Protein kinase CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induces apoptosis and caspase-dependent degradation of haematopoietic lineage cell-specific protein 1 (HS1) in Jurkat cells

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Incubation of Jurkat cells with 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of protein kinase CK2, induces dose- and time-dependent apoptosis as judged by several criteria. TBB-promoted apoptosis is preceded by inhibition of Ser/Thr phosphorylation of haematopoietic lineage cell-specific protein 1 (HS1) and is accompanied by caspase-dependent fragmentation of the same protein. Both effects are also observable if apoptosis is promoted by anti-Fas antibodies and by etoposide. Moreover, *in vitro* experiments show that HS1, once phosphorylated by CK2, becomes refractory to cleavage by caspase-3. These findings, in conjunction with similar data in the literature concerning two other CK2 protein substrates, Bid and Max, suggest that CK2 may play a general anti-apoptotic role through the generation of phosphorylated sites conferring resistance to caspase cleavage.

Key words: casein kinase 2, cell death, protein phosphorylation, proteolysis.

INTRODUCTION

Protein kinase CK2 (an acronym derived from the misnomer 'casein kinase 2') is probably the most pleiotropic Ser/Thr-specific protein kinase known to date. The continuously growing list of CK2 substrates includes at least 200 proteins [1], whose phospho-acceptor sites are specified by multiple acidic residues downstream from the phosphorylatable amino acid, the acidic side chain at position $n+3$ playing the most prominent role [2,3]. Such an extraordinary pleiotropicity may account for the unique property of CK2 of being constitutively active, either in its hetero-tetrameric form, composed of two catalytic (α and/or α') subunits and two regulatory β-subunits, or as an isolated catalytic subunit, without the need of specific stimuli, activators, second messengers or previous phosphorylation, as happens with most protein kinases. In turn, high constitutive activity is suspected to underlie the pathogenic potential of CK2, outlined by a number of observations, notably its elevated activity in tumours (reviewed in [4]), its ability to induce transformation in co-operation with other proto-oncogenes [5–8] and its exploitation as a phosphorylating agent by many viruses [9]. Despite the abundance of coincidental arguments suggesting that CK2 must play crucial functions in cell regulation, little is known about its ability to impinge on specific biochemical events that could provide the molecular rationale for its essential role within the cell [10], and for its oncogenic potential.

Recent experiments, based mainly on the usage of antisense anti-CK2α RNA, suggested that CK2 is an anti-apoptotic agent [11,12]. This could also account, at least partially, for the observation that CK2 is invariably elevated in proliferating tissues, either normal or transformed [9]. Evidence is accumulating concerning the involvement of CK2 in apoptotic events, such as its interaction with Fas-associated factor-1 [13], and its possible role in regulating p53 function [14,15]. Another finding that could link CK2 to apoptosis was provided by showing that haematopoietic lineage cell-specific protein 1 (HS1), implicated in B-cell apoptosis [16–18], besides being tyrosine-phosphorylated can be also phosphorylated at Ser/Thr residues by CK2 [19]. The physiological relevance of this latter event and its functional consequences, however, remain unknown.

Recently the availability of a very selective cell-permeant inhibitor of CK2, 4,5,6,7-tetrabromobenzotriazole (TBB) [20], has provided a new tool for assessing the implication of this kinase in specific cellular functions. Here we take advantage of this reagent to gain information about the role actually played by CK2 in the programmed death of Jurkat cells, a transformed cell line derived from T-cells, and we show that TBB induces apoptosis and deeply affects two modifications of HS1 protein: namely, it prevents its CK2-dependent phosphorylation and promotes its caspase-dependent degradation. These data, in conjunction with those of others [21,22], suggest that CK2 could counteract apoptosis by a general mechanism consisting of the generation of phosphorylated sites refractory to caspase cleavage.

MATERIALS AND METHODS

Materials

TBB was synthesized according to the protocol described in [23], and TBB solutions were made in DMSO (the amount of DMSO added to cell suspension never exceeded 0.5%, v/v). Anti-HS1 antiserum was evoked in rabbit against the whole recombinant human protein. Anti-human Fas-activating antibody was from Upstate Biotechnology, and rabbit anti-poly(ADP-ribose) polymerase (PARP) antibody and protease-inhibitor cocktail were

Abbreviations used: HS1, haematopoietic lineage cell-specific protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; TBB, 4,5,6,7-tetrabromobenzotriazole; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone.

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from Roche. Recombinant CK2 α subunit was expressed and purified as described in [24,25], and recombinant HS1 protein as described in [26]. Active caspase-3 was from Chemicon International, etoposide was from Sigma, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) was from Calbiochem, and enhanced chemiluminescence (ECL) reagent and [^{32}P]P_i were from Amersham Bioscience. CK2 substrate peptide ($R_3AD_2SD_5$) was synthesized as in [27], anti-CK2 antibodies were against residues 66–86 of the human CK2α sequence [25] and monoclonal anti-cytochrome *c* antibodies were from Alexis Biochemicals.

Cell culture and treatment

The human leukaemia Jurkat T-cell line was maintained in RPMI 1640 (Dutch modification; Sigma) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, in an atmosphere containing 5 % CO_2.

Before treatment, cells were washed, resuspended at a density of $\approx 10^6$ cells/ml in medium containing 1 % fetal calf serum, and incubated at 37 °C in the presence of the indicated compounds for various periods of time, as described in the text and Figure legends. Control cells were treated with equal amounts of the solvent.

At the end of the incubations, cells were centrifuged, washed and lysed as indicated below for the specific experiments.

