The *Bacillus subtilis* regulator protein SpoIIE shares functional and structural similarities with eukaryotic protein phosphatases 2C

Ragnar Schroeter a, Susanne Schlisio a, Isabelle Lucet b, Michael Yudkin b, Rainer Borriss a,b,*

a Institut für Biologie, Humboldt Universität Berlin, Chausseestr. 117, 10115 Berlin, Germany
b Department of Biochemistry, University of Oxford, Oxford OX1 3QU, UK

Received 7 December 1998; received in revised form 22 February 1999; accepted 25 February 1999

Abstract

De phosphorylation of SpoIIE-P by SpoIIE is strictly dependent on the presence of the bivalent metal ions Mn$^{2+}$ or Mg$^{2+}$. Replacement by Ala of one of the four Asp residues, invariant in all representatives of protein phosphatase 2C, completely abolished the SpoIIE phosphatase activity in vitro, whilst replacement of the Asp residues by another acidic amino acid, Glu, had varying effects on the activities of the resulting mutated proteins. D610E and D795E exhibited some residual activity while D628E and D745E were without enzymatic activity. The results suggest that the functional model in which metal-associated water molecules are involved in the dephosphorylation reaction catalyzed by human protein phosphatase 2C can also be applied to the bacterial protein phosphatase 2C-like protein. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: SpoIIE; Sporulation; *Bacillus subtilis*; Protein phosphatase 2C

1. Introduction

Protein phosphatase 2C (PP2C)-like enzymes are known to catalyse the Mg$^{2+}$- or Mn$^{2+}$-dependent dephosphorylation of phosphoserine- and phosphothreonine-containing proteins [1,2]. Representatives of eukaryotic PP2C are implicated in several cellular signal transduction pathways governed by reversible protein phosphorylation, e.g. in response to stress.

Some limited sequence similarity to eukaryotic PP2C has been detected in protein phosphatase SpoIIE [1], which activates the transcription of cell type-specific genes in the pre-spore compartment of *Bacillus* cells during an early stage of sporulation (for review see [3]). The structural model of human PP2C established from an X-ray analysis suggested that the residues invariant in prokaryotic and eukaryotic PP2C are involved in metal ion-catalyzed dephosphorylation. It has been proposed that Asp residues 38, 60, 239, 282 together with Glu-37 and Gly-61 coordinate a bivalent metal ion center. Metal-associated water molecules act as a nucleophile to...
attack the phosphorus atom in an $S_N2$ mechanism and as a donor to protonate the seryl leaving group oxygen atom [2]. A structural similarity between the bacterial SpoIE protein, already characterized as a Mn$^{2+}$-dependent SpoIIA-A-P phosphatase [4] and the yeast TD1 protein has been demonstrated. The replacement by Ala of either of the two invariant COOH-terminal Asp residues corresponding to Asp-239 and Asp-282 of human PP2C blocked the function of the protein in vivo [5]. Here, we show by in vitro analysis of mutants that most of the residues proposed to be crucial in human protein phosphatase 2C are also essential in the function of the *Bacillus* SpoIE protein, suggesting that the functional model established for eukaryotic PP2C might also be valid for bacterial PP2C-like proteins.

2. Materials and methods

2.1. Cloning and expression of Bacillus SpoIE

The wild-type spoIE gene was amplified from chromosomal DNA of *Bacillus subtilis* SG38 [6] by the GeneAmp Polymerase Chain Reaction process (GeneAmp XL PCR kit, Perkin Elmer) in such a way as to place a NdeI restriction site at the translation start codon and a BamHI restriction site at the 3' end of the spoIE gene. After confirmation of the sequence of the resulting 2568-bp product, the gene was cloned into the expression vector plasmid pET11a (Novagen) cut with NdeI and BamHI. The ATG start codon of the spoIE gene was thus placed eight nucleotides downstream from the highly efficient SD sequence of the phage T7 major capsid protein encoded by the vector plasmid. The recombinant plasmid pRB1011 encoding the full length spoIE gene was transformed into *Escherichia coli* strain C41 (DE3) [7]. Expression of the cloned spoIE gene, under the control of the T7 promoter, was achieved by IPTG (1 mM) induction of the T7 RNA polymerase gene [8].

2.2. Analysis of wild-type and mutant SpoIE expressed in recombinant *E. coli* cells

Western blot analysis of cell extracts with antibodies raised against SpoIE$_{1-827}$ confirmed that SpoIE$_{1-827}$ was of the size (about 90–92 kDa) expected from its amino acid sequence [2]. Crude extracts were prepared from 4-ml cultures. Cells were resuspended in 0.4 ml lysis buffer consisting of 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 5 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride and passed through a French press (pre-cooled to 4°C). Cell debris were removed by centrifugation (10 min, 10,000×g) and 0.4 ml glycerol was added to the supernatant. Purification of SpoIE$_{1-827}$ was performed from the membrane fraction (I. Lucet, unpublished results) and quantified [16]. SpoIIAA-phosphate, overproduced and purified as previously described [9], was used to assay the protein phosphatase activity in two different ways. The first assay is based on the observation that on non-denaturing polyacrylamide gel electrophoresis, SpoIIAA-P migrates faster than its hydrolysis product SpoIIA [9]. Alternatively, non-radioactive phosphatase assays on microtiter plates, final volume 50 μl, were performed with 100 μM Ser/Thr phosphopeptide (Promega) or 34 μM SpoIIAA-P. This system determines the amount of free phosphate generated during the reaction [10]. The two phosphatase assays were performed at 30°C.

