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Hematopoietic lineage cell specific protein 1 associates with and down-regulates protein kinase CK2

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Abstract The catalytic (α) subunit of protein kinase CK2 and the hematopoietic specific protein 1 (HS1) display opposite effects on Ha-ras induced fibroblast transformation, by enhancing and counteracting it, respectively. Here we show the occurrence of physical association between HS1 and CK2α as judged from both far Western blot and plasmon resonance (BIAcore) analysis. Association of HS1 with CK2α is drastically reduced by the deletion of the HS1 C-terminal region (403–486) containing an SH3 domain. HS1, but not its deletion mutant HS1 Δ324–393, lacking a sequence similar to an acidic stretch of the regulatory β-subunit of CK2, inhibits calmodulin phosphorylation by CK2α. These data indicate that HS1 physically interacts with CK2α and down-regulates its activity by a mechanism similar to the β-subunit.

Key words: Casein kinase-2; HS1; Protein kinase CK2; Transformation; Plasmon resonance

1. Introduction

Protein kinase CK2 (formerly termed 'casein kinase-2' or '-II') is a ubiquitous, constitutively active Ser/Thr specific protein kinase essential for cell viability, thought to play a role in a wide spectrum of cellular function as judged from the huge number and variety of its targets (> 160 known to date) many of which are involved in signal transduction, gene expression, protein synthesis and metabolic regulations (reviewed in Refs. [1–4]). The regulation of CK2 is still enigmatic: its catalytic α-subunit is spontaneously active in the absence of second messengers or phosphorylation events, by virtue of a unique interaction between its N-terminal segment and the activation loop which is kept in its open conformation [5,6]. Two catalytic subunits (α and/or α′) are normally associated with two regulatory β-subunits to form stable heterotetramers which do not dissociate unless under denaturing conditions. The basal activity of the holoenzyme is even higher than that of the catalytic subunit toward many substrates, with a number of notable exceptions however, exemplified by calmodulin, whose phosphorylation is actually inhibited by the β-subunit. Based on these and other observations, it has been postulated that the catalytic subunits of CK2 may also be present in the cell as monomers, where they could play a role distinct from that of the heterotetrameric holoenzyme [7–10]. Pertinent to this may also be the finding that misscheduled expression of CK2 catalytic subunits cooperates with proto-oncogenes in promoting tumors [11–13] and cell transformation [9]. Given this scenario, the recent detection of proteins than can interact with the individual CK2 subunits has opened the possibility that the phosphorylating activity of this kinase may be regulated in a novel way (reviewed in [10]). The growing list of these 'partners' includes proteins that associate with either the β or the catalytic subunits. Among the latter ones are HSP90 [14], nucleolin [15] and protein phosphatase 2A [11].

The recent report that the malignancy of Ha-ras transformed fibroblasts, which is enhanced by CK2α and α′ [9], is conversely suppressed by transfection with apoptosis inducer hematopoietic specific protein 1, HS1 [16] prompted us to check if HS1 could also behave as a partner and a regulator of CK2α. The results of this study are reported here.

2. Materials and methods

[γ-^{32}P]ATP, chemiluminescence reagent (ECL) and secondary antibodies were purchased from Amersham, protease inhibitor cocktail from Boehringer Mannheim; all other chemicals were from Sigma. Monoclonal anti-CK2α antibodies (1AD9) were kindly donated by Dr. O.-G. Issinger (Odense University, Odense, Denmark), while polyclonal anti-CK2β antiserum was prepared by immunization of a rabbit with peptide 53–64 of human CK2β coupled to keyhole limpet hemocyanin (KLH). Proline-rich peptide KGGRSRLPLPPLPPPG and the CK2 substrate peptide RRRADDSDDDDD, were synthesized with an automated peptide synthesizer ABI 431-A (Applied Biosystem) equipped with Fmoc chemistry, according to a protocol described elsewhere [17,18].

Full length (f.l.) HS1, HS1 Δ1–207 and HS1 Δ403–486 were obtained and purified as previously described [19]. HS1 Δ324–393 was obtained by cleavage of pTrcHis HS1 with *Dra*III and *Xho*I followed by ligation of the oligonucleotides 5′GTGGAGGGGGAGTATG-AGGTGC3′ and 5′TCGAGGACCTCATAGTCCCCCTCCACTG-G3′. Expression and purification were performed according to the protocol described in [19].

