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Discovery of a protein phosphatase activity encoded in the genome of bacteriophage λ

Probable identity with open reading frame 221

Patricia T. W. COHEN and Philip COHEN
Department of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland, U.K.

Infection of *Escherichia coli* with phage λgt10 resulted in the appearance of a protein phosphatase with activity towards ^{32}P-labelled casein. Activity reached a maximum near the point of cell lysis and declined thereafter. The phosphatase was stimulated 30-fold by Mn^{2+}, while Mg^{2+} and Ca^{2+} were much less effective. Activity was unaffected by inhibitors 1 and 2, okadaic acid, calmodulin and trifluoperazine, distinguishing it from the major serine/threonine-specific protein phosphatases of eukaryotic cells. The λ phosphatase was also capable of dephosphorylating other substrates in the presence of Mn^{2+}, although activity towards ^{32}P-labelled phosphorylase was 10-fold lower, and activity towards phosphorylase kinase and glycogen synthase 25–50-fold lower than with casein. No casein phosphatase activity was present in either uninfected cells, or in *E. coli* infected with phage λgt11. Since λgt11 lacks part of the open reading frame (*orf*) 221, previously shown to encode a protein with sequence similarity to protein phosphatase-1 and protein phosphatase-2A of mammalian cells [Cohen, Collins, Coulson, Berndt & da Cruz e Silva (1988) Gene **69**, 131–134], the results indicate that ORF221 is the protein phosphatase detected in cells infected with λgt10. Comparison of the sequence of ORF221 with other mammalian protein phosphatases defines three highly conserved regions which are likely to be essential for function. The first of these is deleted in λgt11.

INTRODUCTION

The genome of bacteriophage λ contains at least 62 open reading frames (*orfs*), but only about 50 of these have been assigned a function [1]. The remainder include a series of eight overlapping *orfs* in the *ninR* region of the genome, which lie between the t_{R2} termination signal and gene *Q* [2,3]. One of these (*orf*221) encodes a putative protein of 221 residues and is homologous to a *nin* cistron of bacteriophage φ80 [3]. We recently reported that the *N*-terminal half of the protein encoded by *orf*221, and the homologous gene in φ80, showed 35% identity (49% similarity if highly conservative differences were included) to protein phosphatase-1 (PP-1) or protein phosphatase-2A (PP-2A) [4], two of the major serine/threonine specific protein phosphatases of mammalian cells (reviewed in [5]). In this paper we demonstrate that a protein phosphatase activity is indeed encoded in the genome of bacteriophage λ, present evidence that this enzyme is the protein product of *orf*221, and discuss its possible roles in the propagation of bacteriophage λ.

EXPERIMENTAL

Materials

Inhibitor 1 and inhibitor 2 were purified from rabbit skeletal muscle [6] and calmodulin from sheep brain [7] by Drs. Michael Hubbard and Nicholas Tonks in this laboratory. Okadaic acid was a gift from Dr. Y. Tsukitani (Fujisawa Chemical Company, Japan) and trifluoperazine was obtained from Smith, Kline and French, U.S.A.

Infection of *E. coli* cells with phage λgt10 and λgt11

E. coli (2×10^9 cells) from an overnight culture in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) containing 0.2% maltose at pH 7.5 were resuspended in 0.6 ml of 10 mM-magnesium sulphate. Half of the suspension was incubated for 15 min at 37 °C after the addition of 10^7 plaque-forming units of the appropriate λ phage, while the other half was incubated without phage. Each sample was then diluted into 50 ml of LB medium, pH 7.0, containing 10 mM-magnesium sulphate, and incubated at 37 °C. Aliquots (1.5 ml) were withdrawn every 30 min and the cells pelleted by centrifugation for 5 min at 10000 *g*. After removal of the supernatant, cells were lysed by sonication for 50 s in 0.2 ml of 50 mM-Tris/HCl (pH 7.5) containing 1.0 mM-EDTA, 1.0 mM-EGTA and 0.1% 2-mercaptoethanol, centrifuged for 10 min at 6000 *g* (4 °C) and the supernatant removed for analysis.

