Serum-stimulated cell cycle entry of fibroblasts requires undisturbed phosphorylation and non-phosphorylation interactions of the catalytic subunits of protein kinase CK2

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Abstract Protein kinase CK2 is a pleiotropic Ser/Thr kinase occurring as $\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$ tetramers. A requirement in serum-stimulated cell cycle entry in both the cytoplasm and the nucleus of human fibroblasts for phosphorylation(s) by CK2 has been concluded from stimulation inhibition by microinjected antibodies against the regulatory subunit (β). We have now examined this idea more directly by microinjection-mediated perturbation of phosphorylation and non-phosphorylation interactions of the catalytic subunits (α and α′), and by verifying the supposed matching of the cellular partition of CK2 subunits in the fibroblasts employed. While immunostaining and cell fractionation indicate that the partitions of subunits indeed match each other (with their predominant location in the nucleus in both quiescent and serum-stimulated cells), microinjection of substrate or pseudosubstrate peptides competing for the CK2-mediated phosphorylation in vitro resulted in significant inhibition of serum stimulation when placed into the nucleus but not when placed into the cytoplasm. Also inhibitory were nuclear but not cytoplasmic injections of antibodies against α and α′ that affect neither their kinase activity in vitro nor their complexing to β. The data indicate that the role played by CK2 in serum-stimulated cell cycle entry is predominantly nuclear and more complex than previously assumed, involving not only phosphorylation but also experimentally separable non-phosphorylation interactions by the catalytic subunits.

Key words: Protein kinase CK2 (casein kinase II); Cell cycle signaling; Human lung fibroblast; Microinjection; Serum stimulation; Gene expression

1. Introduction

Protein kinase CK2 (also known as casein kinase II) is a pleiotropic, ubiquitous Ser/Thr kinase vital for eukaryotes. CK2 phosphorylates at sites typically located within acidic stretches of proteins with -Ser/Thr-X-X-Glu/Asp- as a consensus. Glu/Asp are replaceable by phospho-Ser or phospho-Tyr, a property perfectly suited for specific cross-talks to other kinases and thus for the participation of CK2 in regulatory cellular networks. Of the many proteins phosphorylated by CK2, a majority are involved in cell cycle-related signaling and gene expression. CK2 of all eukaryotes investigated so far has a tetrameric structure composed of two catalytic (α) and two regulatory (β) subunits. Humans (and other mammalians) possess an additional catalytic α′ subunit, so that $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ and $\alpha'_2\beta_2$ holoenzymes are found throughout tissues in which αβ or α′β dimers are complexed via β-β interaction [1–4]. Like the quaternary structure of CK2, the primary structure of each of the individual subunits is highly conserved, the conservation manifested even in the structure of the genes [5–7]. The role played by CK2 and the regulation of CK2 in cells remain unclear. No distinct factor(s) are known to which CK2 would respond in a turn-on/off manner; activity seems rather to relate to the presence of polybasic and polyacidic molecules as exemplified by polyamines and heparin, respectively, and to the ionic microenvironment. Subunit β seems to determine the protein spectrum phosphorylated by CK2, and also to what degree the phosphorylations occur [2–4]. Further, there are indications that CK2-mediated effects other than by phosphorylation might happen, and that 'free' subunits, i.e. subunits not complexed into CK2 holoenzymes, might be present in cells [4]. Deviations from the tetrameric stoichiometry beyond certain extents have been suspected to be responsible for a potentiation of fatal pathological situations such as theileriosis or cancer [8–11].