Cell viability assay

Cell viability was detected by means of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT) reagent, a tetrazolium salt that is metabolized by mitochondrial dehydrogenases and produces a purple precipitate in viable cells. Cell suspension (100 μl; 10^5 cells) was incubated in each well of a 96-well plate under different conditions. Then, 1 h before the end of the incubations, 10 μl of MTT solution (5 mg/ml in PBS) was added to each well. Incubations were stopped by addition of 20 μl of lysis solution at pH 4.7, consisting of 20 % (w/v) SDS, 50 % (v/v) *N*,*N*-dimethylformamide, 2 % (v/v) acetic acid and 25 mM HCl. Plates were read for attenuance (*D*) at λ590 nm, in a Titertek Multiskan Plus plate reader (Flow Laboratories).

Detection of PARP fragmentation

Activation of caspases was monitored by detection of the fragmentation of the nuclear protein PARP. Treated cells were lysed by the addition of ice-cold hypotonic buffer consisting of 10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, protease inhibitor cocktail, 10 mM NaF and 1 μM okadaic acid. After 20 min of incubation on ice, 0.6 % (v/v) Nonidet P-40 was added, and the lysates were centrifuged immediately at 14000 ***g*** for 30 s. The supernatants contained the cell soluble fraction, whereas the pellets corresponded to the nuclear fraction and were incubated for 20 min on ice with a hypertonic buffer consisting of 20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, protease inhibitor cocktail, 10 mM NaF and 1 μM okadaic acid. The nuclear extracts, obtained by centrifugation for 10 min at 14000 ***g*** and 4 °C, were used as a source of PARP protein. Aliquots corresponding to 10 μg of proteins were resolved by SDS/PAGE (7.5 % gels), blotted on to Immobilon-P membranes (Millipore) and processed in Western blots with an antibody against full-length PARP, which also recognized the 89 kDa apoptotic fragment.

Detection of cytochrome *c* release

The cytosolic fraction for the detection of cytochrome *c* release was prepared as described in [28]. Briefly, after treatment, cells were lysed by the addition of hypotonic buffer consisting of 20 mM Hepes, pH 7.5, 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and protease inhibitor cocktail, and incubated for 20 min on ice. Lysates were centrifuged for 15 min at 10000 ***g*** at 4 °C, and supernatants further centrifuged for 1 h at 100000 ***g*** at 4 °C. The cytosolic fraction (60 μg of proteins) was analysed by SDS/PAGE (15 % gels) and Western blotting with anti-cytochrome *c* antibodies.

DNA laddering

Extraction of apoptotic DNA was performed by means of the Suicide Track DNA laddering isolation kit from Oncogene Research Products. After treatment, cells were lysed according to the manufacturer's instructions; apoptotic DNA extraction and precipitation were performed following a procedure suggested by the kit's manufacturer for the separation of fragmented DNA from high-molecular-mass chromatin. Samples were subjected to 1.5 % agarose gel electrophoresis, and detection of DNA bands was obtained by staining the gel with 0.5 μg/ml (w/v) ethidium bromide followed by UV illumination.

[^{32}P]P_i cell labelling

Jurkat cells were treated essentially according to [29]: briefly, cells were incubated for 15 h in a phosphate-free Eagle's essential medium with 2 % dialysed fetal calf serum. Carrier-free [^{32}P]P_i was then added (0.7 mCi/ml) and the incubation prolonged for 6 h, with the addition of apoptotic inducers (anti-Fas, etoposide or TBB) for the last 2 h, as indicated. At this point, cell viability was assessed by the MTT method, as described above. Cells were then centrifuged, washed twice and lysed by addition of the following hypotonic buffer: 20 mM Tris/HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 10 % (v/v) glycerol, 0.05 % Nonidet P-40, protease-inhibitor cocktail, 10 mM NaF and 1 μM okadaic acid (Buffer B). The lysate was cleared by centrifugation for 10 min at 14000 ***g***.

HS1 immunoprecipitation

The lysate corresponding to 5×10^6 cells, prelabelled with [^{32}P]P_i, was used for HS1 immunoprecipitation, performed for 2 h with 2.5 μl of anti-HS1 antiserum, as described in [20]. Immunoprecipitates were subjected to SDS/PAGE and blotting on to Immobilon-P membranes. The amount of HS1 precipitated was analysed by Western blotting with the same anti-HS1 antiserum, while the radioactivity incorporated was detected by analysis of the membrane with a Cyclone instrument (Packard).

Endogenous CK2 activity in cell lysate

After treatment with increasing concentrations of TBB, as indicated, cells were recovered by centrifugation, lysed with the hypotonic buffer B, and 2.5–5 μg of proteins from the cleared lysate was used for the assay of CK2 activity, measured by the addition of the specific peptide $R_3AD_2SD_5$ (1 mM) in the presence of 50 mM Tris/HCl, pH 7.5, 12 mM $MgCl_2$, 10 μM [γ-^{33}P]ATP ($\approx$1000 c.p.m./pmol) and 0.1 M NaCl, in a total volume of

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20 μl. Incubation was carried out for 10 min at 30 °C, and stopped by sample spotting on to P-cellulose papers as described elsewhere [27]. To assess the amount of CK2, 10 μg of proteins from the same cell lysates were resolved by SDS/PAGE (11 % gels) and analysed by Western blotting with anti-CK2α antibodies.

The activity of freshly added CK2 in the presence of cell lysate was measured as described above, with the addition of 0.02 μg of recombinant CK2.

Detection of HS1 fragmentation

After treatment, cells were lysed to obtain the total soluble fraction, as described above. Aliquots corresponding to 6 μg of total proteins were separated by SDS/PAGE (11 % gels) and analysed by Western blotting with anti-HS1 antiserum.