Preparation of ^{32}P-labelled substrates (10^6 c.p.m./nmol) and phosphatase assays

^{32}P-casein [8] and ^{32}P-phosphorylase kinase [9] were prepared by phosphorylation with cyclic AMP-dependent protein kinase. ^{32}P-phosphorylase [10] and ^{32}P-glycogen synthase [9,11] were labelled with phosphorylase kinase and glycogen synthase kinase 3, respectively. These four substrates were made by Mr. D. Schelling. Protein phosphatase activity was measured by the release of ^{32}P radioactivity from ^{32}P-labelled substrates as in [8] and [10], at a 6-fold final dilution of the cell extracts;

Abbreviations used: PP, protein phosphatase; *orf*, open reading frame; phosphatase substrates are denoted as, e.g., ^{32}P-casein to mean casein [^{32}P]phosphate.

1 unit of activity was that amount which catalysed the dephosphorylation of 1.0 μmol of substrate in 1 min.

RESULTS AND DISCUSSION

E. coli C600 (*F⁻, thi-1, thr-1, leuB, lacY1, tonA21, supE44*) cells were infected with phage λgt10 (*imm434, b527*) [12] and cell lysates examined at various times for released trichloroacetic acid-soluble ^{32}P radioactivity from ^{32}P-casein. A ^{32}P-releasing activity was detected in infected cells which reached a peak at 2.5 h, close to the time of cell lysis, and declined thereafter (Fig. 1). By contrast, there was no detectable activity in uninfected cells (not shown). Nearly all the released ^{32}P radioactivity could be complexed to molybdate and extracted into butan-2-ol/benzene (1:1, v/v), demonstrating that the product was inorganic phosphate and not trichloroacetic acid-soluble phosphopeptides. Released ^{32}P radioactivity also comigrated with inorganic phosphate during thin layer electrophoresis at pH 1.9 (not shown; performed by Dr. D. G. Campbell). These experiments demonstrated that the activity detected in infected cells was a protein phosphatase and not a proteinase.

The casein phosphatase activity was almost completely dependent on Mn^{2+} and was unaffected by inhibitor-1 (0.1 μM) or inhibitor-2 (0.2 μM) which inactivate mammalian PP-1 [5], okadaic acid (1 μM) which inactivates PP-1 and PP-2A [13,14] and trifluoperazine which inactivates PP-2B. Ca^{2+} (in the presence or absence of calmodulin) or Mg^{2+} were far less effective than Mn^{2+} (Table 1), distinguishing the activity in infected cells from mammalian PP-2B and PP-2C [5].

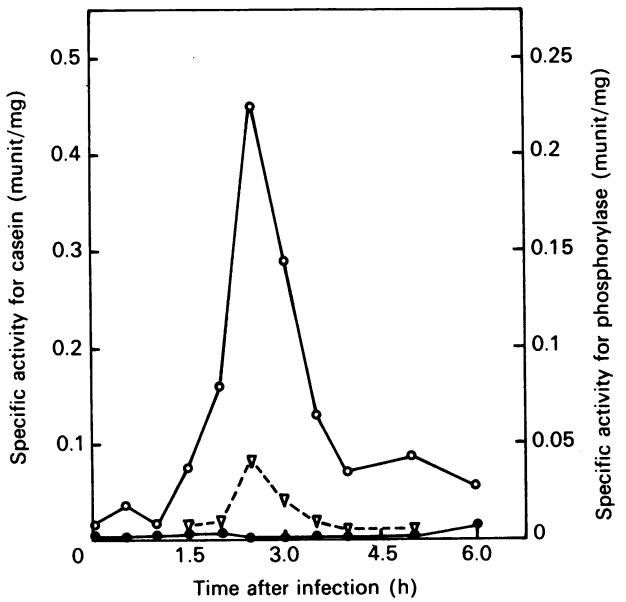

Fig. 1. Protein phosphatase activities in cell extracts prepared from *E. coli* infected with λ phages

(○) Casein phosphatase and (▽) phosphorylase phosphatase specific activities after infection of *E. coli* C600 cells with phage λgt10; (●) casein phosphatase specific activity after infection of *E. coli* Y1088 cells with phage λgt11. Protein phosphatase activities were assayed with 4 μM-^{32}P-casein in the presence of 2 mM-$MnCl_2$. Protein was measured according to Bradford [23]. The time of cell lysis is between 2.5 and 3 h for phage λgt10 and between 2 and 2.5 h for λgt11. There was no detectable casein phosphatase activity in uninfected cells at any time up to 6 h. Similar results were obtained in four different experiments with λgt10 and two with λgt11.