CK2α wild-type (w.t.), CK2α mutant and CK2β were expressed in *E. coli* as described in [20]. Purification of the isolated subunits was obtained by phosphocellulose chromatography, as already described [21]. CK2α K74–77A mutant was generated by an oligonucleotide directed mutagenesis according to the method described in [22].

For the far Western experiments, HS1 or its mutants were blotted to nitrocellulose membranes, which were then blocked with 3% (w/v) bovine serum albumin and incubated with binding buffer (300 mM KCl, 0.1% (v/v) Tween-20 and 20 mM Tris-HCl, pH 7.5) for 30 min at 4°C. A solution of the interacting protein (CK2 α-subunit, β-subunit or both) in the same buffer was then added at a final concentration of 30 μg/ml for each protein and incubated at 4°C for 2 h, with gentle

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Abbreviations: HS1, hematopoietic lineage cell-specific protein; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride; f.l., full length; HBS, HEPES buffer solution; NHS, *N*-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; SH3, Src homology 3 domain; SPR, surface plasmon resonance; w.t., wild-type

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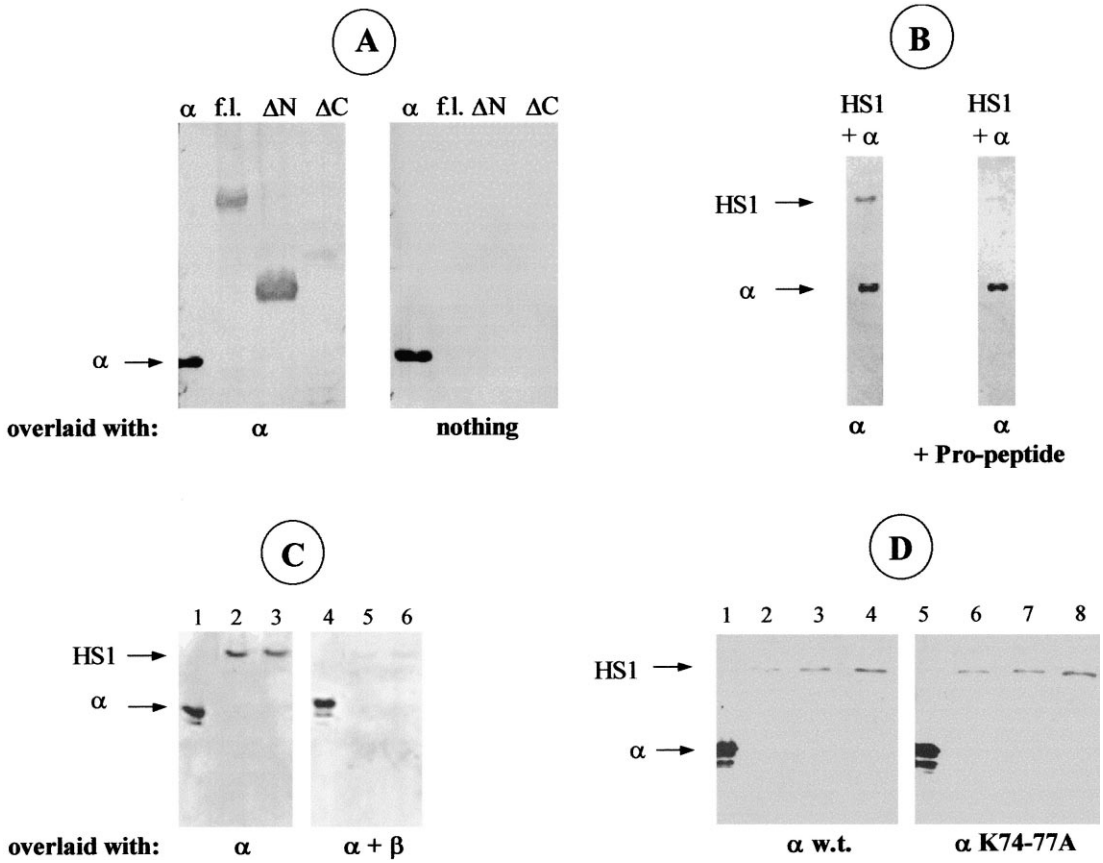

