Casein kinase II phosphorylates Ser^{468} in the PEST domain of the *Drosophila* IκB homologue cactus

Leonard C. Packman, Ken Kubota[1], James Parker, Nicholas J. Gay*

Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

Received 9 October 1996

Abstract Cactus protein is a *Drosophila* homologue of the mammalian IκB family of cytoplasmic anchor proteins. In unstimulated cells they function to retain rel/NFκB transcription factors in the cytoplasm but are rapidly degraded in response to signalling. The destruction of cactus or IκBα allows the rel/NFκB transcription factor to relocalise to the nucleus. Cactus is a phosphoprotein and has in its C-terminus a PEST protein stability domain. In this paper we show that, like mammalian IκBα, the PEST domain of cactus is phosphorylated by casein kinase II. We have localised the site of modification to a single residue, Ser^{468}, and find no evidence for additional phosphorylation sites. The conservation of these sites in mammalian and invertebrate cytoplasmic anchor proteins suggests that phosphorylation by casein kinase II may play a critical functional role, plausibly in the regulation of constitutive or inducible proteolysis.

Key words: IκB cytoplasmic anchor protein; Casein kinase II phosphorylation; Protein stability

1. Introduction

The cactus protein is a *Drosophila* homologue of the mammalian IκB family of cytoplasmic anchor proteins [1,2]. During precellular embryonic development, cactus is present in the cytoplasm as heterotrimeric complexes with the rel/NFκB transcription factor dorsal [3]. At the syncytial blastoderm stage a signal transduction pathway mediated by the transmembrane receptor Toll is activated at ventral positions in the embryo. These signalling events lead to the dissociation of the dorsal/cactus complexes and the migration of dorsal protein into ventral nuclei. This causes a ventral to dorsal gradient of the transcription factor to form, a gradient which directs the subsequent development of dorso-ventral pattern in the cellular embryo [4].

Cactus is a phosphoprotein of M_r 55 kDa [1,2]. The predicted sequence contains an acidic domain in the N-terminus and in the C-terminus six copies of ankyrin repeat sequences followed by a PEST domain. In mammalian IκBα, phosphorylation of two serine residues in the N-terminus in response to signalling targets the protein to the proteasome degradation pathway (see for example [5]). In cactus these residues are conserved [6] and there is evidence that cactus is destabilised in response to signalling [6,7]. Thus the terminal steps in signalling to rel/NFκB transcription factors may be highly conserved in evolution.

*Corresponding author. Fax: (44) (1223) 333345.
E-mail: njg11@mole.bio.cam.ac.uk

[1] *Present address:* Laboratories of Infectious Disease and Immunology, Yamanouchi Pharmaceutical Co., Ltd, Miyuki-ga-Oka 21, Tsukuba, Ibaraki 305, Japan.

Recently, it has been shown that IκBα is phosphorylated constitutively by casein kinase II (CKII) at 4 sites in the PEST protein stability sequences (Ser^{283}, Ser^{289}, Ser^{293} and Thr^{291}) [8–11]. These phosphorylation sites are conserved in the equivalent sequences of cactus and may be required for signal-induced dissociation of the heterotrimeric dorsal/cactus complexes. Thus, constitutive phosphorylation of cactus and IκBα by CKII may be important for regulating signal-induced and signal-independent degradation.

In this paper we show that cactus protein is a substrate for CKII and localise the site of modification. We also show that phosphorylation by CKII does not cause a shift in the electrophoretic mobility of the cactus protein and that a constitutive kinase present in *Drosophila* tissue culture cells, of the same molecular mass as CKII, phosphorylates cactus.

2. Materials and methods

2.1. Expression and purification of maternal/zygotic cactus protein

Maternal/zygotic cactus protein was expressed in *E. coli* from the plasmid pCACTEB (the gift of Dr R. Geisler) [2]. Cactus was purified as previously described [12] except that the gel filtration step was omitted and ion exchange chromatography was carried out in 6 M urea. The preparation did not have significant amounts of purine nucleotide binding or hydrolysis activities.