Using perturbation assays, we had investigated CK2 for an involvement in proliferation stimulation of human fibroblasts by serum, a process highly significant for wound healing [12], and a model for cell cycle re-entry and progression [13]. Perturbation was achieved at the nucleic acid level with antisense oligodeoxynucleotides directed against α and α′, or β [14], and at the protein level with microinjected antibodies. Antibodies specific for β were injected into either the cytoplasm or the nucleus of quiescent cells (serum-deprived cells, i.e. G0 phase cells) prior to stimulation by serum. As a result, stimulation was significantly affected and in a time- and cell compartment-specific manner; injections into cytoplasm inhibited stimulation when carried out during G0 to G1 phase transition, or late in the G1 phase, shortly before reaching S phase. Injections into the nucleus led to a dramatic inhibition when carried out during G0 to G1 phase transition and the immediately adjacent part of the G1 phase [15,16]. Viewing β as a constitutive, phosphorylation-determining part of the tetrameric holoenzyme, these effects had been interpreted as indicative for a role of CK2 holoenzymes and, consequently, for CK2-mediated phosphorylations in serum-stimulated cell cycle entry. However, experimental proof for this interpretation has not been provided, mainly because of a lack of appropriate experimental tools at the time of investigation. In the meantime, substrate-related peptides for CK2 have become available [17], and we have successfully raised and af-

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Abbreviations: ADA, peptide RRRADDADDDDD; ADS, peptide RRRADDSDDDDD; XX, peptide DDRDDRDADADR; BrdU, 5-bromo-2′-deoxyuridine; DTAF, 5-(4,6-dichloro-triazinyl)amino-fluorescein; PBS, phosphate-buffered saline; TRITC, tetramethyl-rhodamine isothiocyanate; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PVDF, polyvinylidene difluoride

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finity purified a series of antibodies also against various epitopes of α and α′ subunits [18,19].

We report here on the microinjection of a CK2 substrate peptide and its pseudosubstrate version (Ser exchanged for Ala) into primary human lung fibroblasts (IMR-90 cells) to compete for CK2-related phosphorylation reactions which should, if the predicted involvement in cell cycle entry occurred, affect the serum stimulation of cells. In order to dissect the kinase function from possible interactions of the catalytic subunits other than by phosphorylation (non-phosphorylation interactions), and to distinguish further between roles of α and α′ in this context, we also carried out injections of antibodies that specifically recognize sequences located downstream or upstream of the catalytic domain areas. Occupation of the sequences by antibodies does not interfere with the complexation of α and α′ to β and has, consequently, no effect on tetramer formation and on kinase activity [18]. The results obtained confirm a role of CK2 in serum-stimulated cell cycle entry, in which the catalytic subunits appear to be involved not only via phosphorylation(s) but, interestingly, also via non-phosphorylation interactions experimentally separable from the kinase function, and in which compartmentation seems to be of importance.

2. Materials and methods

2.1. Cell culture, synchronization, and stimulation to and monitoring of cell cycle re-entry

Human lung primary diploid fibroblasts (IMR-90) were obtained from American Type Culture Collection (Rockville, MD, USA) as passage number 8. The cells were grown on coverslips to approximately 80% confluence in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (FCS; Gibco), at 37°C, in a 5% CO_2 atmosphere. Only cells with passage numbers less than 15 were used. The cells were arrested in the G0 phase of the cell cycle, i.e. rendered quiescent, by withdrawing FCS from the medium for a period of 3–5 days. Stimulation to re-enter and progress through the cell cycle was done by replacing the medium with 20% FCS in DMEM. The reaching of S phase was monitored by the incorporation of 5-bromo-2′-deoxyuridine (BrdU; Sigma) into DNA using indirect immunofluorescence microscopy. The procedure has been extensively characterized [15,16].

2.2. Peptides, antibodies, and recombinant proteins

Peptides representing the CK2 model substrate ADS, pseudosubstrate ADA and control peptide XX, or defined regions of CK2 subunits were synthesized as described previously [18]. Polyclonal antibodies were raised in rabbits against CK2 subunit peptides (anti-α; anti-α′; anti-β), purified, and characterized as reported elsewhere [18]. Recombinant CK2 subunits were prepared following a standard procedure established in our laboratory [20].