Fig. 1. Association of CK2α with HS1 in far Western experiments. Samples were run on SDS-PAGE and transblotted onto nitrocellulose membranes, which were then overlaid by solutions (30 μg/ml) containing the indicated proteins. Membranes were then probed with anti-CK2α monoclonal antibodies. A: 0.1 μg CK2α or 0.6 μg HS1 as f.l. or deletion mutants were loaded as indicated. ΔN: HS1 truncated of region 1–207; ΔC: HS1 truncated of region 403–486. B: 0.2 μg CK2α and 0.2 μg HS1 were loaded simultaneously on each lane. Where present in the overlay step, the proline-rich peptide KGGRSRLPLPPLPPPG (Pro-peptide) was added at 1 mM concentration C: 0.1 μg CK2α (lanes 1 and 4), 0.2 μg (lanes 2 and 5) or 0.4 μg (lanes 3 and 6) of HS1 were loaded. Where present in the overlay step, CK2β (30 μg/ml) was in molar excess as compared to CK2α. D: 0.1 μg CK2α (lanes 1 and 5), or increasing concentrations of HS1 (0.1 μg in lanes 2 and 6; 0.2 μg in lanes 3 and 7; 0.3 μg in lanes 4 and 8) were loaded.

stirring. After the unbound proteins had been removed by three washings with the same buffer, the membranes were incubated with the appropriate antibodies followed by the appropriate biotinylated second antibody. Detection was performed by alkaline phosphatase reaction or alternatively with chemiluminescence. Quantitation and normalization of the bands were obtained by analysis with an ImageMaster VDS apparatus and software (Pharmacia Biotech).

For the surface plasmon resonance (SPR) analysis, a BIAcore X system was used to analyze molecular interactions by means of the SPR phenomenon [23]. CK2α was covalently linked to a Sensor Chip CM5 (BIAcore) (carboxymethylated dextrane surface) using amine coupling chemistry [24], as follows. On the surface, activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) according to the manufacterer's instructions, a CK2α solution (18 μg/ml) in 10 mM sodium acetate, pH 5.5, was applied with a flow rate of 5 μl/min, at 25°C. Unreacted groups on the chip were then inactivated by 1 M ethanolamine-HCl, pH 8.5 and CK2α not covalently bound was removed with 10 mM NaOH. A surface density of 5100 RU (resonance units) was generated. HS1 solutions (500–2500 nM) were injected over the surface with a flow rate of 10 μl/min in running buffer HBS (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20) at 25°C. After injections, HBS replaced the HS1 solutions in a continuous flow of 10 μl/min; surface was regenerated by injection of 10 mM NaOH (20 μl, flow rate of 30 μl/min). All samples were run simultaneously over a flow cell containing a blank surface (without any immobilized protein); each sensorgram (time course of the SPR signal) was subtracted of the response observed in this control flow cell and normalized to a baseline of 0 RU. The kinetic data were interpreted according a simple 1:1 binding model and the rate constants of the interactions were calculated using the SPR kinetic evaluation software BIAevaluation 3.0 (BIAcore). The reported values are the means of 4 separate experiments, with S.E.M. values lower than 30%.

Phosphorylation of calmodulin (3 μM) was achieved by incubation with 20 nM CK2α in the presence of 50 mM Tris-HCl, pH 7.5, 10 μM [γ-^{32}P]ATP, 12 mM $MgCl_2$. Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography and phosphorylation was quantified by analysis with an InstantImager (Packard) apparatus. Peptide phosphorylation assays were performed under the same conditions, using 0.1 mM specific peptide $R_3AD_2SD_5$ as substrate; the degree of phosphorylation was evaluated by the method of phosphocellulose paper [25].