Table 1. Effect of divalent cations on the casein phosphatase activity of phage λgt10

Further details are given in the legend to Fig. 1 and in the Experimental section. Where added, the concentration of calmodulin was 0.2 μM. Similar results were obtained with three different preparations.

Cation	Concentration (mM)	Relative activity (%)
None	0	3
Magnesium	2	7
Magnesium	10	15
Calcium	2	5
Calcium + calmodulin	2	4
Manganese	2	100

Phosphatase activity could also be detected in infected (but not in uninfected) cells with other substrates commonly used to assay mammalian phosphatases, such as glycogen phosphorylase. Activity towards phosphorylase was also Mn^{2+}-dependent and showed a similar rise and fall through the lytic cycle (Fig. 1). ^{32}P-phosphorylase (10 μM) was about 10-fold less effective than ^{32}P-casein (4 μM) as a substrate (Fig. 1), while ^{32}P-phosphorylase kinase (1 μM) and ^{32}P-glycogen synthase (1 μM) were 25–50-fold less effective than casein (not shown).

The maximum specific activity, reached 2.5 h after infection, was 0.45 munit/mg with casein as substrate and in the presence of Mn^{2+} (Fig. 1). This is similar to the casein phosphatase activity of PP-1 and PP-2C in rat liver extracts [15], indicating that the λ enzyme is likely to be a significant protein phosphatase activity *in vivo*.

Although the above experiments demonstrated the appearance of a protein phosphatase activity in *E. coli* during infection by bacteriophage λ, they did not show that it was catalysed by the protein product of *orf*221. *E. coli* Y1088 cells [*supE, supF, metB, trpR, hsdR⁻, hsdM⁺, tonA21, strA, ΔlacU169, proC::Tn5(pMC9)*] were therefore infected with bacteriophage λgt11 (*lac5, cI857, nin5, S100*) [12]. The *nin*5 mutation deletes part of the *ren* gene, the t_{R2} terminator, seven overlapping *orf*s and part of the eighth (*orf*221) in the *ninR* region ([2,3], Fig. 2). No casein phosphatase activity was detected in cells infected by phage λgt11, although replication was slightly faster than with λgt10, cell lysis occurring at 2–2.5 h with λgt11, as compared to 2.5–3 h after infection with λgt10. These experiments localize the phosphatase activity in phage λgt10 to the *ninR* region, or to a section of the *b* region replaced by *lacZ* in λgt11 (genes *lom*, *orf*401, *orf*314, *orf*194 and *Ea*47). In conjunction with the sequence similarity between *orf*221 and PP-1/PP-2A [4], the data strongly suggest that *orf*221 does indeed encode a protein phosphatase. The *nin*5 deletion removes bases 40502–43307 in the map of Daniels *et al.* [1]. Since the *ren* gene is translated in phase 2, and *orf*221 in phase 3, removal of these bases produces a DNA sequence that should encode a fusion protein consisting of the first 74 amino acids of the *ren* product and the last 190 of ORF221.

