

# Targeting of the transcription factor Max during apoptosis: phosphorylation-regulated cleavage by caspase-5 at an unusual glutamic acid residue in position P1

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Max is the central component of the Myc/Max/Mad network of transcription factors that regulate growth, differentiation and apoptosis. Whereas the Myc and Mad genes and proteins are highly regulated, Max expression is constitutive and no post-translational regulation is known. We have found that Max is targeted during Fas-induced apoptosis. Max is first dephosphorylated and subsequently cleaved by caspases. Two specific cleavage sites for caspases in Max were identified, one at IEVE<sup>10</sup>↓S and one at SAFD<sup>135</sup>↓G near the C-terminus, which are cleaved *in vitro* by caspase-5 and caspase-7 respectively. Mutational analysis indicates that both sites are also used *in vivo*. Thus Max represents the first caspase-5 substrate. The unusual cleavage after a glutamic acid residue is observed only with full-length, DNA-binding competent Max protein but not with corresponding peptides, suggesting that structural determinants might be

important for this activity. Furthermore, cleavage by caspase-5 is inhibited by the protein kinase CK2-mediated phosphorylation of Max at Ser-11, a previously mapped phosphorylation site *in vivo*. These findings suggest that Fas-mediated dephosphorylation of Max is required for cleavage by caspase-5. The modifications that occur on Max in response to Fas signalling affect the DNA-binding activity of Max/Max homodimers. Taken together, our findings uncover three distinct processes, namely dephosphorylation and cleavage by caspase-5 and caspase-7, that target Max during Fas-mediated apoptosis, suggesting the regulation of the Myc/Max/Mad network through its central component.

**Key words:** caspase-7, c-Myc, DNA binding, IC<sub>50</sub>, protein kinase CK2.

## INTRODUCTION

Components of the Myc/Max/Mad network function as key regulators of cell behaviour [1,2]. In particular, Myc proto-oncoproteins are expressed primarily in cycling cells, whereas Mad proteins are found in differentiating cells. Consistent with these expression patterns are several lines of evidence demonstrating a role for Myc in stimulating cell cycle progression and proliferation [3]. In addition, the constitutive expression of Myc interferes with differentiation. Together these functions are relevant for the role of Myc in tumorigenesis. Indeed, many if not most human tumours possess elevated Myc levels that can be attributed in some cases to specific genetic alterations [1]. In contrast, Mad proteins negatively regulate cell proliferation and might stimulate differentiation. Thus the relative activities of Myc and Mad in cells are thought to define a molecular switch involved in the control of cell behaviour.

The Myc/Max/Mad network also regulates apoptosis. The deregulated expression of c-Myc sensitizes cells for apoptotic pathways [4,5]. At first sight this function is counterintuitive to the role of Myc in tumour formation. However, it might provide protection against the potentially detrimental activity of Myc as a tumorigenic factor. More recently, and in contrast with the

findings with Myc, Mad proteins were found to interfere with apoptosis. The expression of Mad1 provides partial protection against different apoptotic stimuli, including anti-Fas, TRAIL and UV, in an osteosarcoma cell line [6]. In addition, the absence of Mad1 sensitizes haemopoietic cells to apoptosis-inducing conditions [7]. It has also been shown that thymocytes and neural progenitor cells derived from *mad3*<sup>-/-</sup> mice are more sensitive than wild-type cells to  $\gamma$ -irradiation-induced apoptosis [8]. Taken together, these findings suggest physiological roles for both Myc and Mad proteins in the regulation of apoptosis.

The proteins of the Myc/Max/Mad network belong to the basic region (b)/helix-loop-helix (HLH)/leucine zipper (Zip) family of transcriptional regulators, in which the bHLHZip specifies protein-protein dimerization and sequence-specific DNA binding [9]. Max provides an essential function within the network because it is the common interaction partner for both Myc and Mad proteins. These heterodimers can then bind to specific DNA elements commonly referred to as E boxes. Unlike Myc and Mad proteins, Max is ubiquitously expressed and its absence leads to very early embryonic lethality, supporting its important role in the network [2,10]. Although Myc and Mad proteins recruit complexes that regulate gene transcription at least in part by modulating chromatin structure, no comparable

Abbreviations used: EMSA, electrophoretic mobility-shift assay; bHLHZip, basic region/helix-loop-helix/leucine zipper; ODC, ornithine decarboxylase; PARP, poly(ADP-ribose) polymerase; USF, upstream stimulatory factor.

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activities have been found associated with Max [3]. Nevertheless, overexpression of Max can affect the function of Myc and potentially that of other E-box-binding factors by the ability of Max homodimers to compete for binding to DNA. Max is constitutively phosphorylated *in vivo* at protein kinase CK2 sites [11,12]. This phosphorylation has been shown to affect the DNA-binding properties of Max homodimers and might therefore be of regulatory importance.

Apoptosis is a major mechanism in maintaining appropriate cell numbers in a multicellular organism, in removing autoreactive, virally infected or tumorigenic cells, and in remodelling tissues during development [13]. Apoptosis can be stimulated through several different pathways. An important mechanism is the activation of death receptors, including Fas and tumour necrosis factor receptor I [14,15]. A key event during apoptosis is the activation of a cascade of cysteine proteases, the caspases, that cleave a limited number of cellular proteins [16]. Caspase-mediated cleavage of a few of the more than 100 known substrates has been demonstrated to affect the apoptotic process but the physiological relevance of most of them remains unknown [17]. Among the identified substrates are few transcriptional regulators, including Sp1, STAT1, sterol regulatory element binding protein and serum response factor [18–21], suggesting that the regulation of gene transcription might be relevant during apoptosis [22]. Furthermore, several kinases are caspase substrates [23], indicating that signalling cascades are affected during the apoptotic process. Indeed, a recent study has identified several proteins that seem to be altered in their phosphorylation pattern in response to Fas signalling [24]. Interestingly, both increased and decreased levels of phosphate have been observed. These changes might be due to changes in kinase activity or, alternatively, to altered phosphatase activity in apoptotic cells. However, most proteins analysed were not affected during apoptosis [24]. Together these findings demonstrate that many distinct functions are targeted in response to apoptotic signals by proteases and by reversible protein modifications, including phosphorylation. Furthermore, it is also becoming evident that these effects are specific for a small subpopulation of proteins, whereas many are not modified in any obvious manner.

Because the Myc/Max/Mad network has been implicated in the regulation of apoptosis, we analysed the fate of some of these proteins during Fas-mediated apoptosis in Jurkat cells, a human leukaemic T-cell line. We observed that Max was processed proteolytically during apoptosis. This was unexpected because Max has been viewed in the past as the ubiquitously expressed, inert dimerization partner of the other members of the Myc/Max/Mad network. Two distinct cleavage sites were identified *in vitro* in Max that are used by caspase-5 and caspase-7 respectively and that are also recognized *in vivo*. Of particular interest was the N-terminal cleavage of Max by caspase-5 because it occurred after a glutamic acid residue (Glu-10) rather than an aspartic acid residue and thus provides an example of an alternative cleavage site for a caspase. In addition, cleavage after Glu-10 requires the dephosphorylation of an adjacent serine residue (Ser-11), which is modified by protein kinase CK2. Both the proteolytic cleavage and the dephosphorylation affect the DNA-binding properties of Max. These findings identify several Fas-induced signals that converge on Max and affect its function.