2.3. Microinjection and indirect immunofluorescence analysis

Microinjection of IMR-90 cells was performed with an Automated Injection System (AIS) from Zeiss, essentially as described previously [15,16]. A Flamming/Brown micropipette puller (Model P-87 from Sutter Instruments Co.) was used for production of the capillaries. The settings of the machine were tentatively optimized for efficient nuclear injections. Immediately after microinjection, the cells were stimulated to enter the cell cycle (see above), and BrdU was added to the medium. After 24 h of stimulation, cells were fixed by incubation in prechilled methanol (−20°C) for 10 min, and dipping in acetone, and stored in 70% ethanol for up to 1 week until immunofluorescence analysis was performed as follows. Cells were rehydrated in ice-cold phosphate-buffered saline (PBS) and incubated with 5-(4,6-dichlorotriazinyl)amino fluorescein (DTAF)-labeled anti-rabbit antibody (Dianova) allowing detection of ADS-, ADA- or XX-injected cells by co-injected rabbit preimmune IgG fraction, or cells injected with CK2 subunit antibodies. After washing twice with PBS, the cells were kept for 5 min in the presence of 1.5 M HCl, incubated with antibody against BrdU raised in mice (Boehringer Mannheim), washed three times with PBS and further incubated with a tetramethylrhodamine isothiocyanate (TRITC)-labeled anti-mouse antibody (Dianova). Thereafter, the overall DNA was stained with Hoechst dye 33258 (Serva; final concentration 1 μg/ml) and after two washes with PBS, the coverslip was dipped in water and mounted in Mowiol 4-88 (Hoechst). In all cases, antibodies were diluted in 3% BSA and 0.02% Tween-20 in PBS, and allowed to react with cells for 30 min. Washing was carried out by immersing the coverslips in PBS for 5 min with gentle shaking. Whenever BrdU incorporation into DNA could be detected (see above), the cells were counted as stimulation positive. The inhibition of cell cycle re-entry was calculated using the following formula: [BrdU-positive cells (not injected) minus BrdU-positive cells (injected)]/BrdU-positive cells (not injected). For the sake of clarity, the calculated value was multiplied by 100, thereby corresponding to the % of cells whose re-entry into the cell cycle had been prevented [15,16].

2.4. Miscellaneous

Cell fractionation, SDS-PAGE and Western immunoblotting were performed as described previously [15,18]. For comparison of a given protein sequence with databases, the program HUSAR (German Cancer Research Center, Heidelberg) based on the GCG program package version Unix-7.1 (1992, Copyright Genetics Computer Group, Inc.) was used.

3. Results

3.1. Cellular partition of CK2 subunits α and α′ closely resembles that of β

Affinity purified, monospecific polyclonal antibodies raised in rabbits against peptides representing the C-terminal region 329–343 of subunit α (peptide α329–343) and the completely unrelated region 336–350 of subunit α′ (peptide α′336–350) were used in the present study [18]. The antibodies, anti-α329–343 and anti-α′336–350, showed no cross-reactivity in Western blots with the purified recombinant α and α′ subunits nor with proteins present in lysates of the investigated IMR-90 fibroblasts, and neither of them recognized subunit β (Fig. 1A). The antibodies were able to precipitate α and α′, respectively, and to co-precipitate β. Western blot detection and precipitations were efficiently competed by peptide α329–343 and α′336–350, respectively, and neither of the antibodies inhibited the phosphorylation reaction of CK2 in vitro [18]. The antibodies recognized their targets also as they appeared in the cell, i.e. within their cellular context as opposed to the

Table 1
Effect of microinjected CK2 substrate-related peptides on proliferation stimulation

Microinjection	Inhibition (%)	
	Nucleus	Cytoplasm
ADS	28 ± 8 (5)	−5 ± 14 (4)
ADA	53 ± 11 (5)	4 ± 4 (5)
XX	13 ± 9 (7)	0 ± 10 (7)

Quiescent IMR-90 cells were microinjected into the nucleus or the cytoplasm with peptides RRRADDSDDDDD (ADS, 5 mg/ml), RRRADDADDDDD (ADA, 5 mg/ml), or DDRDDRDADADR (XX, 5 mg/ml) prior to proliferation stimulation. Rabbit pre-immune serum (1.5 mg/ml) was included in injection solutions in order to visualize microinjected cells by anti-rabbit antibodies. After microinjection, cells were stimulated with serum (20% final concentration) and incubated in the presence of BrdU. 24 h post-stimulation, cells were fixed and BrdU incorporation visualized by indirect immunofluorescence. Data represent mean values of inhibition of serum stimulation ± S.E.M.; numbers in parentheses indicate the number of independent experiments (at least 200 microinjected cells each). For details of calculation see Section 2.