2.2. In-gel kinase assay

In order to identify unknown kinases for a specific substrate, an in-gel kinase assay was used [13,14]. In this method, kinases in a cell extract are first fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The gel is polymerised with the substrate under test (1 mg ml^{-1}). Cell extracts were solubilized in SDS sample buffer at 70°C for 10 min and applied to the polyacrylamide gels. Proteins were renatured following electrophoresis. First, SDS was removed by washing the gels at room temperature with gentle shaking, using two changes of 100 ml of 20% isopropanol, 50 mM Tris-HCl (pH 8.0) for 1 h and once in 250 ml of 5 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0) for 1 h. Then the gels were soaked in two changes of 100 ml of 6 M guanidine-HCl, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0) for 1 h at room temperature followed by soaking in five changes of 250 ml of 0.04% Tween 40, 5 mM 2-mercaptoethanol, 50 mM Tris-HCl (pH 8.0) at 4°C over a period of 16 h. In order to detect the positions of kinases in the gel, the gels were pre-incubated in 3.5 ml of 0.1 mM EGTA, 5 mM magnesium acetate, 0.15 mM $CaCl_2$, 2 mM dithiothreitol, 40 mM HEPES-NaOH (pH 8.0) for 30 min at 22°C and incubated in the same volume of 0.1 mM EGTA, 5 mM magnesium acetate, 0.15 mM $CaCl_2$, 50 μM ATP, 50 μCi [γ-^{32}P]ATP for 1 h at the same temperature in a sealed plastic bag. To remove the excess ATP and fix proteins, the gel was incubated for 5–10 min in 5% trichloroacetic acid, 1% sodium pyrophosphate at room temperature. This incubation was repeated 5–7 times. The gels were dried and exposed to Fuji NIF X-ray film or analysed by a Phosphor-imager.

2.3. Gel electrophoresis

Cactus isoforms were separated in 7 or 10% SDS-PAGE with low amounts of cross-linker (see [15]).

PII S0014-5793(96)01324-5

2.4. In vitro phosphorylation by casein kinase II

A homogeneous preparation of type II casein kinase holoenzyme purified from *Drosophila melanogaster* embryos was the kind gift of Dr Claiborne Glover and Dr Ashok Bidwai. The enzyme was diluted with 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 200 mM NaCl, 0.05% Triton X-100, 10% (w/v) glycerol, immediately before use. The reactions were carried out in 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 0.1 M NaCl, 20 μM [γ-^{32}P]ATP (4000 cpm/pmol), 0.1–1 mg/ml substrate protein (see [16]). For electrophoresis, the reactions were terminated by adding SDS-PAGE sample buffer and boiled for 5 min. For the determination of initial rates, either [γ-^{32}P]GTP (Amersham) or [γ-^{32}P]ATP was added at concentrations between 1 and 200 μM. The reactions were stopped after 15 min at 18°C by spotting the entire reaction mixture onto a glass fibre filter and immersing it in 20% trichloroacetic acid, 0.1 M sodium pyrophosphate. The filters were then washed, dried and counted as previously described [16]. The data were fitted to Michaelis-Menten kinetics and values of V_{max} and K_m calculated using the sums of squares method.

2.5. Generation and purification of specific phosphopeptides

Cactus protein was phosphorylated and then digested with chymotrypsin (final concentration 10 μg ml^{-1}) at 37°C for 3 h. The digest was adjusted to 10% acetic acid, centrifuged and the supernatant applied to a reverse phase column (Spherisorb ODS2, 3×100 mm, Chrompack, London, UK) equilibrated with 0.1% trifluoroacetic acid (solvent A). Peptides were eluted with linear gradients of acetonitrile containing 0.1% trifluoroacetic acid (solvent B) as follows: 2 min, 15% B; 40 min, 35% B; 42 min, 80% B; flow 0.3 ml/min. Fractions (300 μl) were collected and a sample (5 μl) counted for radioactivity. Detection was at 214 nm using a diode array detector on a Hewlett Packard 1090M chromatograph. The absorbance and counts profiles are shown in Fig. 4A.

Peptides in fractions 24 (the principal radioactive peak) and fractions 27/28 (the minor radioactive peak) were further purified in a second-dimension high performance liquid chromatography (hplc) step at higher pH. Samples were reduced to 10–20 μl under vacuum, diluted with 200 μl of 50 mM ammonium acetate pH 6 and applied to a Spherisorb ODS2 column (1×100 mm, PhaseSeparations Ltd., Deeside, UK) equilibrated with the same buffer. Peptides were eluted with linear gradients of 50 mM ammonium acetate pH 6 containing 50% acetonitrile (solvent B′) as follows: 2 min, 15% B′; 40 min, 40% B′; 42 min, 100% B′; flow 0.075 ml/min. Fractions (75 μl) were collected and the radioactivity in sample (2 μl) determined using scintillation counting. The absorbance and radioactivity profiles are shown in Fig. 4B.

2.6. Mass spectrometry

The radioactive fractions from the second-dimension hplc runs were analysed for mass using a Kratos MALDI III laser desorption time-of-flight spectrometer in linear mode. Sample (0.5 μl) was mixed with 1 μl matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% aqueous acetonitrile/0.1% trifluoroacetic acid, containing 500 fmol/μl bovine insulin as internal calibrant) and air-dried. Each sample was washed with 5 μl water, dried and analysed in positive and negative ion mode. Masses were calculated from replicate determinations.