## EXPERIMENTAL

### Cell culture and apoptosis induction

Jurkat cells were grown in RPMI 1640 medium containing 5% (v/v) fetal calf serum. COS-7 cells in Dulbecco's modified Eagle's medium and RK13 cells in minimal essential medium, both with

10% (v/v) fetal calf serum. Apoptosis was induced in Jurkat cells ( $10^7$  cells/ml) by an anti-Fas antibody (100 ng/ml IgM, clone CH11; Kamiya Biomedical Co., Seattle, WA, U.S.A.) in serum-free medium. Caspase activity was inhibited by preincubation for 90 min with 100  $\mu$ M Z-VAD-FMK (Kamiya Biomedical Co.) before the induction of apoptosis.

### Transient transfections

Jurkat cells were washed in RPMI 1640. Cell suspension ( $250 \mu$ l;  $6 \times 10^7$  cells/ml) was electroporated with 20  $\mu$ g of plasmid DNA at 960  $\mu$ F and 250 V in a 0.4 cm cuvette (Gene Pulser; Bio-Rad). After incubation on ice for 30 min, the cells were transferred into cell culture medium and grown for 18 h before the induction of apoptosis. COS-7 cells and RK13 cells were transfected as described [25].

### Plasmids and site-directed mutagenesis

pSPmaxp22, pSPmaxp22S11A, pSPmaxp22S2A/S11A, pGex-3Xmax, pGex3XmaxS11A and pGex3XmaxS2A/S11A have been described previously [12]. Point mutants of Max were generated by PCR with specific primers and were verified by sequencing.

### Western blotting and antibodies

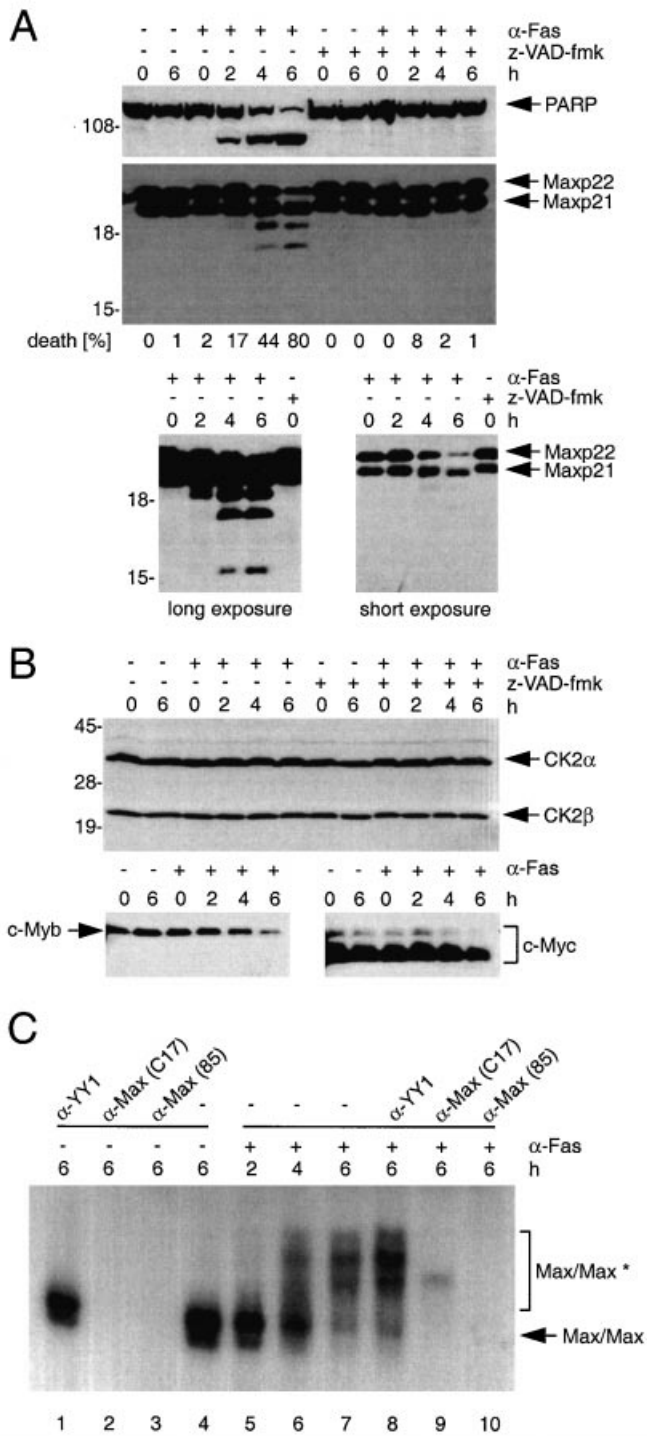
In general, lysates derived from equal cell numbers were separated by SDS/PAGE; Western blot analysis was performed as described [26]. Max was detected with antibodies specific for either residues 37–160 (antiserum 85) [27] or the C-terminal residues 144–160 (C17; Santa Cruz, Biotechnology). Antibodies specific for poly(ADP-ribose) polymerase (PARP) were purchased from Boehringer; antibodies specific for c-Myb (C19) and c-Myc (N267) were from Santa Cruz. Antibodies recognizing YY1 (serum 263) have been described [26] and antibodies specific for the  $\alpha$  and  $\beta$  subunits of CK2 were a gift from D. Litchfield [28].

### Immunoprecipitation, and phosphatase and kinase assays

Transfected COS-7 cells were extracted in 300  $\mu$ l per 10 cm plate of modified buffer F [10 mM Hepes (pH 7.5)/50 mM NaCl/30 mM sodium pyrophosphate/50 mM NaF/100  $\mu$ M  $\text{Na}_3\text{VO}_4$ /0.2% (v/v) Triton X-100/1 mM PMSF/2.5 units/ml pepstatin A/2.5 units/ml leupeptin/0.15 mM benzamide/2.8  $\mu$ g/ml aprotinin] as described [25]. Electroporated apoptotic and non-apoptotic Jurkat cells were lysed in modified buffer F or in buffer AB [20 mM Tris/HCl (pH 7.4)/50 mM NaCl/1 mM EDTA/0.5% (v/v) Nonidet P40/0.5% deoxycorticosterone/0.5% SDS/0.5% aprotinin/0.5% (v/v) PMSF] [25]. Max proteins were immunoprecipitated and washed in either high-stringency buffer AB or low-stringency buffer F or buffer WB [20 mM Hepes (pH 7.4)/150 mM NaCl/1% (v/v) Nonidet P40/0.5% deoxycorticosterone]. Phosphatase and kinase assays were performed as described previously [12]. Immunoprecipitation CK2-kinase assays were performed with glutathione S-transferase-conjugated Max (100 ng per assay) as substrate in the presence of 100  $\mu$ M ATP and 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP at 30 °C.