Western blot situation; immunohistochemical investigations of IMR-90 cells led to clear-cut immunofluorescence signals. For both α and α′, a predominant nuclear location was indicated (Fig. 1B), closely resembling the location of β in previous investigations [2,14–16]. Essentially the same cellular partition was obtained by another approach, cell fractionation followed by Western blot determination of the CK2 subunits. The levels of α and α′ were significantly higher in the nuclear than the cytoplasmic fractions of both proliferating and quiescent cells, and a higher overall level of α and α′ seemed to exist in proliferating than in quiescent cells, β showing corresponding levels and corresponding nuclear-cytoplasmic partition (Fig. 1C).

3.2. Nuclear but not cytoplasmic injection of CK2 peptide substrate and pseudosubstrate interferes with proliferation stimulation

In order to test more directly whether the phosphotransferase function of CK2, i.e. the enzymatic activity of α and α′, has significance for proliferation stimulation, microinjection experiments were carried out with a model peptide substrate and its pseudosubstrate version in which the phosphorylated Ser was exchanged for Ala. These were expected to compete for cellular phosphorylations including those necessary for the serum stimulation to occur, and thus to result in its inhibition. The model substrate chosen was RRRADDSDDDDD (ADS), which is among the CK2 peptide substrates with the lowest K_m and the highest V_{max} [17], and which is widely used for the determination of CK2 activity in cell and tissue homogenates. The pseudosubstrate corresponding to ADS was peptide RRRADDADDDDD (ADA). IMR-90 cells grown on coverslips and arrested in the G0 phase were microinjected with the peptides prior to serum stimulation. Microinjected cells were visualized via co-injected IgG fraction of rabbit pre-immune serum that had no effect on stimulation by itself. As a measure of cell cycle entry, the reaching of S phase (DNA synthesis) was monitored by the incorporation of a detectable DNA precursor BrdU, using indirect immunofluorescence 24 h post-stimulation. As previously shown [16], the cells require roughly 16 h under the conditions used to reach S phase. When injected into the nucleus, ADS significantly inhibited the stimulation by serum (Table 1). By contrast, injection into the cytoplasm had little or no effect. Also ADA was inhibitory upon nuclear injection. The inhibition turned out to be even considerably stronger, i.e. roughly twice as high as with ADS. Cytoplasmic ADA injection had, similar to ADS, little or no effect. As a control, a peptide of the same length composed of the same amino acids but arranged in a random order was used, peptide DDRDDRDADADR (XX). Neither nuclear nor cytoplasmic injection of XX affected proliferation stimulation. Both ADS and ADA, but not XX, inhibited the in vitro kinase activity of recombinant catalytic subunits and reconstituted CK2 holoenzymes (for details of recombinant CK2 and kinase assay, see [20]). The IC_{50} values were calculated to be approximately 0.5–1 mM peptide (data not shown). On the basis of volumes roughly approximated for mammalian cells [21] and for the injected solutions [15,22], the peptide concentrations within nuclei and cytoplasms of injected cells were calculated to reach a similar order of magnitude. It was concluded that an undisturbed cellular performance of the phosphotransferase function of α and α′ is needed for the serum stimulation to occur, and, further, that the phosphorylation reactions catalyzed in the nucleus are of particular importance.

3.3. Nuclear but not cytoplasmic injection of antibodies against α and α′ interferes with proliferation stimulation of cells