2.7. Amino acid sequence analysis

Sequence analysis was performed on an Applied Biosystems Pulsed Liquid Phase Sequencer model 477A. For sequence identification, samples (5–20 pmol) were immobilised on polyvinylidene difluoride membrane using ProSpin columns (Applied Biosystems division of Perkin Elmer, Warrington, UK) and analysed according to the manufacturer's instructions.

To determine the position of the radiolabel, peptide (10–20 pmol) was dried 3 times from a solution of aqueous 0.5% triethylamine, resuspended in 0.1% trifluoroacetic acid/50% acetonitrile and covalently bonded to Sequelon-DITC (Millipore, Watford, UK). This provided attachment through the side chain of a lysine only two residues from the end of the peptide. The peptide was subjected to Edman degradation, but the anilinothiazolinone (ATZ) amino acid was extracted at each cycle using methanol in place of the usual butyl chloride (in which phosphate is not soluble) and delivered to an external fraction collector for radioactive counting. A second sample was sequenced normally to assess repetitive yield.

The yield of ^{32}P at each cycle was normalised to the yield of each PTH-amino acid at that cycle to correct for the loss in signal caused by a low average repetitive yield (86.8%); for 'low yield' PTH-amino acid residues (e.g. T, S, Y), the expected yield was estimated from the average repetitive yield.

3. Results

3.1. Cactus protein is phosphorylated by a protein kinase of M_r 37 kDa

To investigate whether cactus is phosphorylated by protein kinases constitutively present in SL2 cells or induced by the activation of the Toll receptor signal transduction pathway, an in-gel kinase assay system was used. CactEB protein was polymerised into an SDS-PAGE gel and extracts of SL2 cells and SL2Tl10B cells (a stable derivative of SL2 with the F9Toll10B plasmid incorporated [17,18]) were separated in the gel by electrophoresis. Cactus is not phosphorylated by any kinases induced in SL2Tl10B cells but is modified by a protein kinase with a molecular mass of about 37 kDa which is present in all the extracts tested. The cactus sequence contains several consensus sites for CKII and the catalytic subunit of this enzyme has a mass of 37 kDa [16,20]. Thus it is likely that the phosphorylation of cactus detected by this assay is mediated by CKII.

3.2. The cactus protein is a substrate for CKII in vitro

Full-length maternal zygotic cactus protein, casein, bovine serum albumin (BSA) and dorsal were reacted in vitro with embryonic CKII and ^{32}P-labelled ATP (see Section 2). As shown in Fig. 1, casein and cactus are phosphorylated in such reactions whereas dorsal and BSA do not incorporate label. Phosphorylation of cactus by CKII is unaffected by the presence in the reaction mixture of dorsal. The dorsal and cactus proteins bind together under these conditions [12] and thus both free and bound forms of cactus appear to be substrates for CKII.

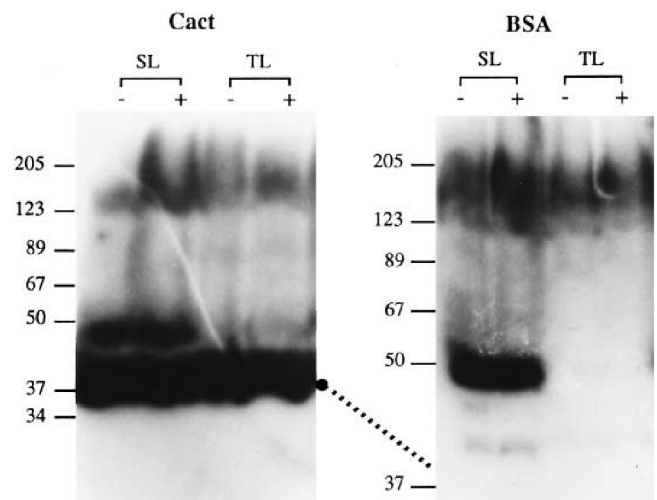


Fig. 1. A kinase activity for cactus in SL2 cells. Extracts were prepared from SL2 (SL) or STL10BH cells [15] (TL) (3×10^6 cells/lane) before (−) or after (+) heat shock and assayed for kinase activity as described in Section 2. The gels were autoradiographed (18 h, −70°C). The kinase activity for cactus is indicated on the right and the equivalent position in the control gel indicated by a dashed line.