In vitro cleavage of HS1 by caspase-3

HS1 was first incubated with 50 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$ and 10 μM [γ-^{33}P]ATP in the presence or absence of CK2 α subunit, as described in [19]. Where present, CK2 induced a phosphorylation stoichiometry of 3 mol of P_i/mol of HS1 protein. Since the phosphorylation mixture was inhibitory for caspase-3 activity, samples were then dialysed against 5 mM Hepes, pH 7.5, 0.1 % (w/v) sucrose and 0.01 % (w/v) Chaps. Aliquots corresponding to 5 ng of HS1 were incubated for 3 h at 37 °C in 20 μl of a buffer consisting of 50 mM Hepes, pH 7.5, 1 % (w/v) sucrose, 0.1 % (w/v) Chaps and 10 mM dithiothreitol in the presence or absence of active caspase-3 (0.5 unit). Samples were subjected to SDS/PAGE (11 % gels) followed by Western blotting with anti-HS1 antibodies. A unit of caspase-3 was defined as the enzyme activity that cleaved 1 nmol of the substrate DEVD-*p*-nitroaniline/h at 37 °C.

RESULTS

TBB promotes Jurkat cell death and apoptosis

As shown in Figure 1, exposure of Jurkat cells to increasing concentrations of TBB has a detrimental effect, both dose- and time-dependent, on cell viability. Based on three distinct criteria, it can be concluded that cell death induced by TBB is, at least in part, accounted for by apoptosis. First, TBB induces the appearance of the apoptotic PARP fragment (Figure 2A); secondly, it causes a significant release of cytochrome *c* from mitochondria, comparable with that induced by the well-known apoptotic agent etoposide (Figure 2B) and thirdly, as shown in Figure 2(C), DNA laddering, similar to that visible after Fas-receptor engagement, is observable upon TBB treatment. Given the remarkable selectivity of TBB towards CK2, it was likely that its apoptotic effect took place through CK2 inhibition. Consistent with this, another CK2 inhibitor, emodin, which is almost as effective as TBB, albeit less specific, also caused Jurkat-cell death, accompanied by apoptotic symptoms (results not shown).

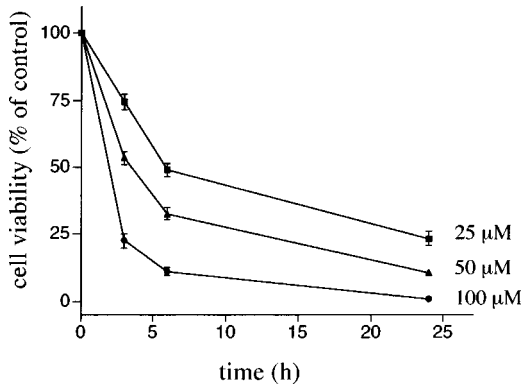

Figure 1 Effect of TBB on cell viability

Jurkat cells were treated for the indicated times with increasing concentrations of TBB. Cell viability was assessed by the MTT method. Control cells were assigned 100% viability, having been incubated with the solvent DMSO (0.5%, v/v). Reported values represent the means ± S.E.M. from five separate experiments.

Ser/Thr phosphorylation of HS1 protein and CK2 activity are inhibited by TBB in the early phase of apoptosis

HS1, a protein whose expression is limited to haematopoietic and lymphoid cells [30], is believed to play a role in receptor-mediated apoptosis, as judged from experiments with HS1-deficient mice [17] and B-lymphoma WEH1-231 cells [16,18]. Although HS1 was first described as a protein becoming tyrosine-phosphorylated upon B-cell receptor cross-linking [31,32], it was later shown to undergo Ser/Thr phosphorylation as well, and CK2 appears to be the first-choice candidate to perform this kind of phosphorylation [19]. HS1 phosphorylation could therefore provide a link to correlate the CK2 inhibitor TBB with apoptotic events. Although we have already shown the inhibition of HS1 phosphorylation by TBB in intact platelets [19] and Jurkat cells [20], those experiments were run in the presence of okadaic acid, a wide-spectrum inhibitor of protein phosphatases which is itself an inducer of apoptosis. In the attempt to detect *in vivo* phosphorylation of HS1, Jurkat cells were incubated with [^{32}P]P_i in the absence of protein phosphatase inhibitors, and HS1 protein was immunoprecipitated from the cell lysate and resolved by SDS/PAGE. As shown in Figure 3, by this procedure HS1 could be detected as a radiolabelled band whose disappearance upon alkali treatment was symptomatic of Ser/Thr, not Tyr phosphorylation (results not shown). As also shown in Figure 3, phospho-radiolabelling of HS1 disappeared if TBB was added to the incubation medium, whereas under the same experimental conditions the amount of HS1 protein, as judged by Western blotting after immunoprecipitation, was nearly unchanged. Interestingly, two other apoptosis inducers, anti-Fas antibody and etoposide, which have no direct effect on CK2, reduced HS1 phosphorylation, suggesting that a fall in HS1 phosphorylation, due to either inhibition of kinase(s) or stimulation of phosphatase(s) [33], is a common event in the early phase of apoptosis triggered by different stimuli.

The occurrence of CK2 inhibition in cells treated with TBB was confirmed by showing that in the lysate of these cells CK2 activity measured with a specific peptide substrate was negligible compared with control cells (Figure 4), although the CK2 protein, as judged from Western blotting, was comparable. Incidentally, these data highlight the persistence of TBB inhibition in diluted cell extracts where the concentration of the inhibitor is expected to fall below the threshold of effectiveness. In fact, these lysates displayed no inhibitory effect towards freshly added exogenous CK2 (results not shown).

