increase in CK2 activity has been reported just prior to and during S-phase in human fibroblasts stimulated with serum (Carroll and Marshak, 1989). Our results in tobacco cells indicate a peak of CK2 activity starting exactly at the same time as the induction of histone H4 expression (G1/S) and reaching its maximal value before or at the point of arrest caused by aphidicolin (early S). Consistent with these findings, delivering of a CK2 inhibitor in G1 blocks the cell cycle progression with a delay (2–3 h) that is exactly coincidental with the time of appearance of CK2 activity in control cells. Thus, a functional CK2 at the G1/S boundary might be a general requirement for eukaryotes. For the rest of S-phase, CK2 activity slowly declines in tobacco cells, although we have no data about the effect of CK2 inhibition at late S-phase. Both in *S. cerevisiae* and mammals, depletion of CK2 at late S does not have any effect on the completion of DNA synthesis.

On the other hand, the results presented in this paper confirm studies in animal cells that CK2 activity increases from a resting to a proliferative state, and show a complex pattern of regulation of CK2 activity. Elevated levels of CK2 activity are associated clearly with the onset of cell proliferation, but they correlate only partly with the mRNA levels of both subunits. A correlation is more apparent between the enzymatic activity and the amount of the β subunit, whereas the α subunit is constitutively present even in the stationary phase. Taken together, these results suggest that the CK2β subunit might have a central role in controlling the level of CK2 activity in plant cells, as has also been reported in animals and yeast. In addition, the results suggest that the regulation of β polypeptide levels by a post-transcriptional mechanism might be important in controlling the exit from the proliferative state. Either a translational repression of mRNA or a rapid continuous degradation of the protein might explain the discrepancy between the presence of the β transcript and the lack of its corresponding polypeptide. In exponentially growing human cells, CK2β subunit is synthesised in excess of the α subunit and a substantial fraction of it is degraded within the first hour (Luscher and Litchfield, 1994). Moreover, abnormally high levels of β subunit have been found in tumoral tissues, suggesting that an excess of β subunit might be deleterious to the cell (Stalter *et al.*, 1994). CK2β polypeptide contains elements conserved in the ubiquitin-mediated degradation pathway, such as the cyclin destruction-like box and PEST-sequence, although the functionality of these sequences has not been proven. Degradation of the β subunit might be necessary for the catalytic subunit to interact with other regulatory molecules and inhibit proliferation. Interestingly, an association of the CK2α subunit with phosphatase 2A, in the absence of the β subunit, has been reported that negatively regulates cell proliferation (Hériché *et al.*, 1997). Thus, the association of the CK2α subunit with different regulatory molecules might be one of the mechanisms used by the enzyme to control cell proliferation rate.

As a first step to investigate the mechanisms responsible for the post-translational regulation of CK2, we performed a comparative study of the evolution of CK2 activity and polyamine content in BY-2 cells. Although our results are still not completely conclusive, they show a fair correlation between peaks of CK2 activity and high values of polyamines content. Taken together, the data from the single- and double-synchronisation experiments show an average of a threefold increase in each one of the three polyamines when the cells go from G2 to M phase and of 1.5–1.8 from G1 to S. In the same phases of the cell cycle, CK2 activity increases fivefold and threefold, respectively. During the growth cycle of BY-2 cells, CK2 activity raises four- to fivefold after rescuing the cells from the stationary phase and free polyamines increase significantly in the same period of time.

We also observed a correlation between high levels of polyamines and the presence of the β polypeptide in the cell extracts. Other authors have reported a marked increase in CK2β levels in the presence of polyamines and postulated a role of these compounds in the stabilisation of the β protein (Shore *et al.*, 1997). A polyamine-binding domain has been mapped in the N-terminal region of the β subunit (Leroy *et al.*, 1997) that resides in close proximity to the destruction-like box. Thus, a polyamine-induced structural change might eventually mask the destruction box, preventing ubiquitination and delaying degradation; hence, a certain threshold of polyamines would be necessary for the β subunit to be stable. Our results from the synchronisation experiments, where we detect steady levels of β polypeptide, support this model since the polyamine's concentration, although oscillatory, never falls to the low levels present in stationary cells. This mechanism would constitute a positive feedback loop for the regulation of CK2 by polyamines, since the tetrameric form, $\alpha_2\beta_2$, is the target of polyamine's allosteric stimulation (Valero *et al.*, 1995). Further studies are necessary to validate this hypothesis and to clarify whether it is the total pool of polyamines (total number of positive charges) or a particular one that counts. For instance, we have noted that during the growth curve, between day 0 and day 3 (where β subunit is undetectable), there is a decrease of 65% in the amount of putrescine and 30% of spermine, while spermidine remains unchanged. It is plausible that the important decrease in putrescine and spermine might explain the instability of the β subunit at that point.

In summary, the results shown in this paper suggest multiple levels of control of CK2 enzymatic activity in relation to the proliferative state of the cells. These include differential accumulation of mRNA levels, post-translational activation of the tetrameric enzyme and post-transcriptional control of β polypeptide levels. Further

studies are necessary to elucidate whether polyamines play a central role in some aspects of CK2 regulation and to fully understand the function of CK2 in cell cycle control. Undoubtedly, identification of protein substrates as targets of CK2 activity at G1/S and M will be invaluable to provide further insights on the signalling pathway of this protein kinase in cell proliferation.