When IMR-90 cells arrested in the G0 phase were microinjected with the antibodies anti-α329–343 or anti-α′336–350 prior to serum stimulation, also a significant proportion of cells was prevented from cell cycle entry (Table 2). Surprisingly, and in contrast to antibodies against β [15,16], the inhibition was restricted to microinjections targeted to the nuclear compartment, while no significant effect was observed when the antibodies were injected into the cytoplasm. The effect of both antibodies could essentially be suppressed by co-injection of peptides α329–343 and α′336–350, respectively. The peptides alone had no effect, supporting the assumption that the interference with proliferation stimulation by the antibodies was specific for processes mediated by α and α′. Control injections with pre-immune serum under the same experimental conditions had no effect. When anti-α329–343 and anti-α′336–350 were injected together, the inhibition did not exceed the extent obtained with either antibody alone (Table 2), and, further, remained the same when the antibodies were injected in a more diluted form (roughly at half the concentrations; data not shown). Thus, the inhibitory action of the antibodies was obviously not additive but each of them already gave a maximum. Because the antibodies were not inhibitory for the phosphorylation of CK2 substrates in vitro (see above), it was concluded that cellular interactions other than by phosphorylation mediated by α and α′ (non-phos-

Table 2
Effect of microinjected antibodies against CK2 subunits α and α′ on proliferation stimulation

Microinjection	Inhibition (%)	
	Nucleus	Cytoplasm
Anti-α329–343	36 ± 15 (10)	−6 ± 10 (5)
Anti-α329–343+α329–343	10 ± 8 (2)	1 ± 6 (3)
Anti-α′336–350	31 ± 18 (4)	0 ± 13 (39)
Anti-α′336–350+α′336–350	0 ± 9 (4)	2 ± 10 (6)
Anti-α329–343+anti-α′336–350	28 ± 10 (6)	2 ± 6 (5)
Rabbit IgG	0 ± 15 (3)	−1 ± 8 (7)

Quiescent human lung fibroblasts (IMR-90) were microinjected into the nucleus or into the cytoplasm with affinity-purified antibodies (anti-α329–343, anti-α′336–350; 2 mg/ml each) in the absence or presence of peptides corresponding to the epitopes recognized by the antibodies (α329–343, α′336–350; 10 mg/ml each) as indicated. Controls received rabbit preimmune serum (rabbit IgG, 3.2 mg/ml). For further details see legend to Table 1. Data represent mean values ± S.E.M.; numbers in parentheses indicate the number of independent experiments (at least 200 microinjected cells each).