Prolonged treatment with TBB promotes caspase-dependent degradation of HS1 protein

The above experiments, showing inhibition of HS1 phosphorylation by TBB, were run under conditions where TBB did not induce any substantial change in the amount of HS1 protein (see Figure 3). Upon prolonged incubation, however, when apoptotic

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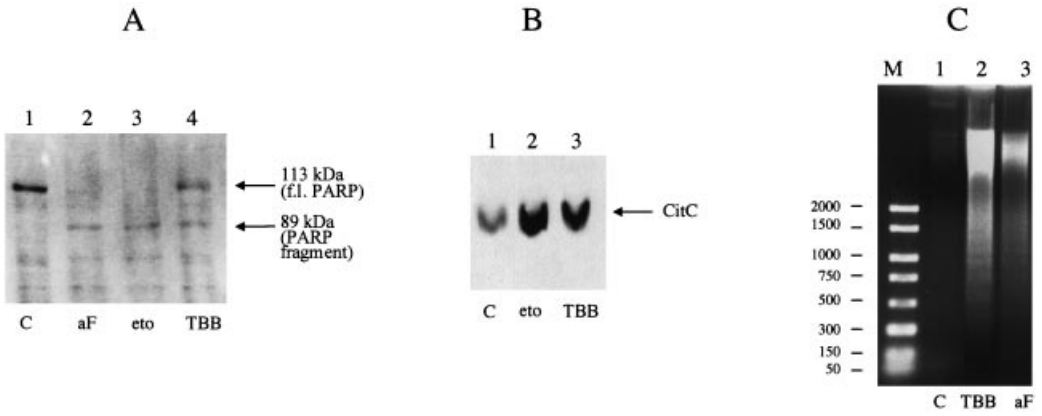

Figure 2 Effect of TBB on PARP degradation, cytochrome *c* release and DNA fragmentation

(**A**) Jurkat cells were treated for 15 h with nothing (C; lane 1), 50 ng/ml anti-Fas (aF; lane 2), 50 μM etoposide (eto; lane 3) or 30 μM TBB (lane 4). Cells were lysed and nuclear extracts (10 μg of proteins) were analysed by Western blotting with an antibody against PARP protein. The arrows indicate the position of full-length (f.l.) PARP and its apoptotic fragment. (**B**) Cells were treated for 6 h with nothing (lane 1), 50 μM etoposide (lane 2), or 30 μM TBB (lane 3). Cells were lysed, and the soluble cytosolic fractions (60 μg of proteins) were analysed by Western blotting with an antibody against cytochrome *c* (CitC). (**C**) Cells were treated for 15 h with nothing (lane 1), 30 μM TBB (lane 2) or 50 ng/ml anti-Fas (lane 3). Fragmented DNA was separated with a protocol that eliminates high-molecular-mass chromatin (see the Materials and methods section) and run on agarose gels; bands were detected by UV illumination after ethidium bromide staining. Markers of the indicated sizes (bp) were loaded in lane M.

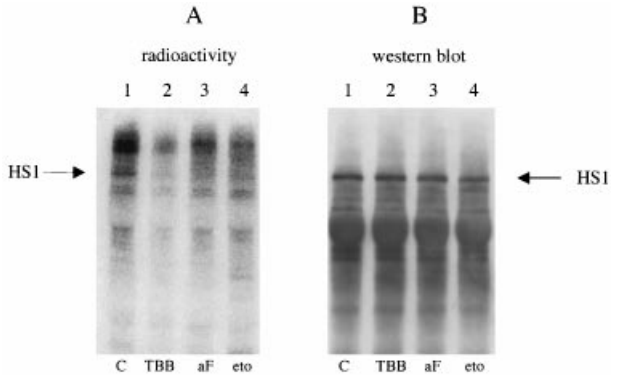

Figure 3 *In vivo* phosphorylation of HS1 protein during apoptosis

Cells were labelled with [^{32}P]P_i and treated for 2 h as follows: control (C; lane 1), 25 μM TBB (lane 2), 50 ng/ml anti-Fas (aF; lane 3) and 50 μM etoposide (eto; lane 4). From cell lysates, HS1 protein was immunoprecipitated, run on SDS/PAGE (11% gels) and blotted on to Immobilon-P. Membranes were then analysed for radioactivity with a Cyclone instrument (**A**) and processed in a Western blot with HS1 antibodies (**B**).

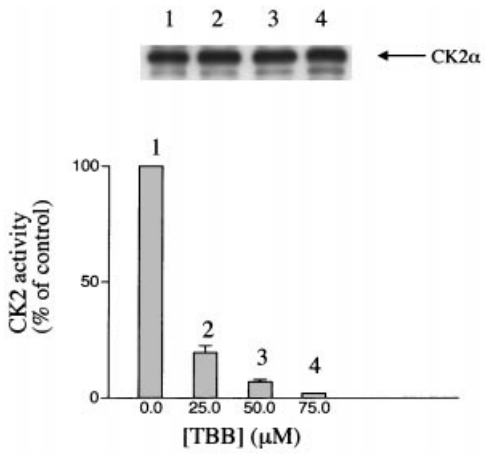

Figure 4 CK2 activity in lysates of cells treated with increasing concentrations of TBB

Cells were treated for 2 h with the indicated concentrations of TBB. Lysates were then assayed for CK2 activity, measured towards the specific peptide $R_3AD_2SD_5$, as described in the Materials and methods section. The activity of the control cells, treated with solvent, was considered to be 100%. Means $\pm$ S.E.M. from five separate experiments are shown. In the inset, the amount of CK2α is shown: 10 μg of protein from control cells (lane 1) or cells treated for 2 h with 25 μM (lane 2), 50 μM (lane 3) or 75 μM (lane 4) TBB were resolved by SDS/PAGE followed by Western blotting with anti-CK2α antibodies.

symptoms become evident, a drastic decrease in HS1 protein was observed, accompanied by the appearance of smaller fragments immunoreactive with anti-HS1 antibodies (Figure 5). After 8 h, the most prominent fragments displayed molecular masses of about 44 and 29 kDa, whereas a larger fragment of about 58 kDa was transiently detectable at shorter times (less than 5 h; results not shown). At variance with the residual full-length HS1 recovered after cell incubation with TBB, which could be readily phosphorylated *in vitro* by exogenous CK2, none of these fragments was susceptible to *in vitro* phosphorylation (results not shown), consistent with their loss of phosphorylation site(s). As in the case of early-phase dephosphorylation (see above), subsequent degradation of HS1 also follows a similar pattern upon either TBB or anti-Fas and etoposide treatment: like TBB, both of these well-known apoptosis promoters induce the fragmentation of HS1 with the appearance of degraded products of a similar size. The time course of HS1 degradation promoted by anti-Fas is shown in Figure 6. Similar curves were obtained with TBB and etoposide (not shown). The correlation between HS1 degradation and apoptotic events was highlighted further by the finding that the caspase inhibitor z-VAD-fmk prevents the fragmentation of HS1, promoted by either TBB or anti-Fas and etoposide (Figure 5).