### Caspase cleavage assays *in vitro* and peptide sequencing

Glutathione S-transferase-conjugated Max and mutants thereof were purified and the glutathione S-transferase portion was cleaved off as described [12]. Caspase-1 to caspase-8 were expressed as N-terminal poly(His) fusion proteins and purified to approx. 90% as described [29]. The enzymes were preactivated



**Figure 1** Caspase-dependent cleavage of Max

Jurkat cells were preincubated with 100  $\mu$ M Z-VAD-FMK or DMSO and then treated with Fas-specific antibodies ( $\alpha$ -Fas) (100 ng/ml) for the indicated times before being harvested in SDS-sample buffer (**A**, **B**) or in modified buffer **F** (**C**). (**A**) PARP and Max (C17 antibodies) were detected by Western blot analysis. Different exposures of part of the Max blot are displayed for better detection of Max cleavage products. In parallel, cells were fixed, stained with Acridine Orange and analysed microscopically for characteristic morphological changes associated with apoptosis, namely chromatin condensation and nuclear fragmentation. For each sample more than 200 cells were inspected. (**B**) The expression of the  $\alpha$  and  $\beta$  subunits of CK2, c-Myb and c-Myc were analysed by Western blotting. (**C**) Lysates of  $10^6$  Jurkat cells were analysed by EMSA with the labelled oligonucleotide CMD. Supershift experiments were performed with antibodies specific for Max ( $\alpha$ -Max) or YY1 ( $\alpha$ -YY1). Max/Max indicates Max homodimers and

for 10 min at 30 °C in caspase buffer [100 mM Hepes (pH 7.5)/20% (v/v) glycerol/0.5 mM EDTA/10 mM dithiothreitol] and cleavage reactions with Max were incubated in caspase buffer at 30 °C in the presence of 0.5  $\mu$ g of BSA in a reaction volume of 20–30  $\mu$ l. The reactions were stopped in SDS-sample buffer and the proteins were analysed by Western blotting.

Peptides were synthesized by standard solid-phase methods (Quality Controlled Biochemicals, Hopkinton, MA, U.S.A.), purified to more than 95% by reverse-phase chromatography and confirmed by MS.  $K_{obs}$  and  $k_{cat}/K_m$  values were determined as described [29].

For sequencing of the cleavage products, recombinant Maxp22 was incubated with caspase-5 or caspase-7 at 30 °C for 15 h. The cleavage products were processed and sequenced on an Applied Biosystems 477A protein sequencer with a 120A online HPLC system [30] or fractionated by reverse-phase HPLC and analysed as described previously [31].

### Electrophoretic mobility-shift assays (EMSA)

EMSA were performed as described [25]. Control or Fas-induced cells ( $2 \times 10^6$  cells/100  $\mu$ l) were lysed in modified buffer F. As probe either an E box consensus oligonucleotide (CMD, 5'-TCAGACCACGTGGTCCGG-3'; the E box is underlined) or a fragment of the first intron of the rat ornithine decarboxylase (ODC) gene, which includes two E boxes, was used [32]. The expression of Max or Max mutants in COS-7 lysates was standardized by Western blotting with antiserum 85 preblocked with the Max peptide 128–160.

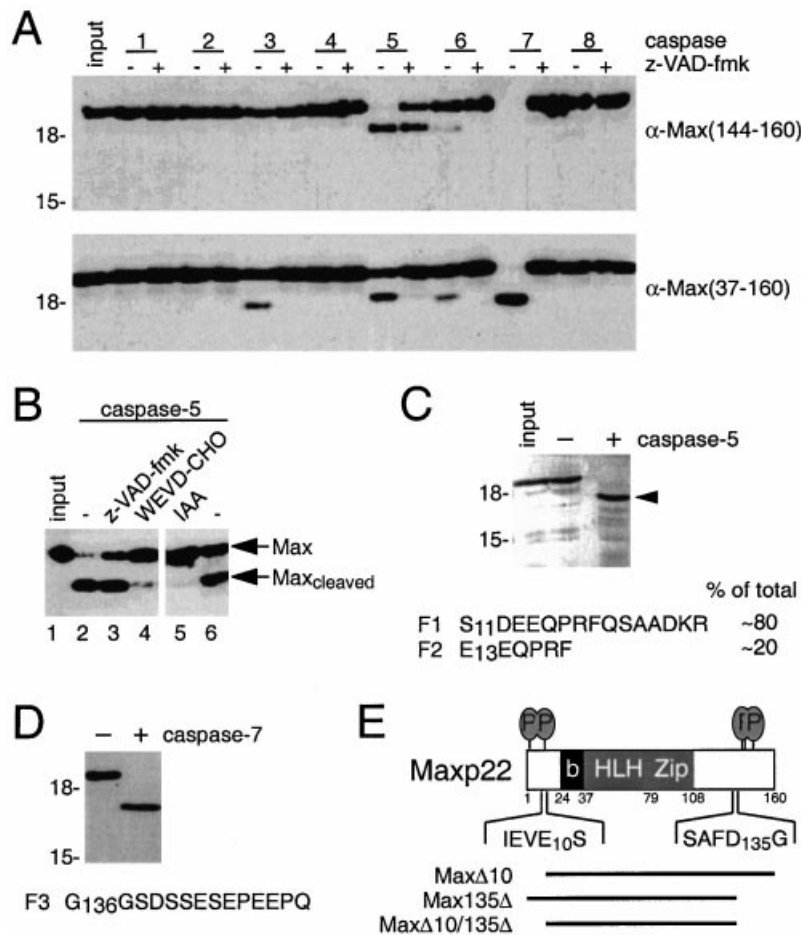
## RESULTS

### Max is cleaved *in vivo* during Fas-mediated apoptosis

During Fas-induced apoptosis in Jurkat cells, both Max isoforms, p22 and p21, are cleaved (Figure 1). Western blot analysis with antibodies specific for the Max C-terminus [C17,  $\alpha$ -Max(144–160)] revealed a 19 kDa product after 2 h and smaller, unstable fragments at later times (Figure 1A, long exposure), suggesting that Max is processed at the N-terminus. A 20 kDa product, concealed by p21 at earlier time points, was visible after 6 h when most of the p21 isoform of Max had been depleted (Figure 1A, short exposure). Staurosporine treatment gave comparable results in Jurkat cells and also in COS-7 and HeLa cells (results not shown). The onset of PARP cleavage slightly preceded that of Max [17] but by 4–6 h similar fractions of each protein were cleaved (Figure 1A, short exposure). Max and PARP cleavage was inhibited by the irreversible caspase inhibitor peptide Z-VAD-FMK ('benzyloxycarbonyl-Val-Ala-D,L-Asp-fluoromethylketone') (Figure 1A). In contrast with Max, c-Myc, c-Myb and the  $\alpha$  and  $\beta$  subunits of CK2 were essentially stable with no cleavage products detectable (Figure 1B). Only late in response to  $\alpha$ -Fas was a decrease in c-Myc and c-Myb observed, probably owing to the decreased expression of these short-lived proteins. Thus our findings identify the central component of the Myc/Max/Mad network, Max, as an early apoptotic target.