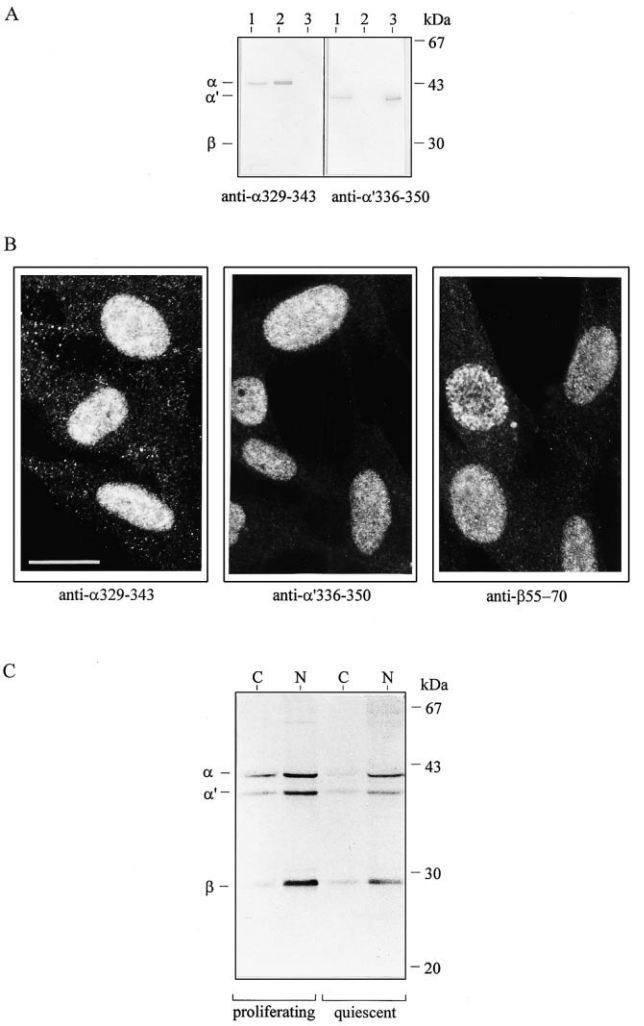

Fig. 1. Distribution of CK2 subunits in IMR-90 cells. A: Specificity of antibodies for the individual CK2 subunits α and α′. 40 μg of cell lysate protein from exponentially growing IMR-90 cells (lane 1), 30 ng each of purified recombinant α and β subunits of CK2 (lane 2), or 50 ng of purified recombinant α′ subunit of CK2 (lane 3) were subjected to SDS-PAGE and blotted to PVDF membrane. The blot was probed with antibodies anti-α329–343 or anti-α′336–350 directed against unrelated sequence sections of α and α′. The positions of CK2 subunits (α, α′ and β) and of molecular mass markers (kDa) are indicated. B: Intracellular distribution of CK2 subunits indicated by indirect immunofluorescence. IMR-90 cells grown on coverslips were fixed with paraformaldehyde, permeabilized with Triton X-100, incubated with rabbit antibodies against the individual CK2 subunits (anti-α329–343, anti-α′336–350, or anti-β55–70), stained with a TRITC-conjugated anti-rabbit IgG antibody, and fluorescence signals detected with a fluorescence microscope (Zeiss). Bar represents 10 μm. C: Intracellular distribution of CK2 subunits indicated by cell fractionation. Approximately 1.5×10^6 exponentially proliferating or quiescent IMR-90 cells were homogenized, separated by centrifugation into a cytoplasmic (C) and a nuclear (N) fraction, and subjected to SDS-PAGE. Individual CK2 subunits were detected by Western blotting using a mixture of antibodies (anti-α329–343, anti-α′336–350, and anti-β55–70). The positions of molecular mass markers (kDa) and of CK2 subunits (α, α′, and β) are indicated.

phorylation interactions) occur and, particularly in the nucleus, are of importance for the serum-stimulated entry of cells into the cell cycle, and that these are not specific for one of the two subunits.

4. Discussion

The inhibition of the proliferation response to serum of quiescent fibroblasts by microinjected CK2 peptide substrate ADS or pseudosubstrate ADA, but not by control peptide XX (isoform of ADS composed of the same amino acids but in a random arrangement), is strong, more direct evidence for a role of CK2-mediated phosphorylation in the process of serum-stimulated cell cycle entry, since both ADS and ADA, but not XX, inhibit in vitro the phosphorylation of substrate proteins by CK2. This provides experimental support for earlier reasoning based on indirect data obtained with microinjected anti-β antibodies. Viewing β as a constitutive, phosphorylation-determining part of the tetrameric CK2 holoenzymes, the inhibitory effects of anti-β on serum stimulation had been interpreted as being indicative for a requirement of phosphorylation by CK2 [15,16,23]. The immunostaining and cell fractionation data presented here are in perfect agreement with this reasoning. The data suggest comparable cytoplasmic-nuclear partition of all three of the CK2 subunits – α, α′, and β – both in quiescent and in proliferating cells, and, because of the well-known extremely high affinity of catalytic and regulatory subunits for each other [1–4], the presence of $\alpha_2\beta_2$, $\alpha'_2\beta_2$, and/or $\alpha\alpha'\beta_2$ tetramers throughout cells. However, this view seems oversimplified. The perturbation effects observed with the injected CK2 substrate and pseudosubstrate peptides do not correlate throughout the cell with those observed with the injected anti-β antibodies; while correlating in the nucleus, they seem to deviate in the cytoplasm. In addition, there are hints that the participation of catalytic subunits might include non-phosphorylation interactions separable experimentally from their kinase function.

