Bovine prion protein as a modulator of protein kinase CK2

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On the basis of far-Western blot and plasmon resonance (BIAcore) experiments, we show here that recombinant bovine prion protein (bPrP) (25–242) strongly interacts with the catalytic α/α' subunits of protein kinase CK2 (also termed 'casein kinase 2'). This association leads to increased phosphotransferase activity of CK2α, tested on calmodulin or specific peptides as substrate. We also show that bPrP counteracts the inhibition of calmodulin phosphorylation promoted by the regulatory β subunits of CK2. A truncated form of bPrP encompassing the C-terminal domain (residues 105–242) interacts with CK2 but does not affect its catalytic activity. The opposite is found with the N-terminal fragment of bPrP (residues 25–116), although the stimulation of catalysis is less efficient than with full-size bPrP. These results disclose the potential of the PrP to modulate the activity of CK2, a pleiotropic protein kinase that is particularly abundant in the brain.

Key words: casein kinase 2, enzymic regulation, protein phosphorylation, surface plasmon resonance, transmissible spongiform encephalopathies.

INTRODUCTION

Protein kinase CK2 (an acronym derived from the misnomer 'casein kinase-2') is probably the most pleiotropic member of the huge family of protein kinases [1]. The long list of its substrates includes at least 200 proteins that share a phosphoacceptor site specified by multiple negatively charged amino acids [2]. CK2 pleiotropicity is further highlighted by the ability to use either GTP or ATP as phosphate donor, and by the fact that CK2, although regarded as a genuine Ser/Thr protein kinase, can also phosphorylate tyrosyl residues under special conditions [3]. Given these premises, the apparent lack of tight control mechanisms of CK2 activity might not be entirely surprising. This feature is exemplified by the fact that the enzyme catalytic (α and α') subunits are constitutively active, either as isolated entities or when assembled with the two regulatory β subunits in the $\alpha_2\beta_2$ holoenzyme. The role of CK2 β subunits seems enigmatic in that, in contrast with regulatory subunits of other kinases, e.g. cAMP-dependent protein kinase and cyclin-dependent kinases, they include distinct functional domains that can depress or up-regulate catalysis [4,5] by a mechanism of α/β interplay that is not yet fully understood. Indeed, attention is currently devoted to cell proteins that, irrespective of being phosphorylated by CK2, are nevertheless able to interact physically with either the catalytic or, more often, the regulatory subunits of the enzyme [6,7]. A model has therefore been proposed in which CK2 activity is subtly modulated by a variety of interchangeable partners [8].

We have recently shown that the recombinant bovine isoform of the prion protein (bPrP), implicated in transmissible spongiform encephalopathies (reviewed in [9]), can be phosphorylated by several protein kinases, including CK2 [10]. In the course of the investigation we noticed that whenever bPrP was added to CK2 in combination with another phosphorylatable substrate, the latter underwent significantly faster phosphorylation. This observation prompted us to assess a possible modulatory role exploited by bPrP on CK2. We show here that bPrP interacts tightly with the catalytic subunits of CK2 and up-regulates their activity.

EXPERIMENTAL

Materials

Recombinant α and β subunits of human CK2 and CK2 holoenzyme ($\alpha_2\beta_2$) were obtained as described previously [11]. Casein kinase 1 (CK1) was either obtained as a recombinant *Xenopus laevis* isoform (CK1α) [12] or purified from rat liver cytosol as described previously [10]. Recombinant piD261 protein kinase was prepared as described [13]. Tyrosine protein kinase Syk, purified from bovine spleen, was kindly provided by Dr A. M. Brunati (Padova, Italy). Inhibitor 2 of protein phosphatase 1 (I-2), HIV-1 Rev protein, human p53, spinach leaf calreticulin and calmodulin were generously supplied by Dr A. DePaoli-Roach (Indianapolis, IN, U.S.A.), Dr V. Ciminale (Padova, Italy), Dr P. Chene (Basel, Switzerland), Dr B. Baldan (Padova, Italy) and Dr E. Carafoli (Padova, Italy) respectively. β-Casein was prepared from bovine milk as described [14].