Because Max is a DNA-binding protein, we tested whether the apoptosis-associated modifications affected the ability of endogenous Max to interact with the E box consensus oligonucleotide CMD. Specific Max complexes were detected in

Max/Max\* refers to complexes containing modified forms of Max. The numbers at the left-hand side of the gels represent the apparent molecular masses (in kDa) of marker proteins run on the same SDS-polyacrylamide gels.



**Figure 2** Cleavage of Max by recombinant caspases

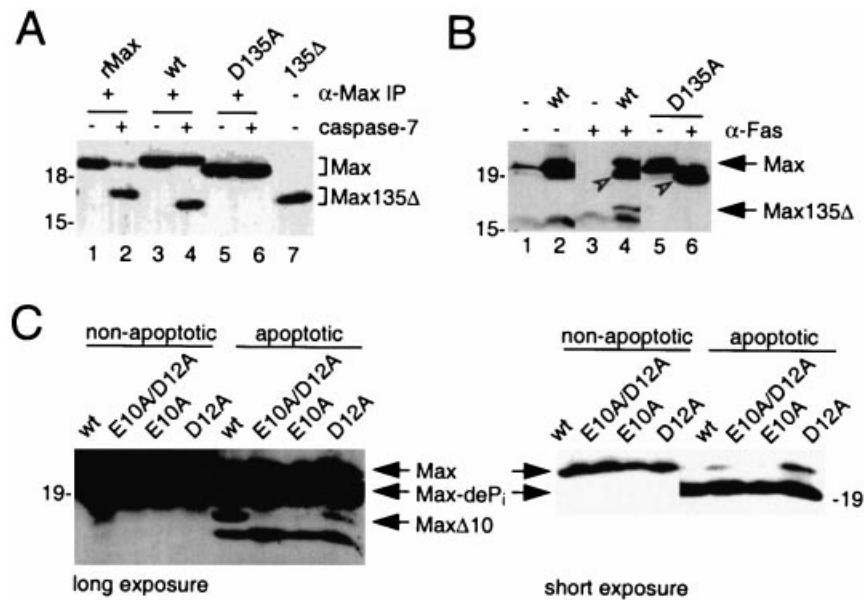
(A) Recombinant Maxp22 (rMaxp22) (15 ng) was treated with caspase-1 to caspase-8 (100 ng of each) at 30 °C for 90 min in the presence or absence of Z-VAD-FMK (100  $\mu$ M) and analysed by Western blotting. Two independent experiments are shown, one developed with antibodies specific for the C-terminus of Max [ $\alpha$ -Max(144–160), C17; top panel], the other with the polyclonal Max serum 85 [ $\alpha$ -Max(37–160), bottom panel]. (B) Caspase-5 (50 ng) was preincubated in the presence or absence of Z-VAD-FMK (50  $\mu$ M) (lane 3) or Ac-WEVD-CHO (50  $\mu$ M) (lane 4) at 30 °C for 30 s. Pretreatment of caspase-5 with 1 mM iodacetamide (IAA) was performed for 30 min on ice (lane 5). Control incubations are shown in lanes 2 and 6. The cleavage reactions with recombinant Max were then performed in the presence of 5 mM dithiothreitol at 30 °C for 30 min. 'Input' refers to 4.5 ng of recombinant Maxp22. Polyclonal Max serum 85 was used for Western blot analysis. (C) Recombinant Maxp22 (4.5  $\mu$ g) was digested with caspase-5 (500 ng), subjected to SDS/PAGE, blotted and stained. The N-terminal amino acids of the Max fragment (arrowhead) were determined by Edman degradation. Two sequences were obtained: fragments 1 (F1) and 2 (F2) represented approx. 80% and 20% of the input respectively. (D) Recombinant Maxp22 (9  $\mu$ g) was digested with caspase-7 (400 ng) and an aliquot was analysed by immunoblotting with the Max-specific serum 85. One peptide, fragment 3 (F3), was recovered by reverse-phase HPLC and subjected to Edman degradation. (E) Schematic representation of Maxp22, indicating the major caspase-5 and caspase-7 cleavage sites in relation to the functional domains of Max. Phosphorylation sites *in vivo*, including the CK2 site at Ser-11, are shown. Max deletion mutants reflecting the predominant cleavage sites are indicated. The numbers at the left-hand side of the gels represent the apparent molecular masses (in kDa) of marker proteins run on the same SDS-polyacrylamide gels.

lysates of control Jurkat cells that co-migrated with Max homodimers from transiently transfected COS-7 cells (Figure 1C, and results not shown). The former were replaced by more slowly migrating complexes when lysates from  $\alpha$ -Fas-stimulated Jurkat cells were analysed (Figure 1C). Supershift experiments with serum 85, recognizing Max(37–160), demonstrated that all complexes with altered mobility contained Max (Figure 1C). Because none of the complexes was supershifted with Mad-specific or Myc-specific antibodies (results not shown), we presume that these were homodimers of differentially modified Max molecules. At least one complex (Figure 1C, lane 9) was resistant to antibodies against the C-terminus (C17), suggesting that this complex contained C-terminally truncated Max. Together with the findings shown above (Figure 1A), this suggests that Max is truncated both N-terminally and C-terminally during Fas-induced apoptosis. At first sight, however, truncation of

Max is not consistent with the observed decrease in the mobility of Max–DNA complexes. As shown below, Max is not only cleaved but also dephosphorylated, which has previously been shown to affect the mobility of Max proteins on both native and denaturing gels [12]. Thus the altered mobilities of Max–DNA complexes reflect not only proteolytic processing but also changes in post-translational modifications.

#### Max is a substrate of caspase-5 and caspase-7 *in vitro*

To obtain information on the enzymes involved in Max cleavage, recombinant Maxp22 was treated with recombinant caspase-1 to caspase-8 [29]. Caspase-5 and caspase-7 cleaved Max efficiently and caspase-6 and caspase-3 cleaved weakly, whereas caspase-1, caspase-2, caspase-4 and caspase-8 did not cleave Max under these conditions (Figure 2A). After treatment with caspase-5 and



**Figure 3** Caspase-5 and caspase-7 cleavage sites are used *in vivo*

(A) Maxp22 (wild-type; wt) and the indicated mutants were expressed in Jurkat cells, immunoprecipitated (IP) with anti-Max ( $\alpha$ -Max) and treated with or without caspase-7. rMaxp22 served as positive control. The Western blot was developed with polyclonal Max serum 85. (B) Jurkat cells were transiently transfected with constructs expressing the indicated proteins, treated with or without Fas-specific antibodies ( $\alpha$ -Fas), and harvested 6 h later in SDS-sample buffer. Western blots were developed with serum 85. Arrowheads in lanes 4 and 5 indicate dephosphorylated Max. (C) Jurkat cells were manipulated as in (B) except for 10 h of anti-Fas treatment. Max proteins were detected with C17. Two exposures of the same blot are shown (long exposure, 10 min; short exposure, 1 s for non-apoptotic cells and 20 s for apoptotic cells). The slightly faster-migrating species of Max is labelled Max-deP<sub>i</sub> because it represents a dephosphorylated, non-cleaved form of Max (see the text for details). MaxΔ10 is not visible on the short exposure. The numbers at the left-hand side of the gels represent the apparent molecular masses (in kDa) of marker proteins run on the same SDS-polyacrylamide gels.

caspase-6, Max fragments were detected with antibodies against the C-terminus (C17), suggesting that these enzymes cleaved near the N-terminus, whereas Max fragments generated by caspase-3 and caspase-7 were detected with serum 85 but not with antibodies against the C-terminus, indicating that these enzymes removed a C-terminal peptide. Pretreatment of caspases with Z-VAD-FMK blocked the activity of caspase-3, caspase-6 and caspase-7 and partially inhibited caspase-5 (Figures 2A and 2B). In addition, cleavage by caspase-5 was blocked by Ac-WEVD-CHO, an inhibitor of caspase-5, as well as by iodoacetamide (Figure 2B). Thus we conclude that caspase-5 and caspase-7 cleaved Max efficiently at two distinct sites *in vitro*.