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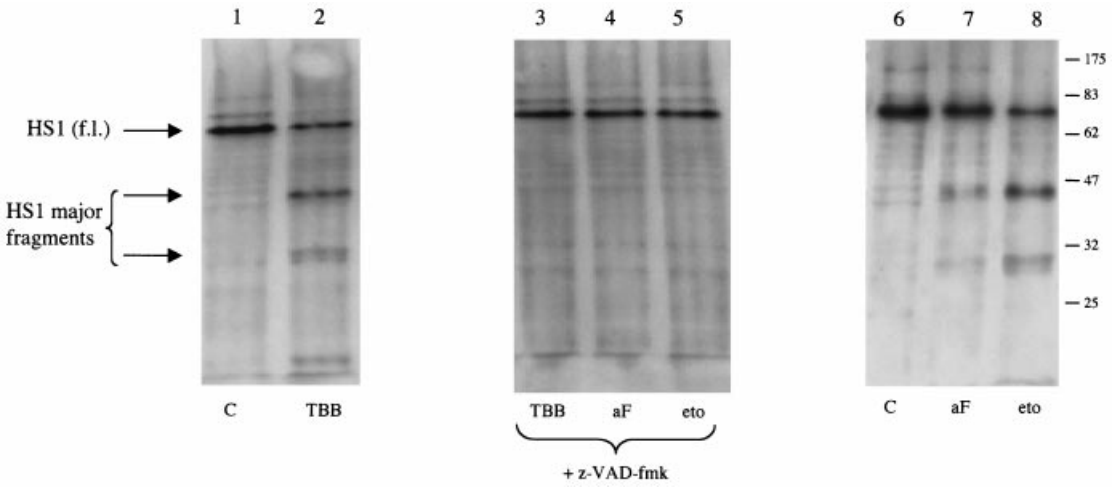

Figure 5 Caspase-dependent fragmentation of HS1 protein

Cells were treated for 8 h with nothing (C; lanes 1 and 6), or 25 μM TBB (lanes 2 and 3), 50 ng/ml anti-Fas (aF; lanes 4 and 7), 50 μM etoposide (eto; lanes 5 and 8). Cells of lanes 3–5 were pretreated for 1 h with 25 μM z-VAD-fmk, before the addition of the apoptotic inducers. Proteins from cell lysates (6 μg) were then subjected to SDS/PAGE (11% gels) and Western blotting with anti-HS1 antibodies, followed by ECL detection. Positions of molecular-mass markers (in kDa) are shown on the right. HS1(f.l.), full-length HS1.

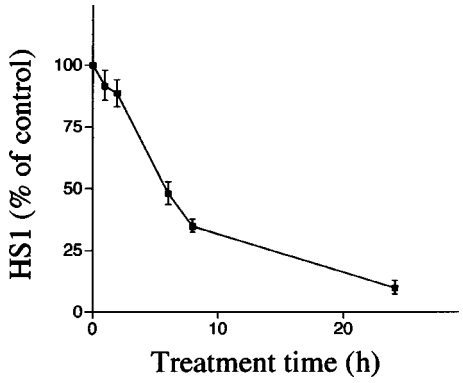

Figure 6 Time course of HS1 degradation

Cells were treated with 50 ng/ml anti-Fas for 1, 2, 6, 8 and 24 h. Analysis of HS1 fragmentation was performed by Western blotting, as described for Figure 5.The amount of full-length HS1 was quantified by detection of the ECL reaction with Kodak Image Station 440CF and analysis by Kodak 1D Image Analysis software. The amount of full-length HS1 from non-treated cells was considered to be 100%. Reported values represent the means $\pm$ S.E.M. from three different experiments.

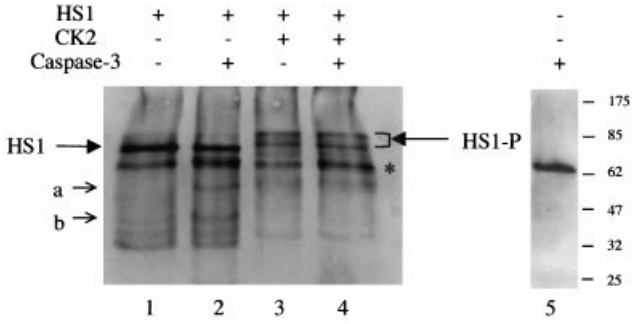

Figure 7 Phosphorylation by CK2 prevents the cleavage of HS1 protein by caspase-3

Recombinant His-tagged HS1 was incubated with $MgATP^{2+}$ in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of recombinant CK2α, as described in the text. A phosphorylation stoichiometry of about 3 mol of P_i/mol of protein was achieved in the presence of CK2. Aliquots corresponding to 5 ng of HS1 were incubated for 3 h at 37 °C with (lanes 2 and 4) or without (lanes 1 and 3) caspase-3, as indicated. Reactions were stopped by addition of SDS/PAGE loading buffer, and analysis was performed by Western blotting with anti-HS1 antibodies. The migration of the bands corresponding to phosphorylated HS1 (HS1-P) is up-shifted compared with the non-phosphorylated protein. The major bands generated from HS1 by caspase-3 are indicated as a and b. The asterisk denotes the position migrated to by both a putative HS1 fragment present in the HS1 preparation and an overlapping contaminant of the caspase-3 preparation, giving an aspecific immunoreaction. This latter is also evident in lane 5, where caspase-3 alone was loaded (separate experiment). Molecular-mass marker positions (in kDa) are shown on the right.

