FEBS Letters 403 (1997) 200–202 FEBS 18156

The regulatory subunit of protein kinase CK2 is a specific A-Raf activator

Carsten Hagemann, Andreas Kalmes, Viktor Wixler[1], Ludmilla Wixler, Tillman Schuster, Ulf R. Rapp*

Institut für med. Strahlenkunde und Zellforschung, Bayerische Julius-Maximillians-Universität, Versbacher Str. 5, D-97078 Würzburg, Germany

Received 11 December 1996

Abstract Two protein kinases that are involved in proliferation and oncogenesis but so far were thought to be functionally independent are Raf and CK2. The Raf signaling pathway is known to play a critical role in such fundamental biological processes as cellular proliferation and differentiation. Abnormal activation of this pathway is potentially oncogenic. Protein kinase CK2 exhibits enhanced levels in solid human tumors and proliferating tissue. In a two-hybrid screen of a mouse-embryo cDNA library we detected an interaction between A-Raf and CK2β subunit. This binding was specific, as no interaction between CK2β and B-Raf or c-Raf-1 was observed. Regions critical for this interaction were localized between residues 550 and 569 in the A-Raf kinase domain. A-Raf kinase activity was enhanced 10-fold upon coexpression with CK2β in Sf9 cells. The α subunit of CK2 abolishes this effect. This is the first demonstration of both a direct Raf-isoform-specific activation and a regulatory role for CK2β independent of the CK2α subunit. The present data thus link two different protein kinases that were thought to work separately in the cell.

Key words: Protein kinase CK2; CK2β subunit; A-Raf kinase; Protein-protein interaction; Two-hybrid system; Kinase assay

1. Introduction

The family of cytoplasmic Raf serine/threonine kinases comprises three members in mammals: c-Raf-1, B-Raf and A-Raf. They have a high degree of similarity, consisting of three conserved regions, CR1, CR2 and CR3. The CR1 region includes the Ras binding domain that is responsible for recruitment of Raf kinases to the cellular membrane and subsequent activation during growth factor stimulation. The CR3 region represents the catalytic domain. Deletion of the regulatory N-terminal half of all three Raf kinases leads to constitutively active oncogenic forms. Whereas an essential role of the ubiquitously expressed c-Raf-1 in the control of cellular proliferation and transformation is well established, little is known about potential specific functions of A-Raf and B-Raf [1]. Besides the preferential mRNA expression of A-Raf in urogenital tissues [2], a differential regulation of A-Raf in comparison to c-Raf-1 was shown during NGF-induced differentiation of pheochromocytoma PC12 cells [3] and in ventricular myocytes' response to growth-promoting stimuli [4]. Little is known about a Raf-isozyme-specific regulation. Therefore, a mouse-embryonic cDNA library was screened using the two-hybrid system with A-Raf as a bait leading to the isolation of CK2β.

CK2β is the regulatory subunit of protein kinase CK2, a ubiquitously expressed serine/threonine protein kinase [5–7]. The CK2 holoenzyme is a heterotetrameric complex ($\alpha_2\beta_2$). Two β subunits dimerize and bind two catalytic α subunits to form the holoenzyme [8,9].

Although there are several reports on a potential role of CK2 in cell proliferation and tumorigenesis [10–12], not very much is known about its regulation and physiological function. There is some evidence that the subunits may also have a function when they occur separated from each other [13–15]. Overexpression of CK2β in *Schizosaccharomyces pombe*, an organism that does not contain a Raf homolog, leads to inhibition of cell growth and cytokinesis, resulting in a multiseptated phenotype [16], whereas CK2α overexpression has no effect.

In multicellular organisms that carry a full complement of Raf enzymes, the effect of β overexpression on cellular phenotype has not yet been evaluated. Here we report that A-Raf specifically binds the regulatory β-subunit of protein kinase CK2 and this leads to activation of A-Raf kinase, independent of CK2α.