The cleavage sites of Max *in vitro* were determined by sequencing the caspase-5 and caspase-7 cleavage products. Cleavage by caspase-5 gave one major and one minor product, indicating hydrolysis after Glu-10 and Asp-12 respectively (Figure 2C). Treatment with caspase-7 revealed one fragment cleaved at Asp-135 (Figure 2D). In summary, we identified two major sites of cleavage, a caspase-5 site at Glu-10, N-terminal to the bHLHZip of Max, and a caspase-7 site at Asp-135, C-terminal to the bHLHZip (Figure 2E).

#### Sites in Max used by caspase-5 and caspase-7 are cleavage sites *in vivo*

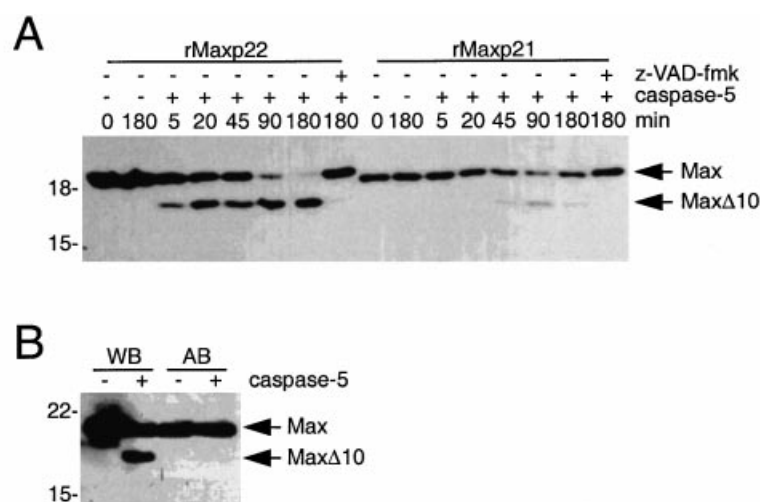
Next we verified the cleavage sites determined *in vitro* by assays both *in vitro* and *in vivo*. Maxp22 and the cleavage-site mutant Maxp22-D135A were expressed in COS-7 cells, immunoprecipitated and treated with caspase-7 *in vitro*. Recombinant Maxp22 (rMaxp22) served as positive control. Whereas Maxp22 was cleaved, Maxp22-D135A was not (Figure 3A). The mobilities of Maxp22-135Δ and caspase-7-cleaved Maxp22 were indis-

tinguishable, which is consistent with cleavage at Asp-135 (Figure 3A). Despite an N-terminal tag, rMaxp22 migrates faster than Maxp22 because the latter is phosphorylated at C-terminal sites (Ser-138, Ser-140, Ser-141, Ser-142 and/or Ser-144) that increase the apparent molecular mass of Maxp22 [12]. Caspase-7-cleaved Maxp22 no longer contained any of these phosphorylation sites and therefore migrated faster than cleaved rMaxp22 with its N-terminal tag (Figure 3A). Maxp22-D135A was also analysed *in vivo* after overexpression in Jurkat cells. Whereas the stimulation of Fas resulted in cleavage of wild-type Maxp22 to a fragment corresponding to Maxp22-135Δ, Maxp22-D135A was not cleaved (Figure 3B). These findings confirm the mapping of the C-terminal Max cleavage site at Asp-135 *in vitro* and *in vivo*.

The use of the N-terminal cleavage sites *in vitro* was evaluated *in vivo* by analysing Max mutants in Fas-treated Jurkat cells. Whereas Maxp22-D12A was cleaved, neither the double mutant Maxp22-E10A/D12A nor Maxp22-E10A was cleaved, indicating that Glu-10 was indeed a caspase cleavage site *in vivo* (Figure 3C). However, Maxp22-E10A/D12A was still cleaved near the C-terminus, producing a fragment that co-migrated with Maxp22-135Δ (results not shown). Taken together, these findings demonstrate that both IEVE(10)-S and SAFD(135)-G are independent Max cleavage sites *in vivo*.

#### Cleavage at Glu-10 requires a native conformation of Max

The unusual finding that Max was cleaved after a glutamic acid residue both *in vivo* and *in vitro* led us to address in more detail the cleavage at Glu-10. We compared the cleavage of rMaxp22 to rMaxp21 by caspase-5 *in vitro*. Maxp21 is derived from an alternatively spliced message and differs from Maxp22 by a small deletion of nine residues ( $\Delta$ 13–21) towards the C-terminus from



**Figure 4** Structural requirements for cleavage by caspase-5 at Glu-10

(A) rMaxp22 and rMaxp21 (15 ng of each) were treated with or without caspase-5 at 30 °C for the indicated durations in the presence or absence of Z-VAD-FMK (100  $\mu$ M) and analysed by Western blotting. Max was stained with C17. (B) Recombinant Maxp22 (28 ng) was immunoprecipitated with Max-specific antibodies (C17) and washed in low-stringency buffer WB or high-stringency buffer AB, then washed extensively in buffer WB and caspase assay buffer. Subsequent cleavage occurred with caspase-5 (50 ng) at 30 °C for 3 h. The Western blot was developed with serum 85. The numbers at the left-hand side of the gels represent the apparent molecular masses (in kDa) of marker proteins run on the same SDS-polyacrylamide gels.

the caspase-5 cleavage site. This difference might influence the structure of the cleavage site and thus caspase accessibility and/or activity towards the unconventional site with a glutamic acid residue in the P1 position. We observed that rMaxp21 was cleaved less efficiently than rMaxp22 by caspase-5 (Figure 4A). Furthermore, we compared the cleavage of immunoprecipitated rMaxp22 washed in either a low-stringency (WB) or a high-stringency (AB) buffer. The latter is known to denature proteins partly and to abolish Max/Max dimer formation and as a result to abrogate its DNA binding. After this partial denaturing the immunoprecipitates were washed extensively in buffer WB and in caspase buffer to remove detergents before the cleavage reaction. Only Maxp22 prepared in buffer WB was cleaved by caspase-5, whereas Maxp22 prepared in buffer AB was resistant to cleavage (Figure 4B). These findings support the interpretation that the structural integrity of Max favours cleavage after Glu-10 in Maxp22.