Full-length bPrP isoform (25–242) and its 25–116 and 105–242 truncated derivatives were generated from *Escherichia coli* and purified as described [10]. The synthetic peptide encompassing the amino acid stretch 153–168 of bPrP was obtained as in [10]. Mouse recombinant full-length PrP (23–231) was kindly provided by Dr J. Stöckel (Martinsried, Germany). The numbering of amino acid residues always relates to the bPrP sequence.

Far-Western blot analysis and antibodies

Immobilization of proteins on nitrocellulose membranes, renaturation, incubation with ligands, and subsequent immunolabelling were performed essentially as described previously [5]. Rabbit polyclonal antibodies raised against the 66–86 and 53–64 amino acid stretches of the α and β subunits of human CK2 respectively were used.

Abbreviations used: bPrP, bovine PrP; CK1, casein kinase 1; CK2, 'casein kinase 2'; I-2, inhibitor 2 of protein phosphatase 1; PrP, prion protein; PrP^c, cellular PrP; PrP^{Sc}, pathological isoform of PrP; SPR, surface plasmon resonance.

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BIAcore analysis

A BIAcore X system was used to analyse molecular interactions by means of surface plasmon resonance (SPR) [15]. bPrP or the α subunit of CK2 was covalently linked to a Sensor Chip CM5 (carboxymethylated dextran surface), with the use of amine-coupling chemistry [16], as described [17]. A surface density of 920 and 820 resonance units was generated for CK2α or bPrP respectively. Solutions of the interacting protein ('analyte') were injected over the surface at 25 °C with a flow rate of 10 μl/min in HBS running buffer [10 mM Hepes (pH 7.4)/150 mM NaCl/3 mM EDTA/0.005% (v/v) surfactant P20]. After injection, analyte solutions were replaced by HBS at a continuous flow rate of 10 μl/min. Surface regeneration was accomplished by injecting 10 mM HCl (1 min contact time). All analyte solutions were run simultaneously over a control flow cell containing a blank surface (with no immobilized protein). Each sensorgram (time course of the SPR signal) was subtracted for the response observed in the control flow cell, and normalized to a baseline of 0 resonance units. To determine the stoichiometry of the bPrP–CK2α interaction, high concentrations (2 and 4 μM) of CK2α were passed for 20 min over the sensor chip with immobilized bPrP (100 μl injections at a flow rate of 5 μl/min) to obtain a saturating response. Kinetic data were interpreted with a simple 1:1 binding model, and interaction rate constants were calculated by using the BIAevaluation 3.0 SPR kinetic software (BIAcore). Reported values are the means for four separate experiments; S.E.M. values were lower than 30%.

Phosphorylation assay

In a final volume of 25 μl of 50 mM Tris/HCl (pH 7.5)/12 mM $MgCl_2$/100 mM NaCl, phosphorylation reactions were performed by incubating, for 10 min at 37 °C, CK2 (20 m-units) with calmodulin, or the synthetic peptide $R_3A_2DSD_5$ (where single letter amino acid notation has been used). Reactions were started by adding 25 μM [γ-^{32}P]ATP (specific radioactivity approx. 1000 c.p.m./pmol) and stopped by cooling samples on ice. The incorporation of ^{32}P into calmodulin was evaluated by subjecting samples to SDS/PAGE, staining and autoradiography, or by direct scanning of the gel on an Instant Imager Apparatus (Canberra Packard). Phosphorylation of the synthetic peptide was evaluated by the phosphocellulose paper procedure [18].

RESULTS

The far-Western blot technique [19] was first applied to test the ability of full-length recombinant bPrP (25–242), and its N- (25–116) and C-terminal (105–242) fragments, to interact with either the α or β subunit of CK2. For success with this assay after SDS/PAGE, the immobilized protein on the immunoblotted membrane must undergo renaturation, to permit its specific interaction with the native overlaid ligand. Given that, under these conditions, denaturation of CK2α is irreversible [5], our experiments were run by treating transblotted bPrP with a solution of native CK2α. As shown in Figure 1(A), a strong signal between CK2α and full-size bPrP was clearly evident (lane 4), supporting the conclusion that bPrP is renatured to a good extent. Of the truncated forms of bPrP, only the C-terminal fragment retained the ability to interact (Figure 1A, lanes 5 and 6). In contrast, no significant association between the various bPrP forms and the β subunit of CK2 was detected by this procedure (results not shown).

