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Asymmetric Expression of Protein Kinase CK2 Subunits in Human Kidney Tumors*

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Renal clear cell carcinomas and the corresponding ipsilateral control tissues were investigated for protein kinase CK2 activity and subunit ratio. The average protein kinase CK2 activity from 21 different kidney samples was 318 U/mg and that from the corresponding tumors 610 U/mg. The subunit ratio of protein kinase CK2α in tumors/normal tissue (T/N) was 1.58 and that of the protein kinase CK2ß (T/N) was 2.65. The data suggest that the generally described increase in protein kinase CK2 activity in tumor cells may to some extent result from a deregulation in subunit biosynthesis or degradation. This at least partly owing to the presence of excess enzymatically active protein kinase α-subunit but also to a significantly higher presence of the non-catalytic β-subunit.

Protein kinase CK2 is a tetrameric enzyme with a subunit structure of $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$. The α-subunit exhibits protein kinase activity. The β-subunit is non-catalytic. The activity of protein kinase CK2 has previously been shown to be elevated in cells and tissues with a high mitotic index, e.g. in colon crypt cells, and in rapidly proliferating tumor cells (1-3). The elevated enzyme activity was at least in part due to larger amounts of enzyme molecules present in these cells as shown by immunoblotting using phosphatase-linked secondary antibodies and BCIP and NBT as detection reagents (1). However, the detection limit of this method did not allow precise densitometric analyses of protein kinase CK2 and its subunits in unfractionated crude cellular extracts; in particular the protein kinase CK2β subunit was not always detectable (1, 4). Therefore, no clear cut answer concerning the exact ratio of protein kinase CK2 subunits in tumors was obtained. With the availability of the chemoluminescence (CL) detection method, it has become possible to detect both protein kinase CK2 subunits in crude cellular extracts and to analyse the obtained fluorographs by densitometry. Highly-purified recombinant or native protein kinase CK2 holoenzyme, where the protein kinase CK2 subunits are present in stoichiometric amounts, was used as a reference.

Material and Methods

Biopsy Material - Tumors were obtained immediately after surgery. The material was either used directly or from liquid nitrogen stocks. Tumors were classified according to the current TMN

*The former casein kinase 2 (CK2) is referred to in this text as "protein kinase CK2".

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classification. For comparative studies throughout the investigations, biopsy material from the ipsilateral tissue was used.

Tissue Extraction - Tumor material was suspended in 20 mM Tris/HCl, pH 8.0; 100 mM NaCl, 0.5 mM $PhMeSO_2F$, 7 mM 2-mercaptoethanol, then cut into small pieces and homogenized in an Ultraturrax followed by sonication.This material was centrifuged at 10,000 x g for 20 min at 4°C to remove cellular debris. The resulting supernatant was used for protein kinase CK2 activity determination and immunoblot analysis. Protein determination was carried out using the BioRad protein reagent. Aliquots of 300 µg protein were removed, dialysed against 20 mM ammonium hydrogencarbonate, lyophilised and then dissolved in 20 µL SDS sample buffer.

SDS PAGE Analysis - In order to ensure equal protein loading of the gels, a match gel was run; usually one tenth of the total protein volume was applied. After the run, the gels were stained with Coomassie blue. The individual samples were matched visually and if adjustments had to be made, a second match gel was run to verify the changes. Usually, 100-200 µg of protein were used for the immunoblotting analysis.

Anti Protein Kinase CK2-Specific Antibodies - The antibodies used were polyclonal and raised in rabbits against gel-derived recombinant human subunits. The antibodies were made monospecific by affinity purification as described in detail (5). Monospecific antibodies only detected protein kinase CK2 subunits in crude extracts.