2. Materials and methods

2.1. Screening a two-hybrid library with A-Raf as a bait and direct interaction tests

A 14.5 day mouse-embryo cDNA library with 1.92×10^6 independent inserts cloned in pPC67 as fusion with the Gal4 activation domain [17] was cotransformed with A-Raf cloned in pPC97 as a fusion with the Gal4 DNA-binding domain into the yeast strain HF7c [18]. Positive clones were monitored by growth on medium lacking histidine and activity of the lacZ-reporter gene in filter assays. Replica filters of transformants were frozen in liquid nitrogen and incubated in Z buffer (16.1 g/l $Na_2HPO_4{\cdot}7H_2O$, 5.5 g/l $NaH_2PO_4{\cdot}H_2O$, 0.75 g/l KCl, 0.246 g/l $MgSO_4{\cdot}7H_2O$) which contained 0.27 ml 2-mercaptoethanol and 1.67 ml X-gal (20 mg/ml in dimethylformamide) per 100 ml at 30°C for up to 12 h.

Of 9.9×10^6 transformants screened, 57 clones were His and lacZ positive and further analysed by sequencing and colony hybridization.

For direct interaction tests yeast strain HF7c was cotransformed with Raf deletion constructs cloned in pPC97 and the CK2β full length clone isolated from the mouse library. Transformants were then handled as described above.

2.2. Expression in insect cells

Sf9 cells were grown in IPL-41 Insect Medium under standard culture conditions. 10^7 Sf9 cells were infected with baculoviruses (10 MOI/cell) expressing CK2α, CK2β and A-, B- or c-Raf-1 genes. After 48 h cells were washed twice with PBS and lysed in Triton buffer (50 mM Tris-HCl pH 8.0, 137 mM NaCl, 1% Triton X-100, 10 mM Na-pyrophosphate, 25 mM Na-glycerophosphate, 2 mM EDTA, 2 mM

*Corresponding author. Fax: (49) (931) 201 3835.

[1]*Present address:* Inst. für Biochemie II der Universität Köln, Joseph-Stelzmann Str. 52, D-50931 Köln, Germany.

PII S0014-5793(97)00011-2

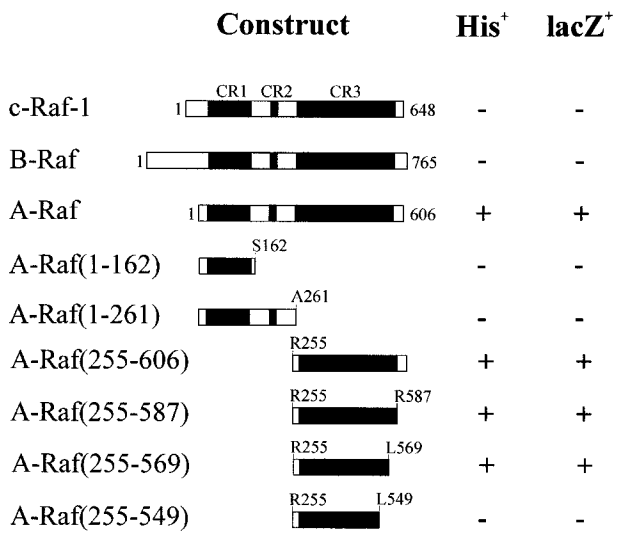

Fig. 1. Interaction of Raf isozymes with CK2β and mapping of the binding sites. Two hybrid tests of different Raf constructs with CK2β. In addition to the constructs shown, the A-Raf deletion constructs A-Raf(1–491), A-Raf(1–428), A-Raf(1–310), and A-Raf(255–530) were tested with negative results. CR, conserved regions; His^+, growth on medium lacking histidine; $lacZ^+$, activity of the lacZ reporter gene.

EGTA, 10% glycerol, 0.1% 2-mercaptoethanol, 1 mM Na-vanadate, 25 mM NaF, 0.01% leupeptin, 0.01% aprotinin, 2 mM pepstatin). The lysates were cleared by centrifugation at 10000 rpm for 5 min.