Experimental procedures

Cells and cell cycle synchronisation

The tobacco BY-2 cell suspension was cultivated and synchronised as described by Nagata *et al.* (1992). Synchronisation was achieved by a 24 h subculture of stationary phase cells (7-days-old) in a medium containing 3 μg ml^{-1} aphidicolin (Sigma) followed by extensive washes. To obtain highly synchronised cells starting from metaphase, 3.6 μM propyzamide (Rohm and Haas Co., USA) was added 5 h after the release from the aphidicolin block and then removed after a 5 h treatment period. Mitotic index was determined by staining with 10 μg ml^{-1} DAPI (Sigma) in the presence of 1% Triton X-100, and DNA synthesis by pulse labelling with [^{3}H]-thymidine (Amersham Pharmacia Biotech). Cell viability was monitored by staining with 0.1 mg ml^{-1} FDA (Sigma). The 4,5,6,7-tetrabromobenzotriazole was a gift from Dr David Shugar (Polish Academy of Sciences, Warsaw, Poland) and was used at a final concentration of 35 μM.

Immunochemical procedures

For the antibodies production, *ATCKA2* (Mizoguchi *et al.*, 1993) and *CKB2* (Collinge and Walker, 1994) coding regions were PCR-amplified and fused to the coding region of the glutathione-S-transferase (GST) into the expression vector pGEX-3X (Amersham Pharmacia Biotech). Expression of the two recombinant proteins, GST-CK2α′ and GST-CK2β′, was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37°C. The insoluble fraction, where most of both proteins were found, was solubilised with Laemmli buffer at 100°C and submitted to SDS-PAGE electrophoresis. The bands of the expected size were excised from the gel and electroeluted, at 30 V, overnight, in 25 mM TrisHCl, 250 mM glycine, 0.1% SDS, pH 8.3, precipitated with acetone at −20°C, and redissolved in PBS. Antibodies were raised by inoculating rabbits using conventional methods (Harlow and Lane, 1988). For Western blotting, proteins were electrophoresed on 10% or 12.5% SDS-PAGE gels, transferred to immobilon-P membranes (Millipore) and incubated with antisera against GST-CK2α′ (dilution 1:5000) or GST-CK2β′ (dilution 1:1000), respectively. The immunocomplexes were revealed using the Immun-Star detection kit system (BioRad). Loading of equal amounts of proteins was controlled by Bradford analysis (BioRad) and gel and membrane staining. Competition experiments to prove the specificity of the antibodies were performed by pre-incubation of the antibodies with 30 μg of the same protein used for rabbit inoculation, for 1 h at room temperature.

Protein extracts, CK2 activity and polyamine analysis

Proteins were extracted by sonication of the BY-2 cell pellets in the presence of cold 50 mM TrisHCl pH 7.5 buffer, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.25 mM sucrose and 10% glycerol. CK2 enzymatic assays in crude extracts were performed as described by Espunya and Martinez (1997), using either 1 mg ml^{-1} of dephosphorylated β-casein (Sigma) or 0.32 mM of specific peptide RRRDDDTDDD (Neosystem) as substrate. [γ-^{32}P]GTP and [γ-^{32}P]ATP (0.126 mM, 1000 cpm pmol^{-1}) (Amersham Pharmacia Biotech) were used as phosphate donors for β-casein and the specific peptide, respectively. For determination of free polyamines, cell pellets were sonicated in the presence of 0.32N perchloric acid and 1,6-diaminohexane (Sigma) as internal standard, and polyamines assayed in the supernatant as dansyl derivatives by HPLC (Martínez *et al.*, 1991).

Cloning of tobacco CK2 homologues

Degenerated oligonucleotides corresponding to conserved regions of both CK2α and CK2β polypeptides were used for amplification from cDNA prepared from exponentially growing BY-2 cells. For the CK2α homologue, the upstream primer was 5′-GANTAYTGGGAYTAYGAR-3′ and the downstream primer 5′-ATCATNACRTTRTGNGGY-3′ (residues 17–22 and 153–159, respectively, of the ATCKA1 sequence). For the CK2β homologue, the upstream primer was 5′-GANGAYTAYATHCARGAY-3′ and the downstream primer 5′-CCRTANARCATYTCNGC-3′ (residues 117–122 and 166–171, respectively, of the CKB1 sequence). PCR was performed at 95°C for 1 min, 46°C for 2 min and 72°C for 6 min, for 25 cycles. The PCR-fragments were cloned in pBluescript II KS (Stratagene) and sequenced.

Northern blots

RNA was prepared by the guanidium thiocyanate method (Sambrook *et al.*, 1989), separated on 1% agarose formaldehyde gels and transferred onto Hybond N membranes (Amersham Pharmacia Biotech). The CK2α probe corresponded to the *ntcka11* clone, and the CK2β probe to the *ntckb62* clone. The histone H4 probe corresponded to the coding region of *Arabidopsis H4A748* gene (Reichheld *et al.*, 1995). The translation elongation factor (EF-1α) cDNA, which expresses constitutively, was used as probe for loading control. Hybridisation was performed at 42°C in a buffer containing 6×SSC, 5×Denhardt's reagent, 0.5% SDS, 10% dextran sulphate, 100 μg ml^{-1} salmon sperm DNA and 50% formamide, followed by washes at 42°C in 0.5×SSC, 0.1% SDS. The results were visualised and quantified using a GS-525 Molecular Imager System (BioRad).

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