From these findings we predicted that peptides modelled after the Max Glu-10 cleavage site would not be a substrate for caspase-5 because such peptides would most probably not adopt a native Max conformation; this is indeed what we observed. A peptide corresponding to residues 7–16 of Maxp22 was not cleaved by caspase-5 *in vitro* (results not shown). In contrast, the equivalent peptide in which Glu-10 was replaced by Asp was cleaved (Figure 5D). We also tested several tetrapeptides for their ability to compete with the fluorogenic substrate Ac-LEHD-AMC (in which AMC stands for 7-amino-4-methylcoumarin) for caspase-5 and Ac-DEVD-AMC for caspase-3 and caspase-7 (Table 1). Peptides with a glutamic acid residue in the P1 position competed significantly less well than the respective peptides with an aspartic acid residue in P1. This was true for caspase-5 as well as for caspase-3 and caspase-7. From these findings we suggest that cleavage by caspase-5 after an unusual glutamic acid residue at position 10 in Max requires structural determinants that are present in native Max but not in partly denatured Max or in peptides.

#### Max is dephosphorylated during Fas-induced apoptosis

Max is a highly phosphorylated protein [12]. Sites modified *in vivo* have been identified near the C-terminus (see above) and at Ser-2 and Ser-11. Dephosphorylation of the C-terminal sites in particular results in an increased mobility of Max on SDS/PAGE. The analysis of endogenous or transiently expressed Max in apoptotic cells revealed an intermediate form (named Max-deP<sub>i</sub>) reminiscent of dephosphorylated Max (Figures 1A and 3B). Dephosphorylation is also consistent with the lower mobility of the processed forms of Max in EMSAs (Figure 1C) [12]. Thus from these considerations it seemed possible that Max was not only cleaved but also dephosphorylated during apoptosis. Indeed, the intermediate form of Maxp22 co-migrated with phosphatase-treated Maxp22, whereas Maxp22 from apoptotic cells was not affected by phosphatase treatment (Figure 5A). The intermediate form was also generated from Maxp22-E10A/D12A both after Fas signalling and phosphatase treatment, demonstrating that it did not correspond to Maxp22-Δ10 (Figure 5A). Conversely, CK2 phosphorylation of Maxp22 isolated from apoptotic cells resulted in a decreased mobility that was indistinguishable from Maxp22 derived from control cells (Figure 5A). Max-deP<sub>i</sub> was also generated from several additional N-terminal Max mutants in apoptotic cells but not from Maxp22-Δ135, the latter being consistent with the loss of the C-terminal CK2 sites that are most critical for the altered mobility of Max on SDS/PAGE (results not shown). Significant levels of Max-deP<sub>i</sub> were detected before the appearance of the two primary cleavage products (Figures 3B and 3C). Thus Max was dephosphorylated at least at its C-terminal sites early during Fas-mediated apoptosis.

#### Max cleavage by caspase-5 is affected by phosphorylation

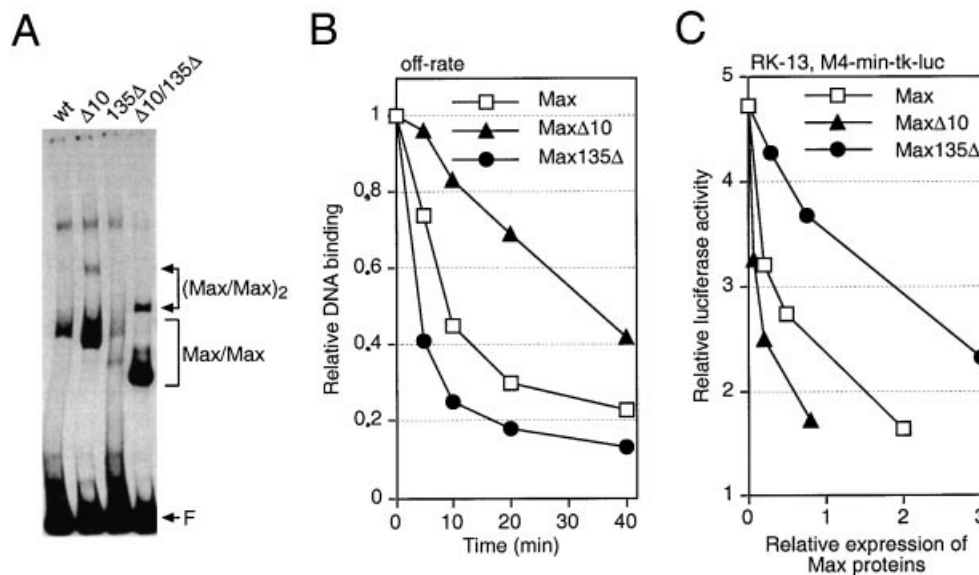
Immunoprecipitated Maxp22 was cleaved by caspase-7 *in vitro* (Figure 3A) but not by caspase-5 (Figure 5B). Furthermore and in contrast with the latter, immunoprecipitated rMaxp22 was a



**Table 1** Inhibition of caspases by peptide aldehydes

The following substrates were used: Ac-DEVD-AMC (20  $\mu$ M) for caspase-3 (250 pM) and caspase-7 (250 pM), and Ac-LEHD-AMC (20  $\mu$ M) for caspase-5 (500 pM). Abbreviations: AMC, 7-amino-4-methylcoumarin; n.i., no inhibition observed with peptide concentrations up to 10  $\mu$ M. Peptides are shown in single-letter amino acid codes.

Enzyme	IC <sub>50</sub> of peptide aldehydes (nM)					
	DEVD	DEVE	IEVD	IEVE	WEHD	WEHE
Caspase-3	1	100	100	n.i.	15 000	n.i.
Caspase-5	250	5000	250	15 000	50	8000
Caspase-7	25	280	700	n.i.	n.i.	n.i.

**Figure 6** Analysis of the DNA-binding activity of N-terminal and C-terminal deletion mutants of Max

(A) Maxp22 and Maxp22 deletion mutants were expressed in COS-7 cells and harvested in modified buffer F. The same amounts of Max proteins were analysed by EMSA, with ODC as probe. Max/Max and (Max/Max)<sub>2</sub> indicate occupation of one or both E boxes respectively. F, free probe; wt, wild-type. (B) Off-rate experiments with COS-7-derived Maxp22 and Maxp22 mutants were performed. Protein extracts were preincubated with labelled probe before the addition of a 200-fold excess of unlabelled oligonucleotides. At the time points indicated the samples were loaded on a running gel and the amount of bound labelled oligonucleotide was measured on a PhosphorImager. The relative amount of bound oligonucleotide is displayed. (C) RK13 cells were transiently transfected with a USF1 expression plasmid (2  $\mu$ g) and a luciferase reporter plasmid (pODCwt-tk-luc, 2  $\mu$ g) containing 151 bp of the ODC gene with two E boxes [32]. Increasing amounts of constructs (0.2, 1 and 2  $\mu$ g) expressing the indicated Max proteins were co-transfected. The relative luciferase activity was determined 36 h after transfection. Because the different Max proteins were expressed unequally, the amounts of transfected expression plasmids were adjusted for the relative amounts of proteins expressed as determined by Western blot analysis from parallel plates. This is indicated as 'relative expression of Max proteins'. A representative experiment of four, performed in triplicate, is shown.