2.3. Preparation of SpoIE mutants

The spoIE mutations were introduced by the Quik Change® (Stratagene) method of site-directed mutagenesis, with plasmid pRB1011 as the template for amplification by *Pfu* DNA polymerase. The primers used to introduce the appropriate substitutions are listed in Table 1.

3. Results and discussion

3.1. Substrate specificity and dependence on Mg$^{2+}$ or Mn$^{2+}$ ions

Cell extracts prepared from recombinant *E. coli* cells (pRB1011) contained the functional SpoIE protein as revealed by their ability to hydrolyse phosphorylated SpoIIA-P. The phosphopeptide RRA(pT)VA, which serves as a substrate for eukaryotic Ser/Thr protein phosphatases 2A, 2B and 2C [11], was not hydrolysed (Fig. 1). This strict substrate
specificity is in line with the finding that SpoIIE did not dephosphorylate RsbV-P, a homolog of SpoIIAA that controls the activity of the B. subtilis stress factor σ^II [4]. The reaction is dependent on the presence of Mn^{2+}. If Mn^{2+} ions are replaced by Mg^{2+}, a 10-20-fold higher concentration of ions is needed to give the same enzymatic activity (Fig. 2).

Dependence on Mn^{2+} or Mg^{2+} is characteristic for protein serine/threonine phosphatases of the type 2C family, suggesting that despite the fact that their primary structures are only slightly similar, the two enzymes do belong to one family. It has already been reported that the dephosphorylation of SpoIIAA-P, catalyzed by SpoIE_{231-927}, a fragment lacking the membrane spanning N-terminal domain, depends on Mn^{2+} [4]. The activity of eukaryotic PP2C has been shown to be five times higher in the presence of Mn^{2+} than in the presence of Mg^{2+} [12].

### Table 1

<table>
<thead>
<tr>
<th>Required mutation in SpoIIE</th>
<th>Equivalent residue in huPP2C</th>
<th>Amino acid/codon changed</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>K602A Arg-33? Arg-33?</td>
<td>Lys-602→Ala</td>
<td>5′-TGCTCAATTCGACCAGCAGCCAGCC (FW)</td>
<td>5′-TGGCTATTCGACCAGCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>K602R Arg-33? Arg-33?</td>
<td>Lys-602→Arg</td>
<td>5′-AGGCCGGCCGCGGGGAGGCCAGCAGGCC (FW)</td>
<td>5′-AGGCCGGCCGCGGGGAGGCCAGCAGGCC (REV)</td>
</tr>
<tr>
<td>D610A Asp-38 Asp-38</td>
<td>Asp-610→Ala</td>
<td>5′-ATGCTGACCTGAGCAGCCGACAGCCAGCC (FW)</td>
<td>5′-ATGCTGACCTGAGCAGCCGACAGCCAGCC (REV)</td>
</tr>
<tr>
<td>D610E Asp-38 Asp-38</td>
<td>Asp-610→Glut</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>G629A Gly-61 Gly-61</td>
<td>Gly-62→Ala</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>T679A Thr-128 Thr-128</td>
<td>Thr-679→Ala</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>D746A Asp-239 Asp-239</td>
<td>Asp-746→Ala</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>D746E Asp-239 Asp-239</td>
<td>Asp-746→Glut</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>G747A Gly-240 Gly-240</td>
<td>Gly-747→Ala</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>D795A Asp-282 Asp-282</td>
<td>Asp-795→Ala</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
<tr>
<td>D795E Asp-282 Asp-282</td>
<td>Asp-795→Glut</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (FW)</td>
<td>5′-ATGGCAGCAGCAGCCGACAGCCAGCCAGCC (REV)</td>
</tr>
</tbody>
</table>

*Mismatches introduced into the nucleotide sequence are underlined.

3.2. Mutants with replacements in invariant residues

A comparison of PP2C and the C-terminal domain of SpoIIE reveals only 10 identical residues which are all clustered within the putative active site area. The residues in SpoIIE that are equivalent to residues presumed to have a functional importance in human PP2C [2] were chosen for replacement by in vitro mutagenesis. In addition, invariant residues equivalent to Thr-128 and Gly-240 were replaced by Ala. Mutations were confirmed by a sequence analysis and the resulting plasmids were transformed into E. coli C41(DE3) in order to express the mutated SpoIIE proteins. Western blot analysis with anti-SpoIIE polyclonal antibodies [13] revealed no apparent differences in the extent of expression of wild-type and mutated SpoIIE (Fig. 3A). Some examples of the activities of the mutated proteins are shown in Fig. 3B. The assay of SpoIIAA-P
dephosphorylating activity revealed that residues equivalent to Asp-38, Asp-60, Asp-239 and Asp-282 of the human PP2C are important for the enzymatic activity. Replacement by Ala of any one of these residues in SpoIIE (Asp-610, Asp-628, Asp-746 or Asp-795) results in inactive or nearly inactive mutant proteins. However, replacement of the same residues by Glu results in mutated proteins with varying residual activities. D628E and D746E were without a detectable activity, but D610E and D795E display some residual activity. Gly-747, which is adjacent to Asp-746, also seems to be crucial for the enzymatic activity. G747A was significantly reduced in its enzymatic activity whereas T679A and G629A were not affected. Replacement by Ala or Arg of Lys-602, which was thought to be equivalent to Arg-33 in human PP2C, was also without an effect on the enzymatic activity (Fig. 3C).