Because CK2 phosphorylates Ser^{154} of bPrP [10], one might argue that the observed physical association reflects merely an enzyme–substrate interaction. However, two findings militate against this possibility. The first is that murine PrP interacted equally well with CK2α (Figure 1B, compare lanes 3 and 4), although this isoform is not phosphorylated by CK2 because it lacks the sequence of the phosphorylatable consensus site [10]. Secondly, a synthetic peptide substrate, encompassing the entire bovine consensus sequence for CK2, failed to counteract the association between bPrP and CK2α, even when added in large molar excess (Figure 1B, compare lanes 3 and 5). In addition, as judged from far-Western blot analysis, it should be noted that several protein substrates of CK2, notably I-2, calmodulin and Rev protein of HIV-1, whose molecular sizes are similar to that of bPrP (Figure 1C), and p53, calreticulin and β-casein (results not shown), failed to interact with CK2α.

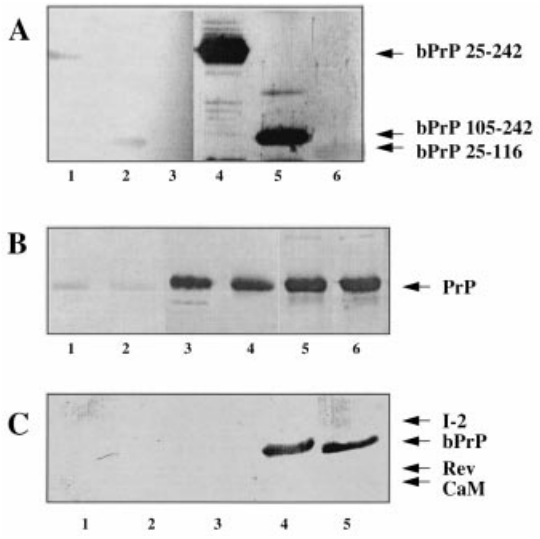

Figure 1 Detection of specific CK2α–bPrP interaction by far-Western blotting

(**A**) bPrP, full size (25–242) (lanes 1 and 4) and its C-terminal (105–242) (lanes 2 and 5) or N-terminal (25–116) (lanes 3 and 6) fragments (1 μg of each), were immobilized on nitrocellulose membranes after SDS/PAGE. Membranes were then renatured [5] and incubated in the absence (lanes 1–3) or presence (lanes 4–6) of CK2α (20 μg/ml). After the removal of unbound molecules, bound CK2α was detected by polyclonal antibody against CK2α. (**B**) Under conditions similar to those described in (**A**), bovine (lanes 1, 3, 5 and 6) and mouse PrP (lanes 2 and 4) were incubated in the absence (lanes 1 and 2) or in the presence of CK2α alone (lanes 3 and 4), CK2α plus a 500-fold molar excess (with respect to bPrP) of the synthetic bPrP peptide 153–168 (lane 5), and a CK2α mutant form bearing an N-terminal deletion ($\alpha\Delta$N) (lane 6). (**C**) Under conditions similar to those described in (**A**), three known CK2 substrates, I-2 (lane 1), HIV-1 Rev protein (lane 2) and calmodulin (lane 3) were immobilized on the nitrocellulose membrane and tested for their ability to interact with CK2α. bPrP overlaid by either CK2α (lane 4), or α' (lane 5) was included as positive control.

To corroborate the reliability of data obtained by far-Western blotting, molecular interaction between full-size bPrP and CK2 subunits was tested by plasmon resonance (BIAcore) analysis (Figure 2), performed by passing analytes over a sensor surface of immobilized bPrP. A strong interaction was observed with both the α and α' catalytic subunits of CK2, whereas the β subunit evoked a much weaker signal (Figure 2A). No interaction was detected when CK2α/α' were replaced by another acidophilic protein kinase, CK1 (α isoform) (results not shown). When native CK2 holoenzyme was tested, interaction with bPrP was still evident (Figure 2A), which is consistent with the conclusion that β subunits do not prevent bPrP–CK2α association. From the sensorgrams of Figure 2(B), the K_d of 5.33×10^{-7} M (K_{diss} 9.70×10^{-4} s^{-1}; K_{ass} 1.82×10^{3} M$^{-1} \cdot$ s^{-1}) calculated for the