3. Results

The finding that HS1 [26] and CK2α display opposite effects on the Ha-ras induced fibroblast transformation [9,16] prompted us to check the possibility that there might be physical and/or functional interactions between these two proteins, possibly resulting in an attenuation of CK2α oncogenic potential. Physical interactions between HS1 and CK2 were clearly detectable by far Western blot experiments, in which HS1 and its deletion mutants were resolved by SDS-PAGE and transblotted, and the membranes were overlaid with

solutions containing either CK2 subunits or CK2 holoenzyme. Bound CK2 was then detected by using proper antibodies. As shown in Fig. 1A a strong signal is detected when the α-subunit of CK2 is used in the overlay step, while no association was observed with the β-subunit (not shown). The N-terminal truncation of HS1 did not prevent interaction, which conversely was nearly abolished by the C-terminal truncation depriving HS1 of its Src homology 3 (SH3) domain. Moreover, the addition of a proline-rich peptide known to interact with Src SH3 domain [27], along with CK2α during the overlay step, counteracted the association of α-subunit to HS1 (Fig. 1B). The interaction is not evident when the β-subunit is added in a molar excess to the α-subunit during the overlay step, indicating that the formation of the $\alpha_2\beta_2$ tetramers prevents the association of α with HS1 (Fig. 1C). It should be noted that HS1 is also appreciably phosphorylated by CK2α, in a manner which is counteracted by the β-subunit (unpublished data). Interestingly, however, the CK2α mutant K74–77A, which is severely defective in substrate recognition but not in β-subunit binding [21], still interacts with HS1 as efficiently as α w.t. (Fig. 1D), consistent with the concept that physical interaction occurs through a mechanism which is distinct from that of substrate recognition.

In the attempt to determine the kinetic parameters of the HS1-CK2α interaction, a SPR approach was applied. CK2α was immobilized on the sensor surface and HS1 was passed over the sensor surface at a flow rate of 10 ml/min. The sensorgram, corroborating the concept that indeed HS1 interacts with the α-subunit of CK2, is shown in Fig. 2. The dissociation and association rate constants were calculated from the curve as described in Section 2; they were found to be 4.46×10^{-3} s^{-1} and 2.24×10^{4} M^{-1} s^{-1}, respectively. The equilibrium dissociation constant (K_D) was 1.99×10^{-7} M. For sake of comparison, the values calculated with the β-subunit (instead of HS1) were: $K_{diss}=9.30\times10^{-4}$ s^{-1}, $K_{ass}=2.27\times10^{4}$ M^{-1} s^{-1}, $K_D=4.08\times10^{-8}$ M.

In order to see whether interaction with HS1 might affect CK2α catalytic activity, experiments were performed using calmodulin as phosphorylatable substrate. Calmodulin was chosen because it is a specific substrate of the catalytic subunit, unaffected by CK2 holoenzyme, usable thereafter as an indicator of free CK2α activity [9]. As shown in Fig. 3A, by adding HS1 to CK2α an almost complete inhibition of calmodulin phosphorylation is observed. Such a dose dependent inhibition of calmodulin phosphorylation is reminiscent of that promoted by the addition of CK2 β-subunit (Fig. 3C), in that both are promoted by concentrations that are nearly

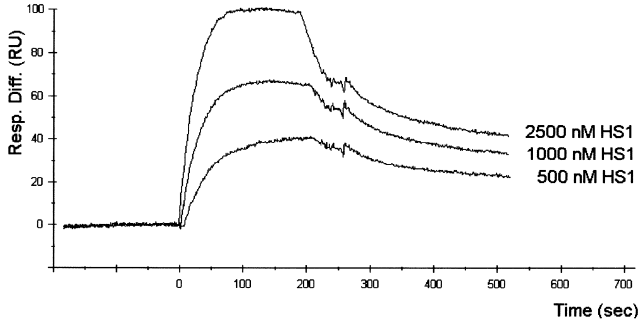

Fig. 2. Detection of CK2α/HS1 association by SPR signal. The interaction was measured with the SPR sensor system BIAcore X. Shown are representative sensorgrams obtained by the injection of 35 μl of HS1 at the indicated concentrations at a flow rate of 10 μl/min over a sensor surface containing 5100 RU of immobilized CK2α. The response obtained with a control sensor surface (without CK2α) was subtracted to each sensorgram (see Section 2 for details). Resp. Diff., response difference; RU, resonance units.