### 3.3. A proposed reaction mechanism of the reaction catalyzed by SpoIIE

We have confirmed that the bivalent metal ions Mn\(^{2+}\) or Mg\(^{2+}\) are essential for the phosphatase activity of SpoIIE, as has been demonstrated for the homologous eukaryotic protein phosphatases 2C [2,12]. However, fewer amino acid residues seem to be functionally involved in SpoIIE than in human PP2C. No functional importance was detected for residues equivalent to Arg-33 and Thr-128, for example.

In the model proposed for human PP2C, five acidic amino acids coordinate two Mn\(^{2+}\) ions at the catalytic site. Four of them, Asp-38, Asp-60, Asp-239 and Asp-282, are invariant in all eukaryotic and prokaryotic protein phosphatases 2C presently known [14]. Recently, it has been shown that substitutions in residues equivalent to Asp-60 and Asp-239 completely abolish the activity of mouse PP2C \(\beta\) in vitro [12]. The results described here confirm that the four invariant Asp are also essential in the prokaryotic relatives of PP2C, while replacement of Gly-629...
(which is equivalent to Gly-61) by Ala did not affect the enzymatic activity of SpoIIE. However, this last result does not necessarily exclude the possibility that Gly-629 is functionally important, since the carbonyl oxygen atom in G629A, proposed to form a hydrogen bond to the metal ion center, remains at approximately the same position. It was inferred from the molecular architecture of human PP2C that Glu-37 was involved in an indirect coordination of the second metal ion via a water molecule [2]. However, that residue is not conserved in prokaryotic PP2C. The replacement of Glu-37 by Ala in mouse PP2C has only minor effects on the activity of mouse PP2C [12], suggesting that Glu-37 is not crucial for the functioning of PP2C. Thr-128 is invariant in all PP2C presently known and it has been proposed that it fixes the position of Asp-60 by hydrogen bonding with the main chain NH [2]. However, replacement of the equivalent Thr-679 by Ala did not affect the kinetics of the enzyme. On the other hand, Gly-747, which is adjacent to Asp-746 (equivalent to Asp-239), seems to be necessary for the proper coordination of the metal ion binding center since its
replacement by Ala did significantly reduce the phosphatase activity.

4. Conclusions

These studies confirm the molecular mechanism for metal ion-catalyzed dephosphorylation proposed for human PP2C [2] also for its prokaryotic counterpart Bacillus SpoIIE. The main features of this model are metal bound water molecules that act as nucleophile and proton donor and a binuclear metal binding center which coordinates and stabilises the more nucleophilic hydroxide ion species. SpoIIAA-P dephosphorylation is likely to be achieved by protonation of the oxygen of the P-O scissile bond that links the phosphate group with Ser-58 of SpoIIAA [15]. No amino acid residues coordinate the phosphate ion either directly or via indirect water-mediated contacts. The only invariant catalytic site residues among the PP2C-like protein phosphatases participate in metal ion coordination. Most crucial among the four invariant Asp residues in SpoIIE are Asp-628 and Asp-746. Asp-628, the equivalent of Asp-60 in human PP2C, coordinates both metal ions via bidentate binding and Asp-746, the equivalent of D239 in human PP2C, forms a direct coordination to one metal ion and a second water-mediated contact (Fig. 4). A Glu residue in either of these positions would be unable to maintain both of the interactions in which Asp is involved. By contrast, Asp-610 and Asp-795 (the equivalents of Asp-38 and Asp-282) each form only one coordination bond to the binuclear metal binding center. It follows that D628E and D746E would be extremely sensitive to slight steric changes of their reactive side chains.

Acknowledgments

The first cloning and expression experiments of SpoIIE were performed during a research stay of R.B. in the laboratory of M.D.Y, Oxford. R.B. wishes to thank the members of the laboratories of M.D.Y and of Jeff Errington, Dunn School of Pathology, for the generous support and hospitality. We are especially indebted to Dr Andrea Feucht, Dunn School of Pathology, Oxford for generously supplying purified SpoIIE\textsubscript{323–827} and antibodies raised against the SpoIIE\textsubscript{323–827} protein. We thank David Barford for critical comments on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft, the Medical Research Council and the Biotechnology and Biological Sciences Research Council.

References

Bifunctional protein required for asymmetric cell division and cell specific transcription in Bacillus subtilis. Genes Dev. 10, 794-803.