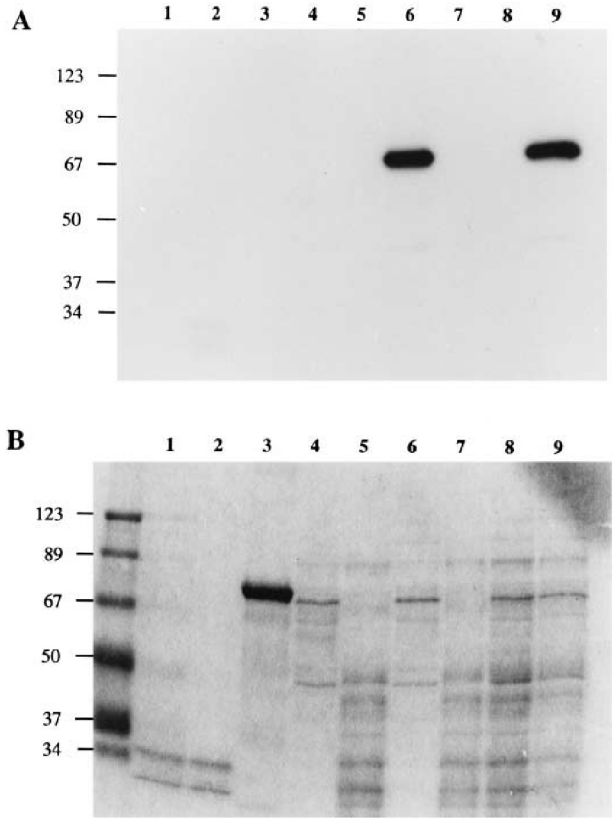


Fig. 2. Cactus is a substrate for casein kinase II in vitro. A: In vitro phosphorylation assay (see Section 2). 0.1 mg ml^{-1} of protein was added to the reaction mixtures except for BSA (1 mg ml^{-1}). Casein (lanes 1, 2), BSA (lane 3), cactus (lanes 4, 6), dorsal (lanes 5, 7) and a mixture of cactus and dorsal (lanes 8, 9). Samples were incubated for 15 min. with (lanes 2, 3, 6, 7, 9) or without (lanes 1, 4, 5, 8) purified CKII. B: Coomassie stain of the gel used in A.

3.3. CKII can phosphorylate cactus efficiently using either Mg-ATP or Mg-GTP as substrate

An unusual and possibly unique property of casein kinase II is that it can use GTP as phosphoryl donor nearly as efficiently as ATP. To confirm that the protein kinase activity phosphorylating cactus in Fig. 2 above is indeed CKII we have carried out a kinetic analysis using both ATP and GTP as substrates. The results are summarised in Table 1 and show that both purine nucleotides can be used as substrates. The values derived for K_m are closely similar to published figures for the *Drosophila* enzyme using casein as substrate (17 μM and 66 μM respectively). Furthermore, under the conditions used in these experiments the turnover number (k_{cat}) for cactus is approximately 10-fold greater than that for casein, a finding consistent with the more efficient phosphorylation of cactus protein observed in Fig. 2 (lane 12, cf. 16).

Table 1
Kinetics of reaction between CKII and cactus

Substrates	K_m (μM)	V_{max} ($\times 10^{-6}$ μM min^{-1})	k_{cat} (s^{-1})
Cactus/ATP	19	2	0.48
Cactus/GTP	70	1.5	0.36
Casein/ATP	20	0.2	0.05

3.4. Phosphorylation of cactus by CKII does not cause an electrophoretic mobility shift

We showed previously that phosphorylated isoforms of cactus with distinct electrophoretic mobility are generated in vivo subsequent to binding with dorsal [15]. To determine whether these isoforms are caused by CKII phosphorylation we have compared their mobility with that of cactus phosphorylated by CKII in vitro. As shown in Fig. 3, the mobility of the phosphorylated *E. coli* cactus protein is indistinguishable from that of the maternal zygotic isoform detected in SLDL cells (see [15]).

3.5. CKII phosphorylates Ser^{468} of cactus

In order to localise CKII sites we phosphorylated cactus and then digested the protein with chymotrypsin. The resulting peptides were fractionated by reverse phase hplc. In the first dimension the radioactively labelled peptides eluted in a major and a minor peak (see Fig. 4A). These peaks were collected, further purified in a second dimension of reverse phase hplc (see Section 2) (Fig. 4B) and the radioactively labelled fractions were subjected to mass analysis. The major radioactive peak contained a peptide of mass 2960.8 Da ± 1.0 ($n = 6$) and an unambiguous partial N-terminal sequence of GAETVTPPDSDYD; the total amount of peptide in the fraction was approximately 20 pmol. The sequence and mass corresponded well to residues 454–479 containing a single phosphorylation site (expected mass 2960.0 Da), in the PEST region adjacent to the ankyrin repeat domains; the peptide had been generated by a tryptic cut at Arg^{453} and chymotryptic cut at Tyr^{479}; clearly the chymotrypsin preparation used contained some trypsin activity. The position of the modified residue was then determined by establishing the position in the Edman degradation at which radioactivity was released from the peptide (see Section 2 for details). As shown in Fig. 4C, radioactivity was eluted from the sample at cycle