2.3. Immunoprecipitation

900 μg of total cell lysates were used for immunoprecipitation of Raf proteins. The anti-Raf antibodies were raised against synthetic peptides representing the 12 C-terminal residues of A- and c-Raf-1 and the 13 C-terminal residues of B-Raf, respectively. After incubation at 4°C for 2 h immunoprecipitates were washed twice with Triton buffer and once with HEPES buffer (25 mM HEPES pH 7.5, 25 mM Na-glycerophosphate, 1.5 mM EGTA, 5% glycerol, 1 mM DTT) and divided into aliquots for (i) immunocomplex kinase assays (see below) and (ii) immunodetection of Raf. After resolving samples by SDS-PAGE and electroblotting onto nitrocellulose membrane, immunodetections were performed with Raf-specific antisera using the ECL detection system (Amersham).

2.4. Immunocomplex kinase assay

Aliquots of Raf immunocomplexes were resuspended in 30 μl of kinase buffer (25 mM HEPES pH 7.5, 25 mM Na-glycerophosphate, 1.5 mM EGTA, 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 100 μM ATP, 5 μCi [γ-^{32}P]ATP (3000 Ci/mM, Amersham) containing 50 μg/ml of recombinant K97M kinase dead MEK mutant protein and incubated at 30°C for 15 min. After resolving by SDS-PAGE, samples were electroblotted onto nitrocellulose. Quantification of kinase activity and the amount of Raf proteins (using ^{35}S-labelled secondary antibodies) was done using the Bio Imaging Analyzer BAS 2000 (Fuji).

3. Results and discussion

3.1. A-Raf interacts specifically with CK2β

In a two-hybrid screen [19] of a mouse embryonic cDNA library with A-Raf as bait 57 positive clones were detected. Sequencing and colony hybridization revealed that one clone represents H-Ras and 56 were identified as CK2β. Eight of these 56 clones were sequenced, representing two independent clones, one full-length clone and one lacking the 17 N-terminal amino acids. In a parallel screen of the same library with B-Raf as bait no CK2β was found (Hagemann et al., unpublished results).

This was the first indication that CK2β may interact specifically with A-Raf. To test this hypothesis and in order to map interaction sites we performed direct two-hybrid tests using all three Raf isoforms and different A-Raf deletion constructs (Fig. 1). These tests showed that CK2β interacts exclusively with A-Raf but not with c-Raf-1 or B-Raf. From large N- or C-terminal deletion mutants of A-Raf only A-Raf(255–606) showed an interaction with full length CK2β. Further deletions of A-Raf(255–606) from the C-terminal end still showed an interaction when amino acids 255–569 were present, but not when the C-terminus was further deleted until amino acid 549. This means that residues 255–569 of A-Raf are sufficient,