Caspase-dependent degradation of HS1 is counteracted by previous phosphorylation by CK2

The above findings could be explained assuming that HS1 becomes less prone to caspase degradation upon phosphorylation by CK2. To corroborate this hypothesis, we examined the susceptibility of recombinant HS1, either as such or after phosphorylation by CK2, to caspase-3 proteolysis *in vitro*. Caspase-3 was chosen because HS1 includes caspase-3 consensus sites (D/EXXD), and the time course of HS1 degradation *in vivo* is similar to that of PARP, a substrate of caspase-3. As shown in Figure 7, caspase-3 promoted the appearance of at least two bands, running faster than HS1 and recognized by anti-HS1 antibodies (Figure 7, compare lanes 1 and 2). In contrast, the caspase-3 treatment of previously phosphorylated HS1 failed to generate the same fragments (Figure 7, lanes 3 and 4). This indicates that HS1 cleavage by caspase-3 is counteracted by previous phosphorylation of HS1.

DISCUSSION

The recent demonstration that TBB, an ATP-site-directed inhibitor of protein kinase CK2 [23], displays a striking selectivity for just this enzyme amid a panel of more than 30 protein kinases [20], provided a new tool for investigating the biological functions

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of this pleiotropic and in some respects still enigmatic kinase. In this work, we have exploited TBB to gain information about the role of CK2 in apoptosis. Recently Guo et al. [12] have shown that during chemically induced apoptosis of prostate cancer cells CK2 is translocated from the cytoplasm to the nucleus, suggesting that such shuttling may reflect a protective response to chemically mediated apoptosis. Consistent with this hypothesis, cells transfected with CK2 showed an increased resistance to apoptosis that was proportional to the parallel elevation of CK2 in the nuclear matrix. In order to shed light on the mechanism by which CK2 might counteract apoptosis, we have taken advantage of the selectivity of TBB towards CK2 and we have chosen Jurkat cells because of their high content of HS1 protein, implicated in apoptosis of haematopoietic cells [16–18] and shown to be phosphorylated by CK2 [19]. We already knew, moreover, that CK2-dependent phosphorylation of HS1 is inhibited by TBB both *in vitro* and in intact Jurkat cells [20]. Our present data corroborate the concept that TBB efficiently and persistently inhibits endogenous CK2 to such an extent that CK2 from TBB-treated cells is nearly inactive. They also show that, in parallel to CK2 inhibition, TBB induces time- and dose-dependent cell death, which is accounted for, at least in part, by apoptosis, as judged by the appearance of the apoptotic PARP fragment, the release of cytochrome *c* from mitochondria and the generation of DNA laddering comparable with that observable upon induction of apoptosis by anti-Fas antibodies. Collectively taken, these data are fully consistent with the hypothesis that CK2 counteracts apoptosis [12], and they include TBB in the growing list of chemical inducers of apoptosis. The cause–effect link between the two effects of TBB, inhibition of CK2 and induction of apoptosis, is corroborated by the observation that another inhibitor of CK2, emodin, also induces apoptosis and, even more, by the behaviour of HS1, a protein substrate of CK2 involved in apoptosis. As a consequence of TBB treatment, in fact, the Ser/Thr phosphorylation of HS1 is inhibited while its fragmentation is dramatically enhanced. That fragmentation of HS1 is an event related to apoptosis is demonstrated firstly by its susceptibility to the caspase inhibitor z-VAD-fmk, which prevents TBB-induced degradation of HS1, and secondly by the observation that HS1 undergoes a similar fragmentation also in response to classical apoptotic stimuli, like those initiated by anti-Fas and by etoposide. Furthermore, HS1 is cleaved by caspase-3 *in vitro*, whereas it becomes resistant to cleavage if it is previously phosphorylated by CK2.

It can be concluded therefore that HS1 fragmentation is an apoptotic event that is counteracted by CK2-induced phosphorylation and thereafter enhanced by the CK2 inhibitor TBB.

Presently the location of caspase cleavage sites in HS1 can just be a matter of conjecture, and the residues phosphorylated by CK2 have been mapped only approximately [19]. Interestingly, however, one or two threonyl residues in the N-terminal segment of HS1 (Thr-16 and Thr-23) have been shown to account for most of the phospho-radiolabelling catalysed by CK2 [19], and none of the HS1 fragments generated by TBB treatment through a caspase-dependent reaction are susceptible to CK2 phosphorylation. This suggests that all of these fragments have lost the N-terminal region, including the main phosphorylation sites. At least one of the caspase-cleavage sites must also be located in the N-terminal domain of HS1, since the largest fragment, transiently detectable after short TBB treatment (results not shown), displays a molecular mass of about 58 kDa. In HS1 there are several sites conforming to the motif recognized by caspases [34], and one is located in the N-terminal domain, adjacent to Thr-23, suspected to be a target of CK2; it is well established, moreover, that caspases often act on atypical sites. In this respect the recent observation that the transcription factor Max is cleaved by caspase-5 at an atypical site (IEVE) specified by a glutamic acid and that this cleavage is inhibited if an adjacent serine is phosphorylated by CK2 [21] is of special interest. At about the same time, while this article was in preparation, another report appeared showing that Bid, a protein playing an essential role in Fas-mediated apoptosis, becomes refractory to caspase-8 if it is phosphorylated previously by CK2 [22]. Therefore HS1 represents at least the third example of an apoptotic protein whose cleavage by caspases is counteracted by CK2-mediated phosphorylation. It is tempting to speculate that CK2 may play a general role in generating caspase-refractory proteins, which would explain its anti-apoptotic effect, at least in part.