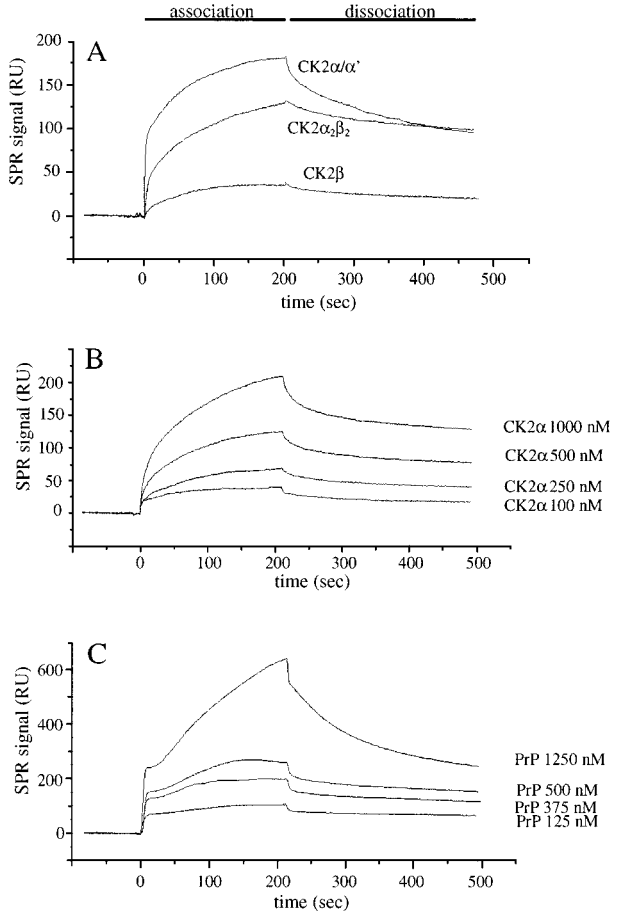

Figure 2 Detection of the interaction of CK2 and full-size bPrP by SPR signal

Shown are representative sensorgrams obtained with a BIAcore X system. Over a sensor surface with a covalently linked protein, 35 μl of a protein ('analyte') in solution was injected at time 0 for a total contact time of 210 s (association phase), after which analytes were replaced by running buffer (dissociation phase). (**A**) To immobilized bPrP, 500 nM CK2 α, α', β, or $\alpha_2\beta_2$ were injected, as indicated. Note that the α and α' subunits of CK2 give superimposable sensorgrams. (**B**) To immobilized bPrP, CK2α was injected at the indicated concentrations. (**C**) To immobilized CK2α, bPrP was injected at the indicated concentrations. Superimposable sensorgrams were obtained if bPrP was replaced by a sample of bPrP previously phosphorylated by prolonged incubation with CK2 holoenzyme and ATP [10]. Abbreviation: RU, resonance units.