Although this paper represents the first report of a

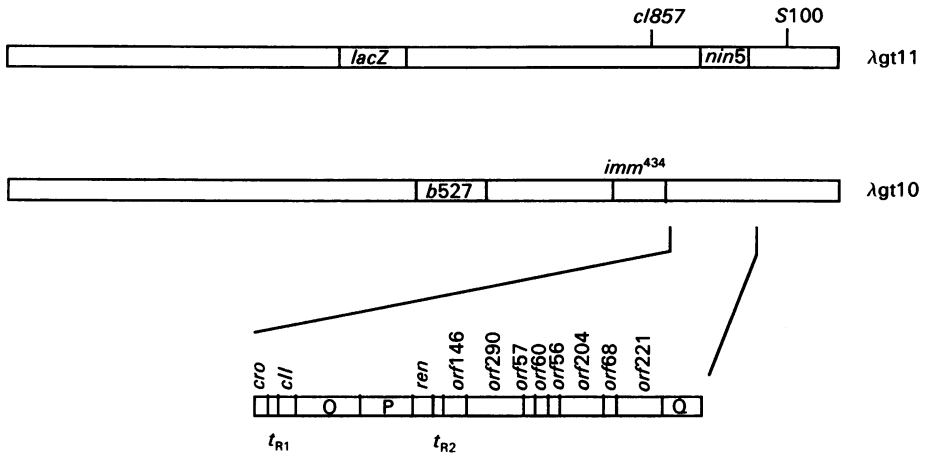

Fig. 2. Gene map of phage λgt10 and λgt11

In λgt10 the *b*527 deletion removes DNA sequences between 49.1% and 57.4% on the wild type λ map. The *imm*^{434} substitution replaces DNA sequences between 72.9% and 79.3%. In λgt11, *cI857* and *S*100 are point mutations in genes *cI* and *S*, respectively, *lacZ* gene replaces sequences between 39.9% and 48%, and *nin*5 deletes sequences between 83.5 and 91.5% (bases 40 502–43 307 on the map of Daniels *et al.* [1]). The region between gene *cro* and gene *Q* in λgt10 is expanded below the gene map. Part of this region is removed by the *nin*5 deletion in λgt11 as discussed in the text.

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R.PP-1α     M S D S E K L N L D S I I G R L L E V Q G S R P G K N V Q L T E N E I R G L C L K S R E I F L S Q P   50
D.PP-1α       M G D V M N I D S I I S R L L E V R G A R P G K N V Q L S E G E I R G L C L K S R E I F L S Q P   48
D.PP-Y          M A V L T T H E I D C I I K E L T S L N G S E C T L K E E L I E R L I Q Q T R E V I K W Q P   46
R.PP-2Aα              M D E K V F T K E L D Q W I E Q L N E C K Q L S E S Q V K S L C E K A K E I L T K E S   43
λORF221                                                                                 M R Y Y E K    6
φ80                                                                                     M S M Y Q R    6

R.PP-1α     I L L E L E A P L K I C G D I H G Q Y Y D L L R L F E Y G G F P P E S N Y L F - L G D Y V D R G K Q   99
D.PP-1α     I L L E L E A P L K I C G D I H G Q Y Y D L L R L F E Y G G F P P E S N Y L F - L G D Y V D R G K Q   97
D.PP-Y      M L L E L Q A P V N I C G D I H G Q F T D L L R I F K A C G F P P K A N Y L F - L G D Y V D R G K Q   95
R.PP-2Aα    N V Q E V R C P V T V C G D V H G Q F H D L M E L F R I G G K S P D T N Y L F - M G D Y V D R G Y Y   92
λORF221     I D G S K Y R N I W V V G D L H G C Y T N L M N K L D T I G F D N K K D L L I S V G D L V D R G A E   56
φ80         I N G A D W R N I F V V G D L H G C Y T L L M N E L E K V S F D P A C D L L I S V G D L V D R G A E   56

R.PP-1α     S L E T I C L L L A Y K I K Y P E N F F L L R G N H E C A S I N R I Y G F Y D E C K R R Y - N I K L  148
D.PP-1α     S L E T I C L L L A Y K I K Y S E N F F L L R G N H E C A S I N R I Y G F Y D E C K R R Y - S I K L  146
D.PP-Y      S L E T I C L L F A Y K V K Y P L N F F L L R G N H E C A S I N K I Y G F Y D E I K R R H - T V R L  144
R.PP-X                                P D R I T L I R G N H E S R Q I T Q V Y G F Y D E C L R K Y G S V T V
R.PP-2Aα    S V E T V T L L V A L K V R Y R E R I T I L R G N H E S R Q I T Q V Y G F Y D E C L R K Y G N A N V  142
λORF221     N V E - - C L E L - - - I T F P - W F R A V R G N H E Q M M I D G L S E R G N V N H W L L - N G G G   99
φ80         N V E - - C L E L - - - I T M P - W F R A V R G N H E Q M M I D G L S E Y G N V N H W L E - N G G V   99