Our data also highlight the physiological relevance of Ser/Thr phosphorylation of HS1. This protein in fact was first discovered as a Tyr phosphoprotein [31,32] susceptible to a sequential multi-phosphorylation catalysed by Syk and Src tyrosine kinases in a concerted way [26,35,36]. It was suggested that tyrosine phosphorylation of HS1 could be related to its apoptotic effect in B-cells, since mutation of the Syk sites prevented translocation of HS1 into nuclei, where it is suspected to act as a transcription factor [37]. Later, it was observed that in platelets HS1 also undergoes Ser/Thr phosphorylation [19], and CK2-mediated phosphorylation of HS1 was shown also to occur *in vitro* and in Jurkat cells [19,20], but the physiological significance of such a phosphorylation remained unclear. Our present data would indicate that, whereas Tyr phosphorylation may dictate the nuclear localization of HS1 [37], its Ser/Thr phosphorylation by CK2 correlates with its implication in apoptosis, by conferring to it resistance to caspase cleavage. This scenario would also be consistent with the observation that the pool of CK2 responsible for its anti-apoptotic effect is the nuclear pool [12]. It is quite possible that the recruitment of CK2 into the nucleus represents an emergency device by which cells counteract apoptosis by rendering HS1, as well as other apoptotic proteins, refractory to caspases and thereafter incompetent for executing programmed cell death. It would be interesting in this respect to assess whether inhibition of HS1 tyrosine phosphorylation, expected to prevent its nuclear translocation, will also affect its TBB-induced fragmentation.

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REFERENCES

1 Pinna, L. A. and Meggio, F. (1997) Protein kinase CK2 ("casein kinase-2") and its implication in cell division and proliferation. Progr. Cell Cycle. Res. **3**, 77–97
2 Pinna, L. A. (1990) Casein kinase 2: an 'eminence grise' in cellular regulation? Biochim. Biophys. Acta **1054**, 267–284
3 Meggio, F., Marin, O. and Pinna, L. A. (1994) Substrate specificity of protein kinase CK2. Cell. Mol. Biol. Res. **40**, 401–409
4 Tawfic, S., Yu, S., Wang, H., Faust, R., Davis, A. and Ahmed, K. (2001) Protein kinase signal in neoplasia. Histol. Histopathol. **16**, 573–582
5 Seldin, D. C. and Leder, P. (1995) Casein kinase II α transgene-induced murine lymphoma: relation to theileriosis in cattle. Science **267**, 894–897
6 Kelliher, M. A., Seldin, D. C. and Leder, P. (1996) Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase II α. EMBO J. **15**, 5160–5166
7 Orlandini, M., Semplici, F., Ferruzzi, R., Meggio, F., Pinna, L. A. and Oliviero, S. (1998) Protein kinase CK2α' is induced by serum as a delayed early gene and cooperates with Hs-ras in fibroblast transformation. J. Biol. Chem. **273**, 21292–21297

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8 Li, D., Dobrowolska, G., Aicher, L. D., Chen, M., Wright, J. H., Drueckes, P., Dunphy, E. L., Munar, E. S. and Krebs, E. G. (1999) Expression of the casein kinase 2 subunits in Chinese hamster ovary and 3T3 L1 cells provides information on the role of the enzyme in cell proliferation and the cell cycle. J. Biol Chem. **274**, 32988–32996

9 Guerra, B. and Issinger, O. G. (1999) Protein kinase CK2 and its role in cellular proliferation, development and pathology. Electrophoresis **20**, 391–408

10 Padmanabha, R., Chen-Wu, J. L., Hanna, D. E. and Glover, C. V. (1990) Isolation, sequencing, and disruption of the yeast CKA2 gene: casein kinase II is essential for viability in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10**, 4089–4099

11 Faust, R. A., Tawfic, S., Davis, A. T., Bubash, L. A. and Ahmed, K. (2000) Antisense oligonucleotides against protein kinase CK2-α inhibit growth of squamous cell carcinoma of the head and neck *in vitro*. Head Neck **22**, 341–346

12 Guo, C., Yu, S., Wang, H., Davis, A. T., Green, J. E. and Ahmed, K. (2000) A potential role of nuclear matrix-associated protein kinase CK2 in protection against drug-induced apoptosis in cancer cells. J. Biol. Chem. **276**, 5992–5999

13 Guerra, B., Boldyreff, B. and Issinger, O. G. (2001) FAS-associated factor 1 interacts with protein kinase CK2 *in vivo* upon apoptosis induction. Int. J. Oncol. **19**, 1117–1126

14 Landesman-Bollag, E., Channavajhala, P. L., Cardif, R. D. and Seldin, D. C. (1998) p53 deficiency and misexpression of protein kinase CK2α collaborate in the development of thymic lymphomas in mice. Oncogene **16**, 2965–2974

15 Sayed, M., Pelech, S., Wong, C., Marotta, A. and Salh, B. (2001) Protein kinase CK2 is involved in G2 arrest and apoptosis following spindle damage in epithelial cells. Oncogene **20**, 6994–7005

16 Benhamou, L. E., Watanabe, T., Kitamura, D., Cazenave, P. A. and Sarthou, P. (1994) Signaling properties of anti-immunoglobulin–resistant variants of WEHI-231 B lymphoma cells. Eur. J. Immunol. **24**, 1993–1999

17 Taniuki, I., Kitamura, K., Maekawa, Y., Fukuda, T., Kishi, H. and Watanabe, T. (1995) Antigen-receptor induced clonal expansion and deletion of lymphocytes are impaired in mice lacking HS1 protein, a substrate of the antigen-receptor-coupled tyrosine kinases. EMBO J. **14**, 3664–3678