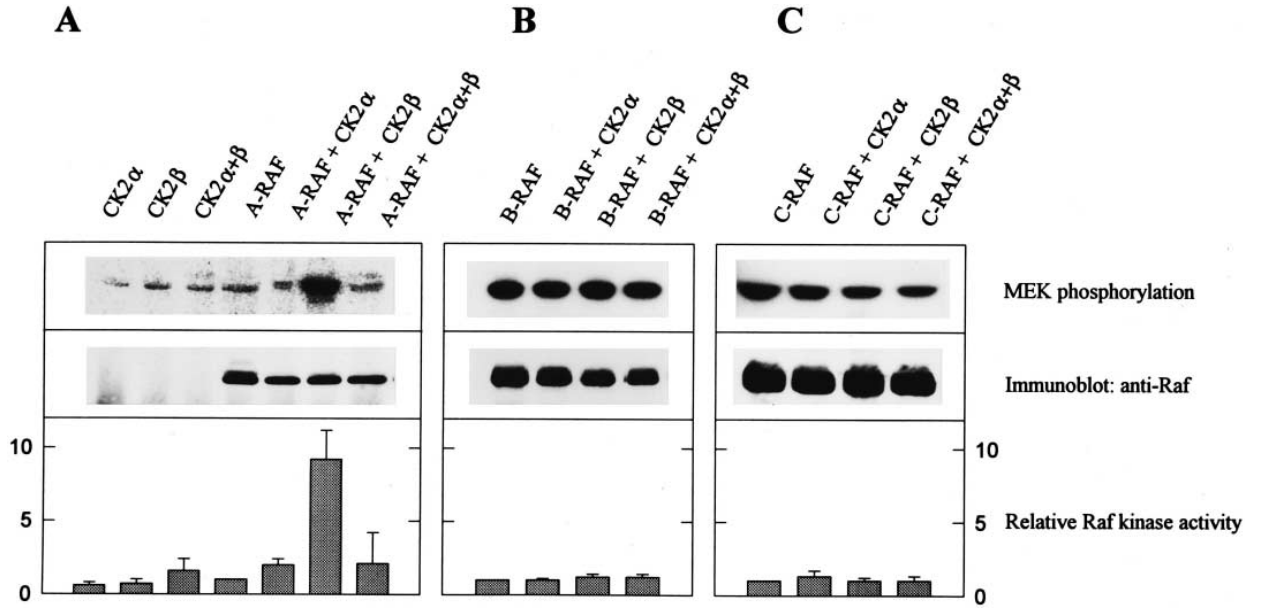

Fig. 2. CK2β subunit activates A-Raf (A), but not B-Raf (B) or c-Raf-1 (C). The amount of precipitated Raf protein was compared within individual panels (A, B, or C) using ^{35}S-labelled secondary antibodies and the variation was ≤25%. Values of Raf kinase activity from triplicate experiments were normalized to the activity of Raf alone, which was arbitrarily chosen as 1.

and residues 550–569 are necessary for binding of CK2β. The present data, however, do not indicate whether residues 550–569 are directly involved in binding, or whether they are necessary for maintaining the correct 3-dimensional structure of the binding site. Amino acids 550 to 569 correspond to the C-terminal half of subdomain XI of the A-Raf kinase domain [20]. This region includes residues which are highly conserved between the different Raf proteins as well as A-Raf-isozyme-specific sequences, which may account for the A-Raf-specific binding. We were not able to find a direct interaction of A-Raf with the α subunit of CK2 (not shown), whereas a trimeric complex between CK2α, CK2β and A-Raf was not excluded.

3.2. CK2β activates A-Raf in immunocomplex kinase assays

In order to address the physiological significance of these observations we asked whether the association with the CK2 subunits would influence the kinase activity of A-Raf. We expressed the individual Raf isozymes together with CK2β, CK2α, or both, in insect cells. Raf was immunoprecipitated using Raf-isozyme-specific antisera and the Raf immunocomplexes were subjected to kinase assays with bacterially expressed MEK as substrate (Fig. 2). Neither the two CK2 subunits alone nor together were able to phosphorylate MEK above basal level. In the case of A-Raf, MEK phosphorylation was significantly enhanced (10-fold) after coexpression of CK2β, but not of CK2α. Coexpression of CK2α, CK2β and A-Raf in a triple infection reduced the A-Raf activity back to basal level. In contrast, MEK phosphorylation by B-Raf and c-Raf-1 was not affected by both CK2β or CK2α, alone or in combination.

The present data clearly demonstrate that the regulatory β subunit of CK2 alone can act as a specific activator of A-Raf. The α subunit abolishes this activation, most probably by competing with A-Raf for binding to CK2β. As CK2β in vivo exists as a dimer that interacts with two CK2α monomers to form the active, heterotetrameric CK2 enzyme [21], it might be possible that heterotetramerization with two A-Raf molecules is responsible for CK2β-mediated A-Raf activation. Activation by protein-protein interaction in the kinase domain is not restricted to A-Raf, as we have observed that the kinase activity of c-Raf-1 was enhanced in vivo and in vitro by binding to the Bcl-2 family protein BAG-1 [22].

