant GST fusion proteins was subjected to SDS-PAGE and subsequent Western blot analysis by using an anti-GST antibody. Signals were quantified by densitometric analysis. The relative values of phosphatase activity were obtained by dividing the values for activities by the respective protein amounts. In the second experiment, soluble GST–mEya (ED) and selected mutants were used at a concentration of approximately 1.1 mg mL^{-1}. The substrate concentrations varied from 0 to 2 mM in a final assay volume of 70 μL. The reaction was started by the addition of 10 μL of the corresponding enzyme. After 0.5, 1, 2, 3 and 5 min the reaction was stopped, and an aliquot (25 μL) of the incubation mixture (duplicate sampling) was used for malachite green phosphate detection in a microtiter plate.

Abbreviations

ED: Eya domain; GST: glutathione S-transferase; HAD: haloacid dehalogenase; *p*NPP: *p*-nitrophenyl phosphate; pY: phosphotyrosine; pS: phosphoserine; pT: phosphothreonine; PTP: protein tyrosine phosphatase; SHP-1: SH2 domain protein tyrosine phosphatase-1.

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Keywords: BOR syndrome · enzymes · haloacid dehalogenase · kinetics · substrate specificity

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