Immunoblotting and Luminol-Based Chemoluminescence Detection (CL) - Protein transfer was performed using a semidry blotting apparatus, 1h at 400 mA, constant current, at room temperature. The transfer was verified using a colored marker mix (Rainbow Marker, Amersham). Fortified nitrocellulose membranes (Schleicher-Schüll) were used. Immunoblots were incubated overnight in the presence of anti protein kinase CK2 antibodies. The first antibody was removed and the immunoblot was washed three times. Finally, the second antibody, donkey/rabbit IgG-peroxidase, was applied for 2h. The detection of the antigens was essentially as described in the CL kit from Boehringer/Mannheim. The films were exposed between 30 sec and 15 min using Kodak X-OMAT AR X-ray film. Although immunoblotting of the tumor extracts and the normal tissue was carried out in parallel on the same membrane, detection of the protein kinase CK2 subunits was done separately with two different antibodies. Hence, only comparison of the homologous subunit ratio (α/α and β/β) was possible (Fig.1). Alternatively, if the heterologous subunit ratio was also of interest an artificial mixture of anti protein kinase CK2 subunit antibodies, where the antibodies are mixed in such a manner that they detect protein kinase CK2 subunits of purified holoenzyme in a 1:1 ratio was used.

Protein Kinase CK2 Activity - An aliquot of the crude cellular supernatant was adjusted to 1 mg/ml and then used for protein kinase CK2 activity determination. At least three different protein concentrations were used for the test. Values in the linear range were used for the calculation of the specific activity, here expressed in units. 1 unit is defined as the amount catalysing the transfer of 1 pmol ^{32}P into the substrate (the synthetic peptide RRRDDDSDDD) in 1 min at 37°C. The assay was essentially carried out as described previously (6). The activity values indicated in table 1 are the result of 2 different independent determinations and have to be multiplied by a factor of 10.

Results and Discussion

The aim of the present investigations was to address the question whether the β-subunit ratio between tumors and normal tissue show a similar imbalance to the ratio found for the α-subunit (1, 2). The question has not been answered so far, owing to technical limitations which have made the detection of the β-subunit very difficult. With the availability of the CL detection technique it is now

possible to detect the β-subunit in unfractionated crude tissue extracts to such an extent that a densitometric analysis can be made.

Fig. 1 shows the CL detection of two tumors (#6, #19) and the corresponding ipsilateral normal tissue. Both tumors are renal clear cell carcinomas, staging and grading are the same, i.e. stage T2 and grade G2 respectively. The normal tissue (N) and the tumor (T) were doubly analysed on SDS PAGE and then transferred to a nitrocellulose membrane. The membrane was cut in two halves. One blot containing tumor and normal tissue extract was incubated with a monospecific anti protein kinase CK2α antibody and the other blot incubated with a monospecific anti protein kinase CK2β antibody. Fig. 1 shows the densitometric analysis of the subunit ratio of the tumors versus their normal tissue. In the cases of patients #5078/93 and #8857/93, the α-subunit ratios were 1.7 and 1.4 respectively. Similar values have been found previously for colonic tumors (1). The finding that the T/N β-subunit ratio is also significantly altered, i.e. 1.8 and 4.0 respectively came as a surprise. Table 1 shows, that this increased presence of the protein kinase CK2β subunit is not limited to a few tumors. A statistical analysis of the T/N subunit ratios α versus β revealed a two-tailed p value of 0.0005, indicating an extremely significant difference between the two groups (Table 1). The average T/N protein kinase CK2α ratio (1.58) is significantly smaller than the value found for the β-subunit (2.65) (Table 1). When the protein kinase CK2 activities from tumors and normal tissues were compared, a statistically significant correlation was found (Table 1), once again confirming earlier results obtained with other human solid tumours (1,2).

The higher level of α-subunit ratio in tumors may account for the higher protein kinase CK2 activity found. This is also what would be expected since the α-subunit is the catalytically active component of the protein kinase CK2 holoenzyme. In order to obtain a clearer picture we have tentatively divided our 21 samples into two groups, i.e. #1-9 (T/N ratio β, 1.1.-2.0) and #10-21(T/N ratio β, 2.5-5.2), based on the observation that the average T/N ratio of the β-subunit is 2.65. Obviously, the T/N ratio of the β-subunit of the two groups is extremely significant. The unpaired Mann-Whitney