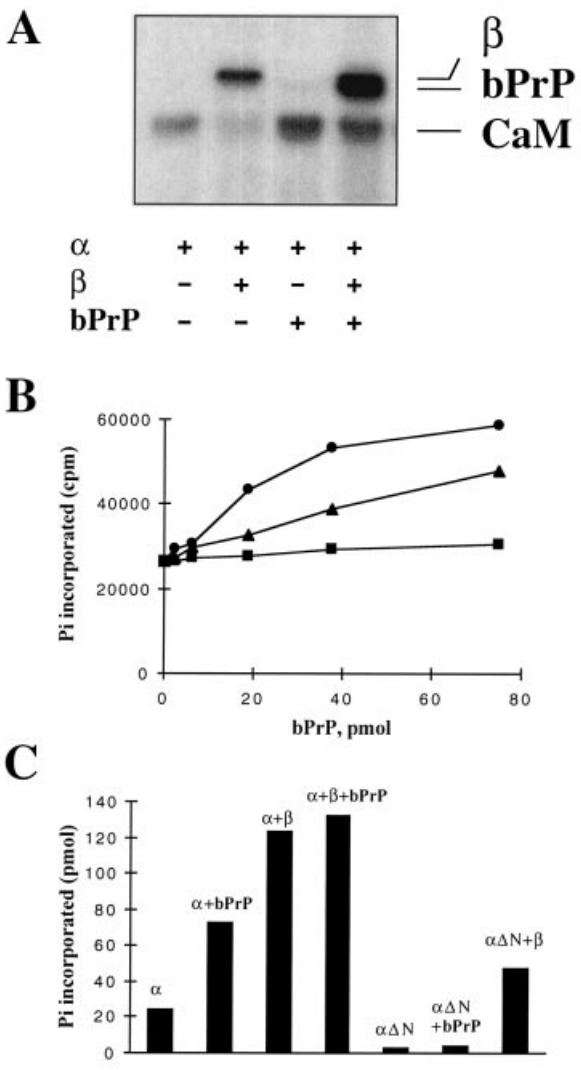

Figure 3 Effect of bPrP on CK2 activity

Activity of CK2 was assayed with calmodulin (**A**) or the synthetic peptide $R_3A_2DSD_5$ (**B**, **C**) as phosphorylatable substrate, under the conditions described in the Experimental section. (**A**) Autoradiograms showing calmodulin (CaM) (10 μM) phosphorylation by CK2α (2 pmol) in the absence or presence of bPrP (25–242) (35 pmol) and of equimolar amounts of the β subunit. The positions of the autophosphorylated β subunit and that of bPrP phosphorylated by CK2 ($\alpha_2\beta_2$) are indicated on the right. (**B**) Phosphorylation of the synthetic $R_3A_2DSD_5$ peptide (100 μM) (expressed as the incorporation of P_i) by CK2α (0.5 pmol) in the presence of increasing amounts of full-size bPrP (●) or its N-terminal (25–116) (▲) or C-terminal (105–242) (■) forms. A superimposable diagram was obtained by replacing full-size bPrP with mouse PrP. (**C**) Bar chart of the phosphorylation activity of CK2α (0.5 pmol), wild-type or mutated (bearing an N-terminal deletion; $\alpha\Delta$N), on synthetic $R_3A_2DSD_5$ peptide (100 μM). Where indicated, equimolar amounts of the β subunit and/or a molar excess (with regard to CK2α) of full-length bPrP (25 pmol) were present. The values shown are means for three independent experiments; S.E.M. values did not exceed 5%.

bPrP–CK2α interaction denotes a rather high binding affinity. These calculations were based on the assumption of a 1:1 stoichiometry of interaction. The validity of this assumption was supported by an analysis of the saturating response obtained when high concentrations of CK2α were injected (see the Experimental section). A high affinity of binding was also observed when CK2α was immobilized and bPrP solutions were injected over the surface, but in this case sensorgrams denoted complex association and dissociation kinetics (Figure 2C) that did not permit precise calculation of the rate constants.

To assess whether interaction with bPrP affected CK2 functionality, we first tested the enzyme phosphorylation capacity on calmodulin as substrate. Calmodulin was chosen in view of its unique features with respect to CK2. Although calmodulin is an apparent physiological substrate of CK2 [20], its phosphorylation by isolated α/α' subunits is known to be markedly decreased in the presence of β subunits [21] (compare the first and second lanes of Figure 3A). Interestingly, not only did full-size bPrP stimulate calmodulin phosphorylation by CK2α (compare the first and third lanes of Figure 3A), it also abolished the negative effect of β subunits present in the reconstituted holoenzyme (compare the second and fourth lanes of Figure 3A). In contrast, the catalytic activity of three other acidophilic protein kinases, two Ser/Thr-specific (CK1 and piD261 [13]) and one Tyr-specific (Syk), were unaffected by bPrP under the conditions used (results not shown).

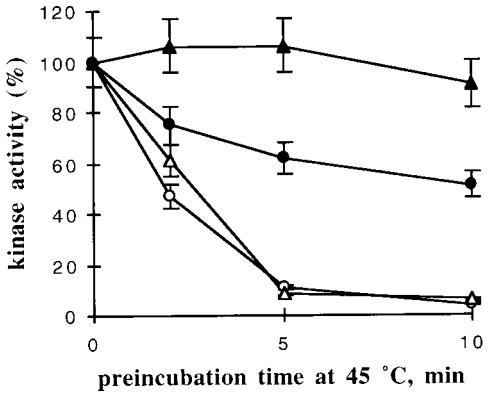

Figure 4 Effect of bPrP on the thermal stability of CK2α and CK2 holoenzyme

Aliquots of CK2α (0.5 pmol) were preincubated at 45 °C for the indicated times, either alone (○) or in the presence of equimolar amounts of β (●), a 50-fold molar excess of bPrP with respect to CK2α (△) or equimolar amounts of CK2β plus a 50-fold molar excess of bPrP (▲). Residual activity, expressed as a percentage of that in samples not subjected to preincubation at 45 °C, was assayed on the specific $R_3A_2DSD_5$ peptide substrate (100 μM), as detailed in the Experimental section.