Cytoplasmic injections of ADS and ADA, unlike cytoplasmic injections of anti-β, surprisingly have little effect on serum stimulation. This seems to question the significance of the anticipated cytoplasmic requirement of phosphorylation by CK2 for serum stimulation and thus for the transport of signals from plasma membrane into nucleus. This was unexpected, since several cytoplasm-located elements of signaling cascades have been shown to be affected by CK2 phosphorylation [1,2,4]. Because ADS has successfully been used as a specific CK2 substrate allowing for determination of CK2's kinase activity (and differentiation from the activity of other kinases) in crude cell and tissue extracts that hardly contain less of unspecific targets for ADA than the cytoplasm of fibroblasts, possible unspecific interactions of ADA with other cellular constituents should not hinder from a detectable inhibition also of CK2-mediated phosphorylation and thus of serum stimulation, as opposed to the zero effect observed. A zero effect, on the other hand, would be obtained when CK2 phosphorylation was involved in signaling, but was connected to the negative control, as exemplified for the MAP kinase pathway by the activation of protein phosphatase 2A due to its CK2-mediated phosphorylation [24]. In this case, inhibition of CK2 phosphorylation by the injected peptides would rather support the maintenance of the pathway. Because continued activity of the MAP kinase pathway seems to be a secured feature of serum stimulation of fibroblasts [12], this would go unrecognized by the experimental approach employed. For the sake of clarity, it should be mentioned that also unrecognized by the employed approach would be involvement of CK2 in other cytoplasmic processes, including

translation, metabolism, vesicle trafficking, cytoskeletal remodeling, etc.

If not based on CK2 phosphorylation, how then to explain the previously observed inhibition of serum stimulation by cytoplasmic injections of anti-β [15,16]? An explanation might come from the pool situation of the CK2 subunits. Certain pools of subunits may be needed at the receiving end of proliferation-regulating pathways, i.e. in the nucleus. Unlike the catalytic subunits, β has no obvious nuclear leader sequence (NLS) and, in addition, seems to be prone to dimerization [25]. As a consequence, while α and α′ might easily translocate into the nucleus, β might not. One might speculate the β-β complexes to be too large for unrestricted cytoplasmic-nuclear translocation (or unsuited for other reasons), and to depend on an interaction with other protein(s) possessing NLS. Injected anti-β might inhibit such interaction(s). The resulting transport inhibition would then hinder proper generation or maintenance of pool(s) of β in the nucleus and cause the observed inhibition of serum stimulation. In fact, the cell fractionation data indicate increased nuclear levels of CK2 subunits in proliferating cells, and, importantly, the cytoplasmic injection of anti-β has been demonstrated to inhibit both the translocation of β into the nucleus (or of β-containing molecular complexes recognized by anti-β) and the proliferation stimulation by serum [15,23]. An alternative explanation might be that β could, in addition to the function as a CK2 regulator, play independent cytoplasmic role(s) linked to signaling in one way or another. Using β as a bait in yeast-based two-hybrid systems, a considerable number of cytoplasmic proteins have been identified as potential interaction partners [26–28]. Some of these might turn out to represent signaling proteins that intimately interact with β, but that do not need phosphorylation by CK2.

Injected into the fibroblasts' nuclei, ADS and ADA significantly interfere with serum stimulation, indicating a requirement for undisturbed phosphorylation by CK2. Compared to cytoplasm, the nuclei possess high levels of all three of the CK2 subunits, as indicated correspondingly by the immunostaining and cell fractionation results. Various roles have been postulated for the nuclear CK2 [1–4]. In the context of the presented data, those linked to transcription appear to be of particular concern, because cell cycle entry does not occur without the expression of genes. Using a cDNA microarray representing more than 8000 human genes, serum stimulation of fibroblasts has been reported to cause diverse temporal profiles of gene expression and repression following an orderly expression program choreography, and the immediate response to be dominated by the expression of genes that encode known or suspected regulators of transcription and other proteins involved in signal transduction, including transcription factors Fos, Jun, and Myc [12]. These represent well-known CK2 substrates [4], and their phosphorylation might relate to functional control [29,30]. Another such CK2 substrate related to serum stimulation is the serum response factor (SRF; p67), a MADS family transcription factor [31] with binding motifs present in the control region of many genes, including immediate early genes, and with transactivational roles that appear to be modulated through phosphorylation by CK2 [32–34]. Thus, both the expression of the genes and the performance of the gene products as gene regulators may relate to the phosphorylation by CK2, including, as shown for the three AP1 binding proteins – Fos, Jun, Nrf1 – the balancing of the action of transcriptionally active proteins [35]. Most of these phosphorylations, however, appear to be catalyzed by CK2 holoenzymes and thus are under the control of subunit β [20]. This is in perfect agreement with the dramatic inhibition obtained previously by nuclear injections of anti-β antibodies [15,16]. Inhibition has been obtained only when injected within the first several hours after serum application, i.e. during G0/G1 phase transition and the early part of the G1 phase, and, moreover, has been shown to be accompanied by expression inhibition of immediate early genes such as fos [36]. In addition, β-mediated non-phosphorylation interactions might have been affected by anti-β injections such as known to occur with p53 [37], increasing the inhibitory effect.