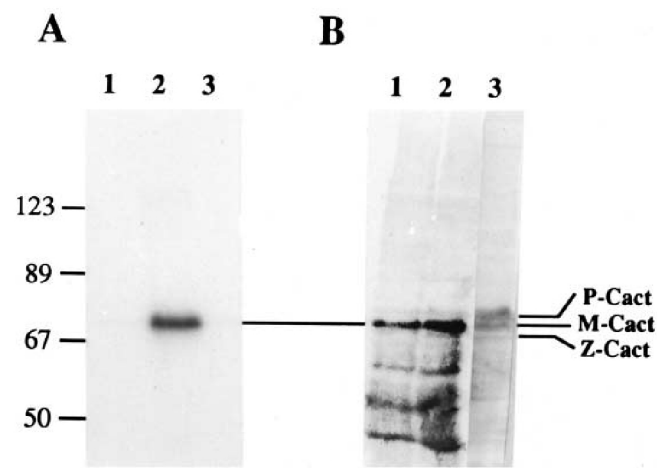
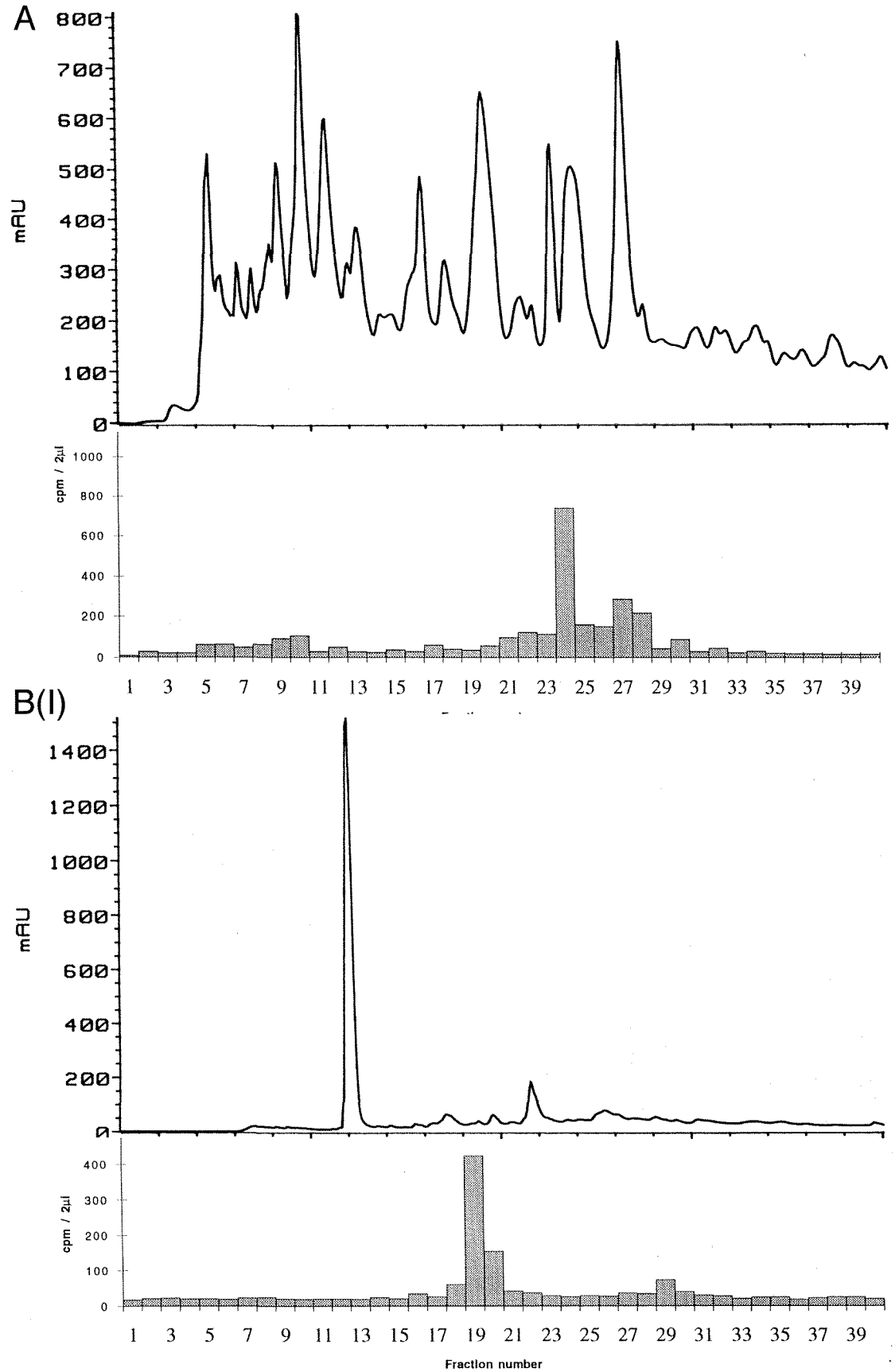