substrate for caspase-5 (Figures 3A and 5B). A contribution of proteins derived from COS-7 cells to the altered digestion profile was excluded because cleavage of immunoprecipitated rMaxp22 was not affected by preincubation in COS-7 extract before treatment with caspase-5 (Figure 5B). One difference between COS-7-cell-derived Maxp22 and rMaxp22 is that the former is phosphorylated and the latter is not. Because Ser-11, the P1' position of the caspase-5 cleavage site, is phosphorylated *in vivo*, we reasoned that phosphorylation of Ser-11 might affect the susceptibility of Max to cleavage by caspase-5. Cleavage by caspase-5 was seen with COS-7-derived Maxp22-S2A/S11A and Maxp22-D12A; the former has mutated N-terminal CK2 phosphorylation sites and the latter has an altered CK2 consensus sequence, so both interfere with phosphorylation at Ser-11 (Figure 5B). Maxp22-E10A with a mutated N-terminal cleavage site was not sensitive to caspase-5 as expected. Next we combined the D12A mutation, which allowed cleavage, and the E10A mutation in Maxp22-E10A/D12A. This mutant was not cleaved

by caspase-5 (Figure 5B). Taken together, these findings provide further evidence for the specificity of caspase-5 for Glu-10 and suggest that cleavage by caspase-5 was inhibited by phosphorylation at Ser-11, whereas cleavage by caspase-7 was independent of phosphorylation.

In support of this interpretation, CK2 phosphorylated Max; however, neither phosphorylated Maxp22-S11A and Maxp22-S2A/S11A nor mock-treated proteins were resistant to cleavage by caspase-5 (Figure 5C). In addition, a peptide modelled on the caspase-5 cleavage site in Max but with an Asp residue in the P1 position was efficiently cleaved by caspase-5; the same peptide phosphorylated at Ser-11 was not hydrolysed (Figure 5D). The findings indicate that cleavage of Max by caspase-5 could be regulated by phosphorylation at Ser-11 and that cleavage of Max was preceded by its dephosphorylation. Because CK2 is the only known nuclear kinase modifying acidic sites [33], it is most likely to be the Max kinase *in vivo*. Decreased Max phosphorylation might be the result of decreased CK2 kinase activity. However,



this seems not to be so, because no change in overall CK2 kinase activity could be measured in response to Fas stimulation (Figure 5E). Other mechanisms, including an altered availability of CK2 and the induction of a Max phosphatase, will have to be considered.

### Cleavage of the N-terminus of Max stimulates DNA binding

We noticed that the overall DNA-binding capacity of Max remained quite constant during apoptosis (Figure 1C). This coincided with a significant loss of Max protein (Figure 1A), suggesting a relative increase in DNA binding of one or several of the modified forms of Max. We therefore measured the DNA-binding activity of Max deletion mutants (Figure 6A). Maxp22- $\Delta$ 10 and Maxp22- $\Delta$ 10/135 $\Delta$  showed increased DNA-binding activity; in Maxp22-135 $\Delta$  it was slightly decreased. To evaluate further the differences in DNA binding, the stability of Max-DNA complexes was determined. In off-rate experiments Maxp22- $\Delta$ 10 associated more stably, whereas Maxp22-135 $\Delta$  bound less well to DNA in comparison with wild-type Maxp22 (Figure 6B). In addition to the effects of these deletions, dephosphorylation of Max stimulates DNA binding [11,12]. Thus during apoptosis both dephosphorylation and truncation of Max affect its DNA binding properties.

To assess a role for these truncations *in vivo* we measured the ability of Maxp22- $\Delta$ 10, Maxp22-135 $\Delta$  and Maxp22 to compete with upstream stimulatory factor (USF) for binding to a reporter gene construct. ODCwt-tk-luc was activated 4–5-fold by USF, a transcription factor that binds the same E box elements as Max (Figure 6C). Because Max homodimers cannot transactivate, their overexpression was expected to inhibit USF-driven reporter activation by competing for the E boxes in the reporter gene. Whereas Maxp22- $\Delta$ 10 was more efficient than wild-type Max in antagonizing USF function, Maxp22-135 $\Delta$  was less so. This is consistent with the effects measured on DNA binding of these different Max proteins. Thus the altered DNA binding capacity of Max mutants measured *in vitro* was also observed *in vivo*.

## DISCUSSION

Signalling through Fas and through related death receptors results in pleiotropic effects in cells that in most cases ultimately trigger apoptosis. We have identified the transcription factor Max as a target for regulation by post-translational modifications during apoptosis. On activation of apoptosis, a complex series of events leads first to dephosphorylation and truncation and then to the complete degradation of Max. N-terminally truncated forms of Max show increased DNA binding activity. Because alterations in Max function have not yet been demonstrated, the present study represents the first example of signalling that primarily affects Max within the Myc/Max/Mad network.

Our analysis *in vitro* revealed that caspase-5 and caspase-7 are most effective in cleaving Max (Figure 2). Because the sites identified *in vitro* are also used *in vivo* (Figure 3), it is likely that caspase-5 and caspase-7 are both expressed in Jurkat cells as determined by reverse-transcriptase-mediated PCR (results not shown) or that enzymes with similar specificity are involved *in vivo*. The caspase-5 cleavage site in Max is unusual because hydrolysis occurs towards the C-terminus from Glu-10 (Figure 2C). This is without precedent because no other caspase-5 substrate has yet been identified and because the requirement for an aspartic residue at the P1 position of substrates has been suggested to be absolute [23]. However, recent evidence indicates that caspase-9 and DRONC, a *Drosophila* caspase, show activity towards sites with a glutamic acid residue at P1 [34,35]. Little

is known about the requirements for this unexpected cleavage pattern. Our findings suggest that proper folding of Max is important for efficient hydrolysis by caspase-5 because partial denaturing of recombinant Max is sufficient to inhibit its cleavage by caspase-5 (Figure 4B). In support of this, peptides spanning the caspase-5 cleavage site of Max are not cleaved *in vitro* by caspase-5 (results not shown) and peptides with a glutamic acid residue at P1 are poor inhibitors of caspase 5 (Table 1). However, replacing this glutamic acid residue by an aspartic acid residue in this peptide resulted in a substrate that was efficiently processed by caspase-5 (Figure 5D). As expected, Maxp22-E10D was also cleaved efficiently both *in vivo* and *in vitro* by caspase-5 (results not shown). Taken together, these findings suggest that the specificity of at least some caspases might be more complex than implied previously by the studies with small peptides [29,36].