The influence of bPrP on CK2 was investigated further by the use of the CK2-specific peptide substrate $R_3A_2DSD_5$. In contrast with calmodulin, this peptide is best phosphorylated in the presence of β subunits. As shown in Figure 3(B), the stimulatory effect of full-size bPrP was evident and dose-dependent. An identical stimulation was observed with mouse PrP, which is refractory to CK2-mediated phosphorylation (results not shown). The stimulatory effect observed in the presence of a 50-fold molar excess of bPrP (0.96 μM) over CK2α is accounted for by an increased V_{max} (270 compared with 45 nmol/min per mg), whereas the K_m remained virtually unchanged (44 μM). We found that optimal stimulation required integrity of bPrP: the N-terminal fragment was less effective and the C-terminal domain had no effect at all (Figure 3B).

The results shown in Figure 3(C) indicate that the increased peptide phosphorylation due to bPrP or the β subunit was not, or was only minimally, additive, as though CK2α lost susceptibility to stimulation by bPrP in the presence of β subunits. That different regulatory mechanisms pertain to the two proteins is substantiated by the use of an N-terminal deleted form (Δ2–12) of the α subunit, defective in catalytic activity. In this case, the mutant activity was restored by the β subunit but not by bPrP (Figure 3C). Nevertheless, the same mutant bound to bPrP as efficiently as the wild-type form (see Figure 1B, lane 6), suggesting that the deleted N-terminal segment of CK2α is implicated in mediating the up-regulatory effect of bPrP, not that of the β subunits.

Note also that, whereas association with the β subunit conferred increasing heat stability on CK2α, this was not true of bPrP (Figure 4). Interestingly, however, the addition of bPrP to the $\alpha_2\beta_2$ holoenzyme increased thermal stability further.

DISCUSSION

Prions are infectious particles responsible for the onset and propagation of transmissible spongiform encephalopathies, or prion diseases, which affect both animals and humans [9]. Prions are composed mainly of a malformed protein (PrP^{Sc}) generated by a post-translational misfolding of a constitutive glycoprotein, cellular PrP (PrP^c) [22–24], found particularly in the central nervous system. Prion infectivity is explained by the capacity of PrP^{Sc} to convert PrP^c into the pathogenic conformation through an essentially autocatalytic mechanism initiated by PrP^{Sc}–PrP^c interaction [25]. However, the precise understanding of the conversion mechanism, its prerequisites and immediate consequences are hampered because the physiological role of PrP^c is still poorly defined. Nevertheless, a few functions have been proposed: for example, an involvement in anti-apoptotic pathways [26] or in the cellular resistance to oxidative stress exploited by the combined superoxide dismutase activity and copper-binding capacity of PrP^c [27,28].

Our results disclose another possible function of PrP, by showing that bPrP interacts with and up-regulates one of the most pleiotropic protein kinases, CK2, known to impinge on many proteins implicated in a variety of cell functions, in particular in signal transduction and gene expression pathways [1,8,29]. It is noteworthy that CK2 is also widely exploited by infectious agents, such as viruses and parasites, to perform the phosphorylation of proteins essential to their life cycle [29]. Although CK2 is almost ubiquitous, it is especially abundant in the brain [30], where it is present in all the regions studied [31]. It is therefore conceivable that PrP^c and CK2 are given the opportunity to come into contact, especially considering that CK2 is one of the few protein kinases whose presence on the outer leaflet of the plasma membrane, where PrP^c also resides, has been demonstrated unambiguously [32]. In this same context it might be pertinent to recall that PrP^c is found in caveolae [33], and that caveolin, the main constituent of caveolae, is both a target and an interacting partner of CK2 [34].