R.PP-1α     W K T F T D C F N C L P V A A I V D E K I F C C H G G L S P D L Q S M E Q I R R I M R P T D V P D Q  198
D.PP-1α     W K T F T D C F N C L P V A A I V D E K I F C C H G G L S P D L T S M E Q I R R I M R P T D V P D Q  196
D.PP-Y      W H N F T D C F N W L P V A A L V G E R I F C C H G G L S P S L R N L Q Q I N H I Q R P T D I P D E  194
R.PP-X      W R Y C T E I F D Y L S L S A I I D G K I F C V H G G L S P S I Q T L D Q I R T I D R K Q E V P H D
R.PP-2Aα    W K Y F T D L F D Y L P L T A L V D G Q I F C L H G G L S P S I D T L D H I R A L D R L Q E V P H E  192
λORF221     W F F N L D Y D K E I L A K A L A H K A D E L P L I I E L V S K D K K Y V I C H A D Y P F D E Y E F  149
φ80         W F F S L D Y E K E V L A K A L V H K S A S L P F V I E L V T A E R K I V I C H A D Y P H N E Y A F  149

R.PP-1α     G L L C D L L W S D P D K D V Q G W G E N D R G V S F T F G A E V V A K F L H K H D L D L I C R A H  248
D.PP-1α     G L L C D L L W S D P D K D T M G W G E N D R G V S F T F G A E V V A K F L Q K H E F D L I C R A H  246
D.PP-Y      G I M C D L L W A D L N H T T K G W G H N D R G V S F T F D K V I V R D F L K A F D L Q L M V R A H  244
R.PP-X      G P M C D L L W S D P E - D T T G W G V S P R G A G Y L F G S D V V A Q F N A A N D I D M I C R A H
R.PP-2Aα    G P M C D L L W S D P D - D R G G W G I S P R G A G Y T F G Q D I S E T F N H A N G L T L V S R A H  241
λORF221     G K P V D H Q Q V I W N R E R I S N S Q N G - I V K E I K G A D T F I - F G H T P A V K P L K F A N  198
φ80         D K P V P K D M V I W N R E R V S D A Q D G - I V S P I A G A D L F I - F G H T P A R Q P L K Y A N  198

R.PP-1α     Q V V E D - G Y E F F A K R Q L Y T L F S A P N Y C G E F D N A G A M M S V D E T L M C S F Q I L K  297
D.PP-1α     Q V V E D - G Y E F F A K R M L V T L F S A P N Y C G E F D N A G A M M S V D D T L M C S F Q I L K  295
D.PP-Y      E V V E D - G Y E F F A N R Q L Y T V F S A P N Y C G M M N N A G G V M S V S T D L I C S F V I I L  293
R.PP-X      Q L V M E - G Y K W H F N E T V L T V W S A P N Y C Y R C G N V A A I L E L D E H L Q K D F I I F E
R.PP-2Aα    Q L V M E - G Y N W C H D R N V V T I F S A P N Y C Y R C G N Q A A I M E L D D T L K Y S F L Q F D  290
λORF221     Q M Y I D T G A V F C G N L T L I Q V Q Q G E G A                                                  221
φ80         Q M Y I D T G A V F C G N L T L V Q V Q Q G G A H A                                                222

R.PP-1α     P A D K N K - G K Y G Q F S G L N P G G R P I T P P R N S A K A K K                                  330
D.PP-1α     P A D K R K K                                                                                      302
D.PP-Y      P C H K Y K - M I A T D A N Q M P T N E E E                                                        314
R.PP-X      A A P Q E T R G I P S K K P V A D Y F L
R.PP2Aα     P A P R R G - E P H V T R R T P D Y F L                                                             309
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Fig. 3. Sequence similarities between rabbit (R), *Drosophila* (D) and bacteriophage protein phosphatases