The regulation of the caspase-dependent cleavage of substrates seems to occur primarily at the level of caspases and their inhibitors. However, kinases have been identified as important regulators of apoptosis [17,37–39]. These kinases might affect various aspects of the apoptotic process, including the regulation of caspases and cleavage of their substrates. In addition, phosphatases have been implicated in the apoptotic process [40–42]. At the level of substrate cleavage, phosphorylation of presenilin-2 at serine residues adjacent to two caspase-3 cleavage sites has been shown to inhibit proteolysis *in vitro* [43]. Similarly, the cleavage of factor I $\kappa$ B- $\alpha$  by caspase-3 is also inhibited by phosphorylation at the P1' position *in vitro* [44]. However, it is not clear whether phosphorylation of the relevant sites is altered *in vivo* during apoptosis. Furthermore, p105Rb and the La autoantigen are dephosphorylated during apoptosis but it is unclear whether dephosphorylation is required for subsequent cleavage [45–47]. Our findings demonstrate that the caspase-dependent hydrolysis of Max is regulated by phosphorylation/dephosphorylation, both *in vivo* and *in vitro* (Figures 3 and 5), providing additional evidence for the importance of these post-translational modifications in regulating specific aspects of apoptosis.

Max is constitutively phosphorylated at multiple sites *in vivo*, all of which, including Ser-11, are modified *in vitro* by protein kinase CK2 [11,12]. CK2 is also the most likely kinase *in vivo* because no other nuclear kinase is known to phosphorylate acidic sites [33]. Cleavage of Max by caspase-5 at Glu-10 requires dephosphorylation at Ser-11 (Figure 5). Consistent with this observation is our finding that Max is dephosphorylated during apoptosis (Figures 3B, 3C and 5A). Taken together, our findings suggest that dephosphorylation of Max is a prerequisite for its N-terminal cleavage. Signal-dependent dephosphorylation of CK2 sites has not been reported previously and it will now be important to define the underlying mechanism. CK2 is a constitutively active kinase with more than 160 identified substrates [33,48]. Originally CK2 was described as a signal-regulated kinase, in particular by different growth factors; however, these findings have been controversial [33,49]. The activation of CK2 in response to stress signals has been reported recently. One study identified p38 mitogen-activated protein kinase, a previously identified stress-regulated kinase, as an activator of CK2 [50]. In another study a DNA damage-induced p53 C-terminal kinase complex, phosphorylating the previously identified *in vitro* CK2 site Ser-392, was purified and found to contain CK2 [51]. These studies indicate that CK2 is regulated during stress but provide no explanation for the apoptosis-associated dephosphorylation of Max. In addition, we did not observe any loss in overall CK2 kinase activity during apoptosis that might explain the dephosphorylation of Max (Figure 5E). Other explanations must therefore be considered. It has been suggested

that the regulation of subcellular distribution of CK2, which potentially affects the accessibility of the kinase to substrates, might be relevant to the modulation of CK2 function [52]. However, in preliminary studies no difference in the subcellular distribution of CK2 was observed when control cells were compared with apoptotic cells (G. Walsemann and B. Lüscher, unpublished work). It is nevertheless plausible that a decreased access of CK2 to Max would result in dephosphorylation. Alternatively, a phosphatase capable of dephosphorylating Max might be activated in response to Fas signalling. One candidate is PP2A, a phosphatase that is cleaved and activated during apoptosis in Jurkat cells and can dephosphorylate CK2 phosphorylated substrates *in vitro* [42,53]. However, treatment of cells with anti-Fas in combination with okadaic acid, an inhibitor of PP2A, did not prevent the dephosphorylation of Max (D. Brandt, A. Krippner-Heidenreich and B. Lüscher, unpublished work). It therefore remains to be determined which phosphatase is involved in dephosphorylating Max during apoptosis.

In view of the large number of reported CK2 substrates and the lack of distinct regulation of most CK2 phosphorylation sites [33], it will also be important to address the question of whether other CK2 substrates are dephosphorylated during apoptosis. In preliminary studies we have found that Nopp140, a CK2 substrate that has been suggested to be involved in nucleocytoplasmic transport [54,55], is also dephosphorylated (N. Herres, A. Krippner-Heidenreich and B. Lüscher, unpublished work). Thus Max might not be the only CK2 substrate that is dephosphorylated during apoptosis. This alteration seems more widespread because recent findings have demonstrated the dephosphorylation of several proteins in response to Fas signalling [24]. Taken together with the findings that during apoptosis several kinases and at least one phosphatase are processed, resulting in modified enzymic activities, this suggests that phosphorylation/dephosphorylation reactions are involved in the regulation of apoptosis.

We were interested to determine whether any of the Max mutants, either non-cleavable forms or deletion mutants, were able to modify the apoptotic response to anti-Fas and to tumour necrosis factor. No significant effects were found in different cell lines (results not shown). This raises the question of the relevance of Max cleavage during apoptosis, which at present we cannot answer. However, several aspects should be considered. One is that at least three distinct events target Max during apoptosis, i.e. dephosphorylation and cleavage by two most probably distinct caspases. We consider it improbable that this complex set of events is not specific. Furthermore, despite the large number of identified substrates [23], many proteins are not cleaved during apoptosis despite their possessing potential caspase cleavage sites, including c-Myc, c-Myb and the  $\alpha$  and  $\beta$  subunits of CK2, as shown here (Figure 1). This suggests a high degree of specificity in substrate recognition. In addition, only few caspase substrates have been shown to be directly involved in specific aspects of apoptosis [17,23]. Are the remaining substrates bystanders, cleaved by coincidence rather than as a result of necessity for executing the apoptotic program? This seems unlikely. Specific aspects of the apoptotic process are presumably covered by the cleavage of several substrates. Therefore affecting the cleavage of one substrate might not result in a significant alteration of the apoptotic process. Thus further work is required to unravel the relevance of Max cleavage, which might need a more detailed analysis of specific aspects of apoptosis.

Transcription has been demonstrated to affect apoptosis by controlling the expression of specific genes, including members of the Bcl-2 family or inhibitors of caspases [56–59]. However, under conditions in which cells undergo apoptosis rapidly and

efficiently, as in the model used here, regulatory mechanisms due to altered transcription are most probably not relevant. Nevertheless, increasing evidence supports the notion that gene transcription is necessary to fine-tune the balance between cell survival and cell death and that for this the caspase-mediated regulation of transcription factors is likely to be relevant [22,60]. Multiple Max isoforms are generated during apoptosis by reversible dephosphorylation and by irreversible cleavage that show altered DNA binding activities and possibly affect the function of the Myc/Max/Mad network and other factors that bind to Myc E boxes, including USF and TFE3 (Figures 1, 3 and 6). In this regard it is important to note that several network members have been associated with the apoptotic process. Myc proteins function as a switch in the decision-making process between life and death [4] and Mad proteins inhibit apoptosis induced by various stimuli [6–8]. Thus it is possible that through affecting Max function the activities of heterodimers with either Myc and/or Mad proteins, or other network members for that matter, might be regulated during apoptosis.

Our study provides a novel insight into the regulation of the central component, Max, of the Myc/Max/Mad network during apoptosis. Although the function of Max cleavage remains to be explained more fully, our findings suggest that the modulation of the Myc/Max/Mad network via Max is an early event during apoptosis. Whereas the partners of Max, Myc and Mad proteins, are highly regulated, little evidence for alterations in Max function has previously been obtained. We have uncovered a complex series of events that target Max, including its dephosphorylation and its cleavage by at least two distinct caspases. Future studies will also have to address the role of modified Max forms in combination with Myc and Mad proteins during apoptosis.

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