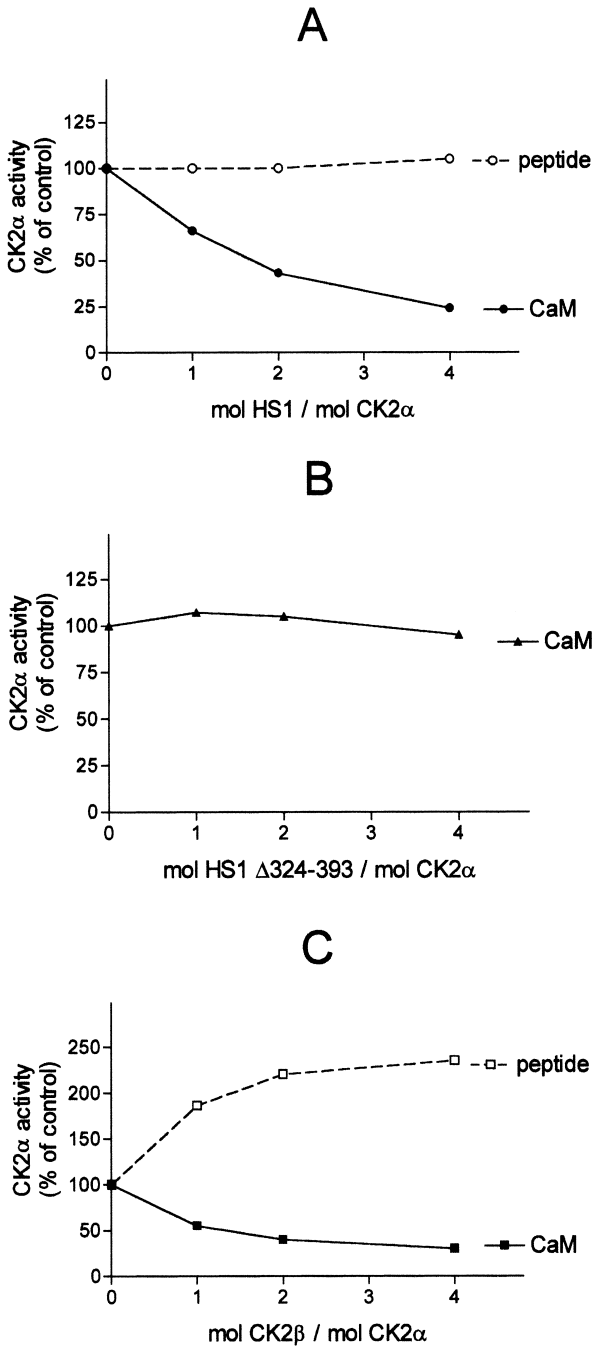

Fig. 3. Effect of HS1 (A), HS1 Δ324–393 (B) or CK2β (C) on the catalytic activity of CK2α. CK2α activity was tested by 10 min incubation at 30°C with 10 μM [γ-^{32}P]ATP, 12 mM $MgCl_2$ using either 3 μM calmodulin (CaM) or 0.1 mM $R_3AD_2SD_5$ peptide as substrate, as indicated. Increasing concentrations of HS1, either w.t. (A) or deleted (Δ324–393) (B), or CK2β (C), were added. CaM phosphorylation was quantified by SDS-PAGE of the samples and analysis with an InstantImager apparatus (Packard). Peptide phosphorylation was evaluated by the method of P-cellulose paper [25]. CK2α activity is expressed as percent of control, in the absence of either HS1 or CK2β (1.2 pmol P_i and 20 pmol P_i with calmodulin and peptide, respectively).

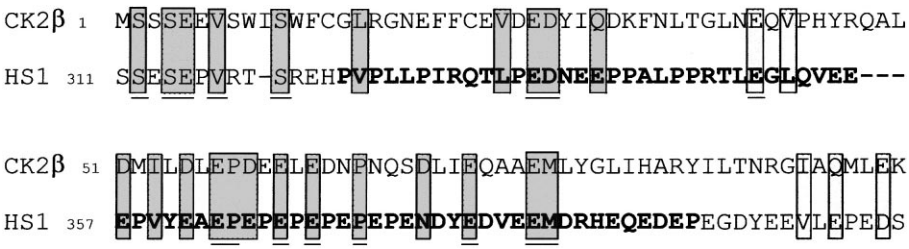

Fig. 4. Alignment of amino acid sequences of CK2β (1–100) and HS1 (311–406). Alignment was obtained by analysis of human HS1 and human CK2β sequences with the Dotter program [31]. Functionally conserved amino acids are boxed, while identities are also underlined. The sequence deleted in HS1 Δ324–393 is bold typed.

equimolar to that of CK2α and both are specifically detectable only using calmodulin as substrate, whereas the phosphorylation of a peptide substrate is either unaffected or even stimulated by HS1 and β-subunit, respectively. The similarity between down-regulation promoted by either HS1 or β-subunit is further highlighted by the observation that a HS1 deletion mutant (Δ324–393), lacking a region similar to the down regulatory domain of CK2 β-subunit (see Fig. 4), has lost its inhibitory activity (Fig. 3B).