Fig. 3. Phosphorylation by casein kinase II does not alter the electrophoretic mobility of cactus. A: Cactus was incubated without (lane 1) or with (lane 2) CKII and separated by SDS-PAGE (7%) alongside an extract from SLDL cells (see [15]). The proteins were transferred to a nitrocellulose membrane and autoradiographed. B: The same membrane used in A was incubated with a rabbit anti-cactus antibody (see [12]) and a secondary antibody conjugated with horseradish peroxidase. The blot was then visualised by incubation with 0.5 mg ml^{-1} diaminobenzidine and 0.06% H_2O_2.


A
mAU
800
700
600
500
400
300
200
100
0
cpm / 2µl
1000
800
600
400
200
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
B(I)
mAU
1400
1200
1000
800
600
400
200
0
cpm / 2µl
400
300
200
100
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39
Fraction number

15, a result which shows unequivocally that the residue modified by CKII is Ser[468].

Analysis of the minor radioactive peak gave a single partial N-terminal sequence of GAETVTPPDSDYDSS and a mass of 2880.6 Da ± 0.9 (n=6), consistent with the mass of the unphosphorylated peptide (2880.0 Da); the total amount of peptide present was ≥200 pmol. As the specific radioactivity of this peptide was only some 5% of that in the major peak, the most likely explanation for the observed data was that this fraction contained mostly unphosphorylated Gly[454]-Tyr[479] and a low level of slightly longer phosphorylated peptide which coeluted on hplc. Careful negative-ion mass spectrometric analysis of the composition of fractions 20 and 21 (Fig. 4B(II)) revealed the presence of a low-level peak of mass 3372.8 Da ± 1.1. The only segment of the protein to which this mass matches (given the proteinase specificity) is Glu[451]-Tyr[479] with a single phosphorylation site (expected mass 3373.5 Da). Sequence analysis of fraction 20 gave a partial sequence of EKRG underlying the main GAETVT sequence at a relative abundance of approx. 2%. The total abundance of the longer peptide did not therefore exceed approx. 5 pmol and represented insufficient material to allow localisation of the radioactivity by Edman degradation. However, given that this sequence is clearly an incompletely di-