Our data suggest a new mode of isozyme-specific Raf kinase regulation, which depends on the availability of free CK2β. There is some evidence that isolated Ck2 subunits are present in cells [13], as rapidly proliferating tissue culture cells and human kidney tumors synthesize the β subunit in excess of the α subunit [14,15]. Since A-Raf shows highest expression levels in urogenital tissue [2], CK2β mediated A-Raf activation might be involved in the formation of these tumors.

Acknowledgements: We would like to thank P. Chevray and D. Nathans for plasmids pPC86, pPC97 and the mouse cDNA library, U. Hemmann and O.-G. Issinger for CK2α cDNA and CK2β recombinant baculovirus, W.-H. Thomas for constructing pPC97-c-raf and R. Krug for sequencing. This work has been supported by grants of the Deutsche Forschungsgemeinschaft (DFG, RA642/1-1 and SFB 172).

References

[1] Daum, G., Eisenmann-Tappe, I., Fries, H.W., Troppmair, J. and Rapp, U.R. (1994) Trends Biochem. Sci. 19, 474–480.
[2] Storm, S.M., Cleveland, J.L. and Rapp, U.R. (1990) Oncogene 5, 345–351.
[3] Wixler, V., Smola, U., Schuler, M. and Rapp, U.R. (1996) FEBS Lett. 385, 131–137.
[4] Bogoyevitch, M.A., Marshall, C.J. and Sugden, P.H. (1995) J. Biol. Chem. 270, 26303–26310.
[5] Pinna, L.A. (1990) Biochim. Biophys. Acta 1054, 267–284.
[6] Issinger, O.-G. (1993) Pharmacol. Ther. 59, 1–30.
[7] Allende, J.E. and Allende, C.C. (1995) FASEB J. 9, 313–323.
[8] Grankowski, N., Boldyreff, B. and Issinger, O.-G. (1991) Eur. J. Biochem. 198, 25–30.
[9] Meggio, F., Boldyreff, B., Marin, O., Pinna, L.A. and Issinger, O.-G. (1992) Eur. J. Biochem. 204, 293–297.
[10] Hanna, D.E., Rethinaswamy, A. and Glover, C.V. (1995) J. Biol. Chem. 270, 25905–25914.
[11] Pepperkok, R., Lorenz, P., Ansorge, W. and Pyerin, W. (1994) J. Biol. Chem. 269, 6986–6991.
[12] Seldin, D.C. and Leder, P. (1995) Science 267, 894–897.
[13] Stigare, J., Buddelmeir, N., Pigon, A. and Egyhazi, E. (1993) Mol. Cell. Biochem. 129, 77–85.
[14] Lüscher, B. and Litchfield, D.W. (1994) Eur. J. Biochem. 220, 521–526.
[15] Stalter, G., Siemer, S., Becht, E., Ziegler, M., Remberger, K. and Issinger, O.-G. (1994) Biochem. Biophys. Res. Commun. 202, 141–147.
[16] Roussou, I. and Draetta, G. (1994) Mol. Cell. Biol. 14, 576–586.
[17] Chevray, P.M. and Nathans, D. (1992) Proc. Natl. Acad. Sci. USA 89, 5789–5793.
[18] Feilotter, H.E., Hannon, G.J., Ruddell, C.J. and Beach, D. (1994) Nucl. Acids Res. 22, 1502–1503.
[19] Chien, C.-T., Bartel, P.L., Sternglanz, R. and Fields, S. (1991) Proc. Natl. Acad. Sci. USA 88, 9578–9582.
[20] Hanks, S.K. and Quinn, A.M. (1991) Methods Enzymol. 200, 38–62.
[21] Boldyreff, B., Mietens, U. and Issinger, O.-G. (1996) FEBS Lett. 379, 153–156.
[22] Wang, H.-G., Takayama, S., Rapp, U.R. and Reed, J.C. (1996) Proc. Natl. Acad. Sci. USA 93, 7063–7068.