4. Discussion

The data presented here may provide a rationale to explain the opposite effects of CK2 α/α′ and HS1 protein on Ha-ras induced malignant transformation. Assuming that the oncogenic potential of CK2 is due to imbalanced expression of its catalytic subunits [9] which are in excess over the β-subunit, resulting in the phosphorylation of a subset of substrates which are otherwise unaffected by the $\alpha_2\beta_2$ holoenzyme, it can be speculated that any partner of α, capable to surrogate the negative function of β by binding to α and down-regulating its activity, will counteract its oncogenic potential. Here we show that HS1 display both functions: it binds to CK2α, as judged from both far Western blots and BIAcore analysis, and, by doing that, it down-regulates kinase activity monitored using calmodulin as substrate. The K_D constant calculated from BIAcore analysis is consistent with high affinity binding of HS1 to CK2α. It should be recalled that association with cellular partners is considered a crucial device by which CK2 activity, which is not subjected to any kind of tight control, can be subtly modulated [10]. While a pletora of partners of the β-subunit have been detected, mostly through the two-hybrid system approach [28] much fewer are the known partners of the α-subunit, namely HSP90 [14], nucleolin [15] and protein phosphatase 2A [11]. The binding of HSP90 and nucleolin is counteracted by heparin, consistent with the concept that they bind to the same site. However a CK2α mutant (K74–77A) which has lost the ability to bind heparin [29] still interacts with HS1, consistent with a mode of binding different from that of HSP90 and nucleolin.

Efficient binding of HS1 to CK2α is dependent on its C-terminal region, including an SH3 domain, whose truncation drastically reduces the ability to interact with CK2α. The intervention of an SH3 domain is also supported by the effect of a proline-rich peptide known to interact with the Src SH3 domain, whose addition counteracts the association between HS1 and CK2α (see Fig. 1B). This suggests that the SH3 domain of HS1 either directly interacts with CK2α, where a proline-rich domain, including a P-X-X-P SH3 binding motif, is present in its C-terminal region, or facilitates the binding indirectly, by conferring to HS1 a conformation prone to interaction. It should be noted in this respect that a proline-rich domain potentially suitable for interacting with the SH3 domain is also present in HS1 itself. It seems that the latter possibility is more likely, since the CK2α′ subunit, where the C-terminal proline-rich domain of the α-subunit is lacking, still associates with HS1, albeit somewhat less strongly (unpublished).

Apparently, down-regulation of CK2α by HS1 and physical association between the two proteins rely on different structural modules: efficient down-regulation depends on a stretch in the Pro/Glu-rich domain of HS1 which displays significant homology to the N-terminal acidic segment of the β-subunit, which is known to mediate the inhibitory effect on catalytic activity [30] (see Fig. 4). Its deletion, in fact, suppresses the ability of HS1 to down-regulate CK2α, but not to associate with it (not shown). It is expected, therefore, that CK2α down-regulation by HS1 takes place through the same pseudo-substrate mechanism operating in the case of the β-subunit.

In conclusion, our data are consistent with the view that one of the modes HS1 displays its anti-proliferative functions in hematopoietic cells is to 'buffer' CK2α/α′ subunits in excess, (i.e. not combined with the β-subunit) and to neutralize their activity toward specific protein targets, whose phosphorylation may contribute to the onset of cell proliferation and transformation. In vivo studies will be required to validate this hypothesis, e.g. by assessing whether co-expression of HS1 and of its mutants interferes with the observed cooperative effect of CK2α in inducing fibroblast transformation [9].