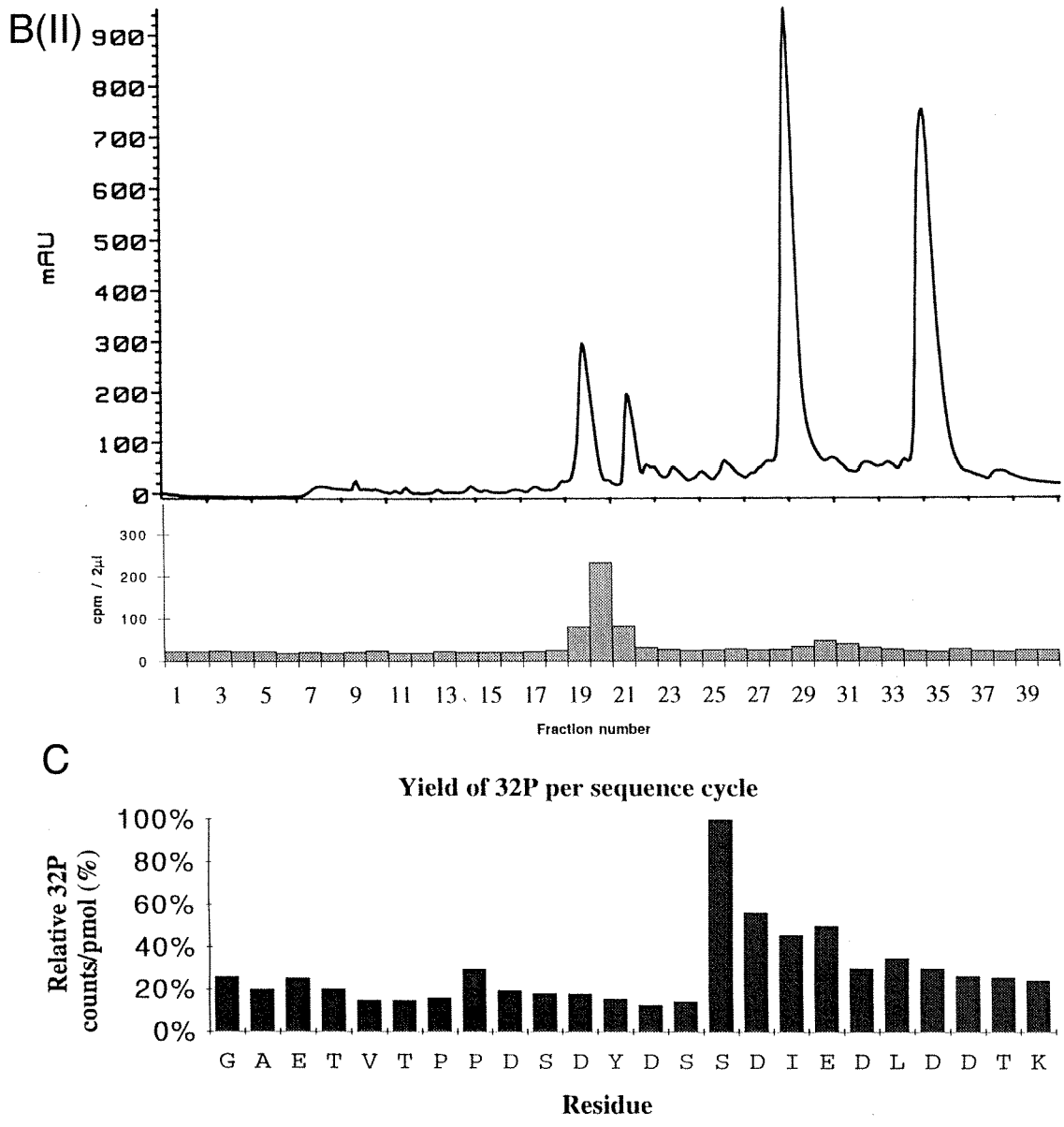


Fig. 4. Localisation of the CKII phosphorylation site in cactus. A: [^{32}P]Phosphorylated cactus was digested with chymotrypsin and the peptides separated by reverse phase hplc. Counting for radioactivity showed a major peak in fraction 24 and a minor peak in fractions 27/28. Each was further purified as shown in B. B: Second-dimension hplc of fractions 24 (I) and 27/28 (II) from A at pH 6 in 50 mM ammonium acetate and a gradient of 0–20% acetonitrile. The highly acidic peptides move to a much earlier position in the gradient at the higher pH compared to A. C: Radiosequence analysis of the major labelled peak. The yield of ^{32}P at each cycle is normalised to the yield of each PTH-amino acid as described in the text and displayed as relative counts per pmol. Ser[468] is clearly the labelled site. The sequence does not extend beyond the site of attachment to the membrane (Lys[477]).

gested form of Gly^{454}-Tyr^{479}, containing a 3-residue N-terminal extension, it is likely that the phosphorylation site in this peptide remains at Ser^{468}.

4. Discussion

In this paper we show, using an in-gel kinase assay, that a constitutive protein kinase of 37 kDa can modify cactus. The molecular mass of the catalytic α subunit of CKII is 37 kDa [20] and thus it is likely that CKII is being detected in this assay. In contrast to identical experiments where dorsal was used as substrate, no inducible protein kinase was detected. Although there is evidence that degradation of cactus is signal-dependent in the embryo [7], phosphorylation by an inducible protein kinase has not been shown. It is possible that destruction of cactus in response to signal involves activation of a protease or phosphatase and that direct phosphorylation of cactus is not required.

We also show here that cactus protein is an excellent substrate for CKII in vitro and have localised the site of phosphorylation to Ser^{468}. This appears to be the only residue modified to any great extent by CKII. Although a second minor peak of radioactivity is separated by the first dimension of hplc (Fig. 4A), our data suggest that this corresponds to a slightly longer partial chymotryptic peptide with one residue phosphorylated. We find no evidence that any of the other consensus CKII sites in the PEST domain (Thr^{459}, Ser^{463} and Ser^{467}) are phosphorylated. Furthermore, the sequence surrounding Ser^{468} has the most favourable consensus, with a glutamic acid being present three residues C-terminal to the modified serine (see [19]). It has been shown recently that the related mammalian cytoplasmic anchor protein IκBα is also phosphorylated by CKII at four sites within the C-terminal PEST region [9–11]. If the cactus and IκBα sequences are aligned using the computer program PILEUP, Ser^{468} of cactus is seen to correspond to Ser^{283} in IκBα, one of the residues phosphorylated by CKII. The other three potential CKII sites in cactus do not align with phosphorylated residues in IκBα.