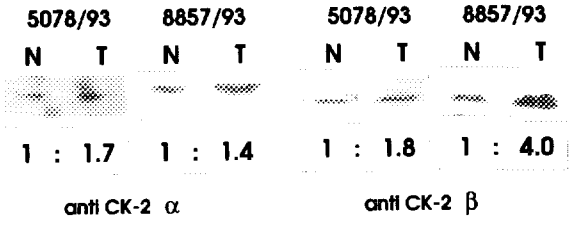

Figure 1. Fluorographs from immunoblots after CL detection. Two tumors (T) and the ipsilateral control tissue (N) analysed on 12.5% SDS PAGE are shown. The numbers on top indicate the patient number. The blots were reacted with either anti protein kinase CK2α antibodies and anti protein kinase CK2β antibodies. The signal intensity on the fluorographs was calculated by densitometry using the Millipore BioImage programme. The numbers on the bottom part of the figure show the ratios obtained. For each of the 4 blots the densitometric values obtained were normalized so that the lowest signal, usually associated with the normal tissue, was set to 1.

Table 1. Comparison of the T/N subunit ratio and the CK-2 activity from tumors and control tissues from 21 renal clear cell carcinomas. CK-2 activity values x 10. Tumors are sorted according to increasing T/N ratio of the β-subunit.

Nr	Patient	Staging	Grading	T/N Ratio[a]		CK-2 Activity[b]		Activity Ratio
				α	β	N	T	
1	13152/93	T2	G4	0.9	1.1	53	92	1.7
2	20496/93	T2	G2	1.3	1.4	18	35	1.9
3	6588/93	T3a	G2-3	1.4	1.5	18	29	1.6
4	14419/93	T3b	G2	1.1	1.5	24	32	1.3
5	17468/91	T2	G2	1.0	1.6	23	39	1.7
6	5078/93	T2	G2	1.7	1.8	30	42	1.4
7	4015/93	T4	G2	1.8	1.9	21	72	3.4
8	19486/93	T2	G2	2.8	1.9	30	66	2.1
9	20964/91	T2	G2	1.1	2.0	27	50	1.9
10	16452/91	T3a	G2	1.1	2.5	73	72	1.1
11	12836/93	T1	G2	2.0	2.5	29	51	1.8
12	7340/93	T2	G2	1.6	2.8	23	79	3.4
13	19649/91	T2	G2	2.0	3.0	21	32	1.5
14	10193/93	T3	G2	1.9	3.0	18	32	1.8
15	13376/93	T2	G2	1.2	3.0	37	59	1.6
16	19014/93	T2	G3	1.6	3.1	92	172	1.8
17	8339/93	T3a	G2	1.8	3.5	32	82	2.5
18	16451/91	T3a	G2	2.2	3.9	22	46	2.1
19	8857/93	T2	G2	1.4	4.0	35	82	2.3
20	6401/93	T3	G3	2.1	4.6	15	48	3.1
21	13051/93	T1	G2	1.3	5.2	27	71	2.6

Paired non parametric test (Mann-Whitney or Wilcoxan). Mean ± S.D.:
[a]T/N ratio: α-subunit (n=21): 1.58 ± 0.47 versus β-subunit (n=21): 2.65 ± 1.1; P<0.0005.
[b]CK-2 activity: Normal (n=21): 31.80 ± 19.06 versus Tumor (n=21): 61.0 ± 32.04; P<0.0419.

test yields a two-tailed P value of 0.0002, whereby a P value of $p > 0.05$ is considered nonsignificant. Such a comparison with respect to the T/N α-subunit ratio and the protein kinase CK2 activity does not lead to signifiant differences; here the P values are $p > 0.1$ in both cases. We conclude from these results, that the high β-subunit level found in the tumors is only in part associated with the higher protein kinase CK2 activity found in the tumors inasmuch as part of the β-subunit is needed for the formation of holoenzyme. The excess β-subunit which is found in the vast majority of the tumors is probably involved in other functions.

It was shown recently that in exponentially growing tissue cultured cells, the β-subunit was synthesized in excess of the α-subunit. A substantial fraction of the newly synthesized β-subunit was degraded within the first hour (7). In the light of our findings in tumors, it is tempting to speculate, that the degradation is a very important regulatory mechanism and that inhibition of this degradation would lead to an increase of the β-subunit which could have deleterious effects on the cell.