We show here that non-phosphorylation interactions may not only occur via β. Non-phosphorylation interactions appear to occur also at the catalytic subunits of CK2 that can experimentally be separated from their phosphorylation function. This is reflected by the inhibition of serum stimulation due to nuclear injections of the antibodies anti-α329–343 and anti-α′336–350. These had been raised and are specifically directed against unique sequences downstream of the catalytic domain regions, find their targets under conditions of Western blotting, immunohistochemistry, and immunoprecipitations [18], are competitively inhibited by the peptides used as antigens, and, consequently, affect neither the complexation of α and α′ to β, i.e. proper holoenzyme formation, nor the in vitro kinase activity of holoenzymes or of the individual catalytic subunits [18,19]. Because it is the same in extent with both antibodies and not additive in their effects, the inhibition of serum stimulation should relate to features inherent in both α and α′, i.e. to features specific for catalytic subunits generally. Another such feature(s) appears to exist upstream of kinase domain areas at position 15–27 of α (α′ position 16–28; three mismatches to α), because nuclear injection of anti-α15–27, an antibody directed against both α and α′, also inhibits serum stimulation to a comparable extent (data not shown). At present, the meaning and abundance of these non-phosphorylation interactions of the catalytic subunits is not clear. The bound antibodies might prevent interactions that occur in addition to phosphorylation, or interactions that facilitate phosphorylation, or both. Facilitating phosphorylation could be via anchoring of CK2 to specific nuclear substrates that may otherwise not become CK2 substrates. A related example would be the anchoring of the c-Jun N-terminal kinase (JNK) to the delta region of c-Jun, a region physically distinct from the phosphoacceptor region within its activation domain, that facilitates phosphoacceptor region translocation into the catalytic cleft of JNK leading to phosphorylation and subsequent dissociation of the kinase from its substrate [38]. If this was the case, the non-phosphorylation interaction would be part of the phosphorylation process. However, although seemingly obvious, currently we have neither data that would substantiate this idea, nor can we exclude CK2 interactions that are related to serum stimulation but concern cellular constituents other than CK2 substrates and thus occur in addition to phosphorylation.

The non-phosphorylation interaction(s) even by the catalytic subunits add one more feature to the already complex cellular scenario with CK2. Obviously, viewing CK2 as a housekeeping enzyme that functions exclusively as phosphotransferase and in a tetrameric form throughout the cell is inadequate. The presented data rather strengthen the view

of an occurrence of various pools with specific cellular locations and functions, pools that contain not only holoenzymes. The existence of such pools has been considered previously [39,40]. Their formation and maintenance may rely on anchoring proteins [41] similar to those known for other protein kinases such as PKC and PKA (Racks and AKAPs, respectively; reviewed in [42]). The lining of structures such as microtubules or the presence of discrete dots at places observed with antibody staining [2,43,44] might be indications. In addition to holoenzymes, the individual subunits might form pools ([39,45], our unpublished data), a problem that is receiving more and more attention. The deviations from the tetrameric stoichiometry appear to have relevance particularly for pathological situations. A most dramatic example is that of T cells transformed by the parasite *Theileria parva* resulting in a leukemia-like deadly disease of cattle, characterized by what seems to be a causal link to extremely high levels of individual α [8]. In transgenic mice, moderate overexpression of α has been shown to result in a stochastic propensity to lymphoma development giving rise to a high incidence of lymphoproliferative disease upon mating α transgenic mice with c-myc or tal-1 transgenic mice [9,10], or with p53 knockout mice [11].

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