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References

[1] Pinna, L.A. (1990) Biochim. Biophys. Acta 1054, 267–284.
[2] Tuazon, P.T. and Traugh, J.A. (1991) Adv. Second Messenger Phosphoprotein Res. 23, 123–164.
[3] Allende, J.E. and Allende, C.C. (1995) FASEB J. 9, 313–323.
[4] Pinna, L.A. and Meggio, F. (1997) Progr. Cell. Cycle. Res. 3, 77–97.
[5] Niefind, K., Guerra, B., Pinna, L.A., Issinger, O.-G. and Schomburg, D. (1998) EMBO J. 17, 2451–2462.
[6] Sarno, S., Marin, O., Ghisellini, P., Meggio, F. and Pinna, L.A. (1998) FEBS Lett. 441, 29–33.

[7] Dobrowolska, G., Meggio, F., Szczegielniak, F., Muszynska, G. and Pinna, L.A. (1992) Eur. J. Biochem. 204, 299–303.
[8] Stigare, J., Buddelmijer, N., Pigon, A. and Egyhazi, E. (1994) Cell. Mol. Biol. Res. 40, 463–472.
[9] Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L.A. and Oliviero, S. (1998) J. Biol. Chem. 273, 21292–21297.
[10] Allende, C.C. and Allende, J.E. (1998) J. Cell. Biochem. Suppl. 30-31, 129–136.
[11] Hèrichè, J.-K., Lebrin, F., Rabilloud, T., Leroy, D., Chambaz, E.M. and Goldberg, Y. (1997) Science 276, 952–955.
[12] Seldin, D.C. and Leder, P. (1995) Science 267, 894–897.
[13] Kelliher, M.A., Seldin, D.C. and Leder, P. (1996) EMBO J. 15, 1621–1631.
[14] Miyata, Y. and Yahara, I. (1995) Biochemistry 34, 8123–8129.
[15] Li, D., Dobrowolska, G. and Krebs, E.G. (1996) J. Biol. Chem. 271, 15662–15668.
[16] He, H., Watanabe, T., Zhan, X., Huang, C., Schuuring, E., Fukami, K., Takenawa, T., Kumar, C.C., Simpson, R.J. and Maruta, H. (1998) Mol. Cell. Biol. 18, 3829–3837.
[17] Marin, O., Meggio, F. and Pinna, L.A. (1994) Biochem. Biophys. Res. Commun. 198, 898–905.
[18] Marin, O., Meggio, F., Sarno, S. and Pinna, L.A. (1997) Biochemistry 36, 7192–7198.
[19] Brunati, A.M., Donella-Deana, A., James, P., Quadroni, M., Contri, A., Marin, O. and Pinna, L.A. (1999) J. Biol. Chem. 274, 7557–7564.
[20] Grankowski, N., Boldyreff, B. and Issinger, O.-G. (1991) Eur. J. Biochem. 198, 25–30.
[21] Sarno, S., Vaglio, P., Meggio, F., Issinger, O.-G. and Pinna, L.A. (1996) J. Biol. Chem. 271, 10595–10601.
[22] Boldyreff, B., Meggio, F., Pinna, L.A. and Issinger, O.-G. (1992) Biochem. Biophys. Res. Commun. 188, 228–234.
[23] Malmqvist, M. (1999) Biochem. Soc. Trans. 27, 335–340.
[24] Johnsson, B., Lofas, S. and Lindquist, G. (1991) Anal. Biochem. 198, 268–277.
[25] Glass, D.B., Masaracchia, R.A., Feramisco, J.R. and Kemp, B.E. (1978) Anal. Biochem. 87, 566–575.
[26] Kitamura, D., Kaneko, H., Miyagoe, Y., Ariyasu, T. and Watanabe, T. (1989) Nucleic Acids Res. 17, 9367–9379.
[27] Weng, Z., Thomas, S.M., Rickles, R.J., Taylor, J.A., Brauer, A.W., Seidel-Dugan, C., Michael, W.M., Dreyfuss, G. and Brugge, J.S. (1994) Mol. Cell. Biol. 14, 4509–4521.
[28] Boldyreff, B. and Issinger, O.-G. (1997) FEBS Lett. 379, 153–156.
[29] Vaglio, P., Sarno, S., Marin, O., Meggio, F., Issinger, O.-G. and Pinna, L.A. (1996) FEBS Lett. 380, 25–28.
[30] Meggio, F., Boldyreff, B., Issinger, O.-G. and Pinna, L.A. (1994) Biochemistry 33, 4336–4342.
[31] Sonnhammer, E.L.L. and Durbin, R. (1995) Gene 167, GC1–10.