By probing the binding capacity of the full-length and truncated forms of recombinant bPrP, our results show that the main interaction between bPrP and CK2 involves the catalytic subunit of CK2 on one side and the C-terminal domain of bPrP on the other (Figure 1). However, the domain responsible for up-regulating CK2 seems to reside in another part of bPrP. This is clearly evident from the observation that, although neither the N-terminal nor the C-terminal fragment displays a stimulatory capacity comparable to that of full-size bPrP, significant stimulation is attained by increasing the concentration of the N-terminal but not of the C-terminal fragment of bPrP (Figure 3B). Seemingly, therefore, one or more activator elements of bPrP reside in the N-terminal moiety but they become less effective once dissected from the rest of the molecule, in which the structural features responsible for tight association seem to be located. A similar situation has been outlined for the β subunit of CK2, whose binding and regulatory properties were dissected by generating synthetic fragments of the protein [5]: its N-terminal segment (1–77), including the down-regulatory domain, retained some of its inhibitory potential, although it had lost its high-affinity binding capacity.

Stimulation of CK2α catalytic activity is already detectable with a 40-fold molar excess of bPrP (Figure 3B); however, it is still increasing with up to a 150-fold excess, suggesting that under these conditions CK2α is not yet fully saturated by bPrP. Although the structural element(s) of CK2α that interact physically with bPrP are currently unknown, it might be worth noting that a truncated form of CK2α (Δ2–12), which is still able to interact with bPrP (Figure 1), has lost its ability to stimulate catalytic activity (Figure 3C). In contrast, the stimulatory effect of the β subunit is still quite evident with this mutant (Figure 3C). Interestingly, the N-terminal segment of CK2α, which is partly truncated in mutant Δ2–12, has been shown by X-ray structural studies [35] and mutational analysis [36] to have a crucial role in favouring the active conformation of CK2α, because it interacts

with the so-called activation loop and holds it in an open conformation. It is therefore tempting to speculate that bPrP strengthens the interaction between the N-terminal segment and the activation loop, thus stabilizing the open conformation of CK2α. This would also be consistent with the kinetics of bPrP stimulation, characterized by increased V_{max} values, which possibly reflects a larger number of molecules in their active conformation.

Stimulation of CK2 by bPrP is especially evident with the isolated catalytic subunits of CK2 (Figure 3), which are suspected to be endowed with oncogenic [37,38] and anti-apoptotic [39] properties. This might provide an intriguing connection with the suggested involvement of PrP in anti-apoptotic pathways [26]. Indeed, when β subunits of CK2 display their stimulatory capacity towards the synthetic $R_3A_2DSD_5$ peptide, up-regulation by bPrP is hardly detectable (Figure 3C). However, it must be assumed that bPrP still interacts functionally with CK2 holoenzyme as judged by BIAcore analysis (Figure 2A), increased heat stability (Figure 4) and, more importantly, the ability to counteract the down-regulation mediated by the β subunits on calmodulin as substrate (Figure 3A).

In this respect, it is important to note that calmodulin belongs to a subset of CK2 substrates whose phosphorylation *in vivo* is a subject of debate, because it has been shown that these proteins are hardly affected by CK2 holoenzyme. Two possibilities have been envisaged to rationalize this observation: the intervention in the cell of either free catalytic subunits of CK2 or polybasic proteins known to trigger the phosphorylation of calmodulin *in vitro* by CK2 holoenzyme [40]. Our results suggest that bPrP also, or PrP^c in general, could exploit a similar role. In fact although, unlike histones and protamines, PrP cannot be regarded as a polycation in the strict sense, there are two features of the protein that could explain the observed stimulatory ability: the high isoelectric point (9.5) and the presence in the N-domain of the basic sequence Lys^{25}-Lys-Arg-Pro-Lys, which resembles closely the unique motif Lys^{74}-Lys-Lys-Lys-Ile-Lys at the start of helix C of the α subunits of CK2. The implication of this basic element in the down-regulation imposed by the N-terminal acidic stretch of the β subunit is supported by use of CK2 mutants [41] and by the competition ability of synthetic peptides encompassing the basic stretch [42]. In view of this information, it is thus highly feasible that, by a similar mechanism, Lys^{25}-Lys-Arg-Pro-Lys of bPrP does indeed contribute to the de-inhibition of calmodulin phosphorylation by CK2 holoenzyme (Figure 3A). Pertinent to this might be the observation that this basic stretch is conserved in murine PrP, which is also able to interact with and to stimulate CK2α activity, although it has lost its ability to undergo phosphorylation.

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