Identities are boxed and conservative replacements underlined. Only the α isoforms of PP-1 [24] and PP-2A [22] are shown. PP-X is the translated sequence of a cDNA clone isolated from a rabbit liver library, which encodes part of a putative protein phosphatase more closely related to PP-2A than PP-1 [25]. D.PP-Y [26], like D.PP-1 [27], was isolated from a *Drosophila* head library, and is more closely related to PP-1 than PP-2A. The sequence of λORF221 is taken from [2,3] and φ80 from [3] and G. Hobom & R. Grosschedl, personal communication).

protein phosphatase activity encoded in the genome of any bacteriophage, serine/threonine-specific protein kinases are known to be encoded in other bacteriophages, such as T7 [16]. Mutants that lack the T7-encoded protein kinase are able to propagate normally under optimal growth conditions, but exhibit a low or non-existent burst size under suboptimal conditions [16]. The T7 protein kinase phosphorylates many *E. coli* proteins, including DNA-dependent RNA polymerase [16] and RNAase III [17]. Phosphorylation of the former causes inactivation of the polymerase, allowing the T7-encoded RNA polymerase to operate more effectively. Phosphorylation of RNAase III stimulates its activity 4-fold, and is thought to contribute to the early control of host transcription.

It is now clear that protein phosphorylation is an important regulatory mechanism in bacteria [18] and over 100 phosphoproteins have been detected by two-dimensional peptide mapping of *E. coli* extracts [19]. By analogy with the T7 protein kinase, it is likely that the protein phosphatase encoded by λ dephosphorylates certain *E. coli* proteins, altering their activities to allow more effective production of phage RNA or protein under some conditions. Alternatively, or in addition, the λ protein phosphatase may regulate viral transcription. At the start of the lytic life cycle of λ, the immediate early gene *N* is transcribed, which encodes an anti-terminator protein that binds to *E. coli* RNA polymerase, allowing it to read through the phage terminator sequences t_{R1} and t_{R2} into the delayed early genes, which comprise the eight overlapping *orf*s (including *orf*221) and gene *Q*. Gene *Q* itself encodes an anti-terminator protein which allows readthrough into the late genes specifying cell lysis and phage coat proteins [20]. It is therefore of interest that the mutation *byp*, which probably lies within *orf*221, has been shown to increase the transcription of gene *Q* and the late genes in the absence of the *N* gene product [21]. As a consequence the *byp* mutation results in clear plaque morphology and reduced lysogenization frequency. It will clearly be important to determine whether any of the anti-terminator proteins (some of which are encoded in the *E. coli* and some in the phage genome) are phosphorylated *in vivo* and dephosphorylated by ORF221.

The primary structures of the serine/threonine-specific protein phosphatases whose sequences have been determined in our laboratory are compared with the sequences of ORF221 and the protein product of the homologous gene in ϕ80 in Fig. 3. The sequence similarities between ORF221 and PP-1/PP-2A are much greater than those between alkaline phosphatases and PP-1/PP-2A, which are barely significant [22]. In addition to sequence similarities between the mammalian and bacteriophage protein phosphatases over the first 115 residues of the latter [4], similarity can be seen to extend to the *C*-terminal region of ORF221. There are some 11 additional residues where amino acid residues in all seven enzymes are either invariant or highly conservative replacements. Overall, Fig. 3 defines 26 identical and 24 conserved residues that may be essential for the catalytic activity of protein phosphatases. The three most highly conserved regions are Gly^{19}–Gly^{23}, Val^{46}–Gly^{53} and Val^{72}–Glu^{77} of ORF221 (Fig. 3). The absence of the first 28 amino acids of ORF221 in λgt11, including one of the conserved sections (amino acids 19–23), may account for the lack of phosphatase activity. It will clearly be important to apply site-directed mutagenesis to examine the roles of this and the other conserved regions in enzymic function.

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