Phosphorylation of cactus by CKII occurs within regions required for signal-dependent and signal-independent degradation [7]. In the case of IκBα, proteolytic mapping experiments have defined a tripartite domain structure. The ankyrin repeats form a protease-resistant core and the PEST sequences an independently folded domain which interacts closely with p65/RelA [21]. It is interesting to note that the tryptic cleavage site at Arg^{453} in cactus corresponds closely to Glu^{275} in IκBα. This residue is highly sensitive to cleavage by V8 protease and lies in a sequence proposed to form a link between the ankyrin repeat and PEST domains. Thus cactus probably has a domain structure very similar to that of IκBα. It is possible that phosphorylation of Ser^{468} may affect the recognition of the PEST motif by cellular machinery, thereby controlling the degradation of cactus. Since CKII is a constitutive kinase, if it is involved in the regulation of cactus degradation, a possible mechanism is that the PEST sequences are in a phosphorylated state before proteolysis and dephosphorylation is a signal for rapid degradation. In this regard, it should be noted that activation of calcium-dependent protein phosphatases correlates with destabilisation of cactus [6] and dephosphorylation of Ser^{468} would not be evident as CKII does not cause a shift in the electrophoretic mobility of cactus. A similar mechanism has been proposed to modulate the DNA binding activity of c-Jun and c-Myb [22,23]. PEST sequences have not yet been identified with a particular pathway of proteolysis, so it is possible that CKII phosphorylation can affect either the ubiquitin- or trypsin-mediated forms of degradation [21]. As noted above, specific phosphorylation of cactus by CKII is consistent with reports that mammalian IκBα is a substrate for this enzyme [8]. It appears that CKII phosphorylation has been conserved in evolution, further indicating that it has a functional significance. Future studies will concentrate on the relationship between inducible proteolytic degradation and phosphorylation of cactus by CKII.

Acknowledgements: K.K. was supported by a studentship from Glaxo-Japan. N.J.G. is a University Research Fellow of the Royal Society. Thanks to Mike Weldon for protein sequence analysis.

References

[1] Kidd, S. (1992) Cell 71, 623–635.
[2] Geisler, R., Bergmann, A., Hiromi, Y. and Nüsslein-Volhard, C. (1992) Cell 71, 613–621.
[3] Isoda, K. and Nüsslein-Volhard, C. (1994) Proc. Natl. Acad. Sci. USA 91, 5350–5354.
[4] Steward, R. and Govind, S. (1993) Curr. Opin. Genet. Dev. Biol. 3, 556–561.
[5] Chen, Z., Hagler, J., Palombella, V.J., Melandri, F., Scherer, D., Ballard, D. and Maniatis, T. (1995) Genes Dev. 9, 1586–1597.
[6] Kubota, K. and Gay, N.J. (1995) Biochem. Biophys. Res. Commun. 214, 1191–1196.
[7] Belvin, M., Jin, Y. and Anderson, K.V. (1995) Genes Dev. 9, 783–793.
[8] Barroga, C.F., Stevenson, J.K., Schwarz, E.M. and Verma, I.M. (1995) Proc. Natl. Acad. Sci. USA 92, 7637–7641.
[9] Lin, R., Beauparlant, P., Makris, C., Meloche, S. and Hiscott, J. (1996) Mol. Cell Biol. 16, 1401–1409.
[10] McElhinney, J.A., Trushin, S.A., Bren, G.D., Chester, N. and Paya, C.V. (1996) Mol. Cell Biol. 16, 899–906.
[11] Schwarz, E.M., van Antwerp, D. and Verma, I.M. (1996) Mol. Cell. Biol. 16, 3554–3559.
[12] Gay, N.J. and Ntwasa, M. (1993) FEBS Lett. 335, 155–160.
[13] Hutchcroft, J., Anostario, J., Harrison, M. and Geahlen, R. (1991) Methods Enzymol. 200, 417–423.
[14] Kameshita, I. and Fujisawa, H. (1989) Anal. Biochem. 183, 183–190.
[15] Kubota, K. and Gay, N.J. (1995) Nucleic Acids Res. 23, 3111–3118.
[16] Glover, C.V.C., Shelton, E.R. and Brutlag, D.L. (1983) J. Biol. Chem. 258, 3258–3265.
[17] Kubota, K., Keith, F.J. and Gay, N.J. (1993) Biochem. J. 296, 497–503.
[18] Kubota, K., Keith, F.J. and Gay, N.J. (1995) FEBS Lett. 365, 83–86.
[19] Pinna, L.A. (1990) Biochim. Biophys. Acta 1054, 267–284.
[20] Saxena, A., Padmanabha, R. and Glover, C.V.C. (1987) Mol. Cell Biol. 7, 3409–3417.
[21] Jaffray, E., Wood, K.M. and Hay, R.T. (1995) Mol. Cell Biol. 15, 2166–2172.
[22] Lin, A.J., Frost, J., Deng, T., Smeal, N., Al-Alawi, N., Kikkawa, U., Hunter, T., Brenner, D. and Karin, M. (1992) Cell, 70, 777–789.
[23] Oelgeschlager, M., Krieg, J., Luscher-Firzlaff, J.M. and Luscher, B. (1995) Mol. Cell Biol. 15, 5966–5974.