Another report comes to the conclusion that a major portion of nuclear protein kinase CK2α does not form heterooligomeric structures with the β-subunit but binds tightly to nuclear components, whereas the β-subunit is loosely bound to the nuclear structure (8). Our approach cannot differentiate between tight and loose interactions of protein kinase CK2 subunits with other cellular components. We therefore do not know whether all of the α-subunits identified in our immunoblots derive from protein kinase CK2 holoenzyme and whether the excess β-subunit is present in a free form or associated with other proteins or cellular structures, e.g. chromatin. Another report (9) showed that when protein kinase CK2β cDNA was introduced into cultured cells it led to an increase in protein kinase CK2 activity although the amount of α-subunit remained constant. Does this observation imply the presence of inactive free protein kinase CK2α which forms the active holoenzyme as soon as the β-subunit is available, or does it mean that endogenous protein kinase CK2 holoenzyme is not fully active and that raising the concentration of the α- or β-subunit stimulates this latent activity? We know that the switch from quiescence to the active growing state also involves changes in the β-subunit ratio (5, 10).

The observation that overexpressed β-subunit in Xeroderma pigmentosum fibroblasts makes the cells more resistant to UV light lends support for a specific nuclear function for the β-subunit (11). One of the most attractive hypotheses in this direction stems from the demonstration that protein kinase CK2β can bind specifically to the tumor suppressor gene product p53 (12, 13). In addition, it was shown previously (14) that p53 is phosphorylated by protein kinase CK2, although no classical consensus sequence exists at the carboxy terminal.

Whether the phosphorylation or simply the association/interaction of protein kinase CK2β with p53 is of importance has yet to be answered. In any case the evidence for a protein kinase CK2/p53 interaction must be further confirmed by in vitro studies using purified protein kinase CK2β and p53, especially since it is still an open question whether protein kinase CK2β exists as a monomer or a dimer. The answer to this question would be important in order to predict what kind of complex may exist. Recently, it was shown (15) in Saccharomyces pombe that the β-subunit overexpression inhibits cell growth and cytokinesis. On the other hand, disruption of the β gene reduces protein kinase CK2 activity to undetectable levels. Protein kinase CK2α overexpression did not lead to an increase in enzyme activity, only co-expression of protein kinase CK2α and protein kinase CK2β subunits resulted in a large enhancement in protein kinase CK2 activity roughly proportional to the increase in CK2α protein levels (15).

The effects generated by protein kinase CK2β subunit overexpression may not stem from an overall increase in protein kinase CK2 activity but rather be an additional function of this subunit. Evidence for trifunctional properties of protein kinase CK2β has been presented in detail elsewhere (for reviews: 16, 17). The CK2β subunit: (i) confers stability to the holoenzyme (18, 19); (ii) increases enzyme activity (6,20) and determines substrate specificity (21, 22). Especially the latter properties have not been elucidated in great detail and may play an important role in cellular regulation. It was shown only recently (23) that polylysine prevents the autophosphorylation of β^{m} (86% inhibition) inducing a parallel increase of α-subunit autophosphorylation. Polylysine is also a mediator of protein kinase CK2 substrate specificity (21,22). Calmodulin is not phosphorylated by the protein kinase CK2 holoenzyme unless polylysine is added. The recombinant α-subunit, however, spontaneously phosphorylated calmodulin, this phosphorylation actually being inhibited rather than stimulated by polylysine. In the case of pathologically high levels of protein kinase CK2β, as seen in our tumors this lack of control, could then lead to deleterious effects, e.g. by the binding of larger portions of p53. This would lead to an enrichment of cellular p53. Nuclear oncoproteins, e.g. N-myc, c-myc, c-fos, p53, and E1A, are among the most rapidly degraded intracellular proteins and it has been shown that they are degraded by the ubiquitin system (24). An imbalance of even one of these important proteins could lead to severe alterations in growth control, which, together with the increased protein kinase CK2 activity might influence the overall tumor growth. Clearly, more data are needed in order to elucidate more thoroughly the unique role of CK2ß.

Acknowledgments

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