18 Fukuda, T., Kitamura, D., Taniuchi, I., Maekawa, Y., Benhamou, L. E., Sarthou, P. and Watanabe, T. (1995) Restoration of surface IgM-mediated apoptosis in an anti-IgM-resistant variant of WEHI-231 lymphoma cells by HS1, a protein-tyrosine kinase substrate. Proc. Natl. Acad. Sci. U.S.A. **92**, 7302–7306

19 Ruzzene, M., Brunati, A. M., Sarno, S., Marin, O., Donella-Deana, A. and Pinna, L. A. (2000) Ser/Thr phosphorylation of hematopoietic specific protein 1 (HS1). Implication of protein kinase CK2. Eur. J. Biochem. **267**, 3065–3072

20 Sarno, S., Reddy, H., Meggio, F., Ruzzene, M., Davies, S. P., Donella-Deana, A., Shugar, D. and Pinna, L. A. (2001) Selectivity of 4,5,6,7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 ('casein kinase-2'). FEBS Lett. **496**, 44–48

21 Krippner-Heidenreich, A., Talanian, R. V., Sekul, R., Kraft, R., Thole, H., Ottleben, H. and Luscher, B. (2001) Targeting of the transcription factor Max during apoptosis: phosphorylation-regulated cleavage by caspase-5 at an unusual glutamic acid residue in position P1. Biochem. J. **358**, 705–715

22 Desagher, S., Osen-Sand, A., Montessuit, S., Magnenat, E., Vilbois, F., Hochmann, A., Journot, L., Antonsson, B. and Martinou, J. C. (2001) Phosphorylation of Bid by casein kinase I and II regulates its cleavage by caspase 8. Mol. Cell **8**, 601–611

23 Szyszka, R., Grankowski, N., Felczak, K. and Shugar, D. (1995) Halogenated benzimidazoles and benzotriazoles as selective inhibitors of protein kinases CK I and CK II from *Saccharomyces cerevisiae* and other sources. Biochem. Biophys. Res. Commun. **208**, 418–424

24 Grankowski, N., Boldyreff, B. and Issinger, O. G. (1991) Isolation and characterization of recombinant human casein kinase II subunits α and β from bacteria. Eur. J. Biochem. **198**, 25–30

25 Sarno, S., Vaglio, P., Meggio, F., Issinger, O. G. and Pinna, L. A. (1996) Protein kinase CK2 mutants defective in substrate recognition. Purification and kinetic analysis. J. Biol. Chem. **271**, 10595–10601

26 Brunati, A. M., Donella-Deana, A., James, P., Quadroni, M., Contri, A., Marin, O. and Pinna, L. A. (1999) Molecular features underlying the sequential phosphorylation of HS1 protein and its association with c-Fgr protein tyrosine kinase. J. Biol. Chem. **274**, 7557–7564

27 Marin, O., Meggio, F. and Pinna, L. A. (1994) Design and synthesis of two new peptide substrate for the specific and sensitive monitoring of casein-kinase-1 and -2. Biochem. Biophys. Res. Commun. **198**, 898–905

28 Robertson, J. D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2000) Distinct pathways for stimulation of cytochrome *c* release by etoposide. J. Biol. Chem. **275**, 32438–32443

29 Martin, A. G. and Fresno, M. (2000) Tumor necrosis factor-α activation of NF-kappa B requires the phosphorylation of Ser-471 in the transactivation domain of c-Rel. J. Biol. Chem. **275**, 24383–24391

30 Kitamura, D., Kaneko, H., Miyagoe, Y., Ariyasu, T. and Watanabe, T. (1989) Isolation and characterization of a novel human gene expressed specifically in the cells of hematopoietic lineage. Nucleic Acid Res. **17**, 9367–9379

31 Yamanashi, Y., Okada, M., Semba, T., Yamori, T., Umemori, H., Tsunasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T. and Yamamoto, T. (1993) Identification of HS1 protein as a major substrate of protein-tyrosine kinase(s) upon B-cell antigen receptor-mediated signaling. Proc. Natl. Acad. Sci. U.S.A. **90**, 3631–3635

32 Hata, D., Nakamura, T., Kawakami, T., Kawakami, Y., Herren, B. and Mayumi, M. (1993) Tyrosine phosphorylation of MB-1, B-29, and HS1 proteins in human B cells following receptor crosslinking. Immunol. Lett. **40**, 65–71

33 Berndt, N. (1999) Protein dephosphorylation and the intracellular control of the cell number. Front. Biosci. **4**, D22–D42

34 Thornberry, N. A., Chapman, K. T. and Nicholson, D. W. (2000) Determination of caspase specificity using a peptide combinatorial library. Methods Enzymol. **322**, 100–110

35 Brunati, A. M., Ruzzene, M., James, P., Guerra, B. and Pinna, L. A. (1995) Hierarchical phosphorylation of a 50-kDa protein by protein tyrosine kinases TPK-IIB and c-Fgr, and its identification as HS1 hematopoietic-lineage cell-specific protein. Eur. J. Biochem. **229**, 164–170

36 Ruzzene, M., Brunati, A. M., Marin, O., Donella-Deana, A. and Pinna, L. A. (1996) SH2 domains mediate the sequential phosphorylation of HS1 protein by $p72^{syk}$ and Src-related protein tyrosine kinases. Biochemistry **35**, 5327–5332

37 Yamanashi, Y., Fukuda, T., Nishizumi, H., Inazu, T., Higashi, K., Kitamura, D., Ishida, T., Yamamura, H., Watanabe, T. and Yamamoto, T. (1997) Role of tyrosine phosphorylation of HS1 in B cell antigen receptor-mediated apoptosis. J. Exp. Med. **185**, 1387–1392

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