Effect of caspase cleavage-site phosphorylation on proteolysis

József TÖZSÉR^{*1}, Péter BAGOSS^{I*}, Gábor ZAHUCZKY^{*}, Suzanne I. SPECHT[†], Eva MAJEROVA[‡] and Terry D. COPELAND[†]

^{*}Department of Biochemistry and Molecular Biology, Faculty of Medicine, Debrecen University, H-4012 Debrecen, Hungary, [†]Basic Research Laboratory, Center for Cancer Research, National Cancer Institute (NCI) at Frederick, Frederick, MD 21701, U.S.A., and [‡]SAIC-Frederick, Frederick, MD 21701, U.S.A.

Caspases are important mediators of apoptotic cell death. Several cellular protein substrates of caspases contain potential phosphorylation site(s) at the cleavage-site region, and some of these sites have been verified to be phosphorylated. Since phosphorylation may affect substantially the substrate susceptibility towards proteolysis, phosphorylated, non-phosphorylated and substituted oligopeptides representing such cleavage sites were studied as substrates of apoptotic caspases 3, 7 and 8. Peptides containing phosphorylated serine residues at P4 and P1' positions were found to be substantially less susceptible towards proteolysis as compared with the serine-containing analogues, while phosphoserine at P3 did not have a substantial effect. P1 serine as well as P1-phosphorylated, serine-containing analogues

of an oligopeptide representing the poly(ADP-ribose) polymerase cleavage site of caspase-3 were not hydrolysed by any of these enzymes, whereas the P1 aspartate-containing peptides were efficiently hydrolysed. These findings were interpreted with the aid of molecular modelling. Our results suggest that cleavage-site phosphorylation in certain positions could be disadvantageous or detrimental with respect to cleavability by caspases. Cleavage-site phosphorylation may therefore provide a regulatory mechanism to protect substrates from caspase-mediated degradation.

Key words: caspase-3, caspase-7, caspase-8, cleavage site, oligopeptide, substrate specificity.

INTRODUCTION

Proteolysis (an irreversible process) and protein phosphorylation (a reversible covalent modification) are involved in fundamental regulatory mechanisms, including apoptosis, cell-cycle regulation and signal transduction. These post-translational modifications are usually studied separately; only a few studies to date have demonstrated that these two crucial processes may act in a concerted way. Although phosphorylation (or dephosphorylation) at a site outside of a region at which a protease binds directly could alter the protein conformation and might change the accessibility of a cleavage site for a protease, direct phosphorylation at a protease-recognition site might also alter substantially the susceptibility of the site towards proteolysis. Such an effect might provide a direct regulatory tool for proteolytic processing. Substrates containing phosphorylated serine residues adjacent to proline have been shown to decrease the susceptibility to cleavage by proline-specific peptidases [1]. Phosphorylation of vitronectin by protein kinase C at a P1' serine residue (nomenclature of substrate residues and substrate-binding subsites conforms to that described in [2]) also attenuated cleavage by plasmin, and this was proposed to have a physiological regulatory effect [3]. We have previously demonstrated that cleavage of peptides representing retroviral proteinase substrates, and vimentin processing by HIV-1 and avian retroviral proteinases, were also affected by cleavage-site phosphorylation [4]. That study also proved that phosphorylated oligopeptides might serve as excellent tools with which to study the effect of phosphorylation on proteolysis [4].

Apoptosis (or programmed cell death) is also mediated by proteolysis. Caspases are important for the progression of apoptotic cell death [5,6]. These enzymes are activated by specific proteolysis, which occurs either auto-catalytically or by other members of the caspase superfamily [7]. An activated caspase is then able to cleave a number of proteins [8]. Substrate-specificity studies of various caspases have implicated the residue at P1

as being a major determinant of specificity, as well as the critical residue at P4 [9]. On the basis of these specificity studies, caspases can be classified into three specificity groups [8]. Group I caspases (caspases 1, 4, 5 and 13) are tolerant at S4, but prefer bulky hydrophobic amino acid residues. Group II caspases (caspases 2, 3 and 7) are substantially more stringent at S4, requiring an aspartate at P4. Group III caspases (caspases 6, 8, 9 and 10), on the other hand, prefer branched-chain aliphatic amino acids at P4 [8].

Several caspase-cleavage sites contain potential or verified serine/threonine phosphorylation sites, as listed in Table 1. In the present study, we have examined how phosphorylation of peptides representing known caspase-cleavage sites alters their cleavability by apoptotic effector caspases 3 and 7, and the initiator caspase-8. We have investigated how P3 serine and P1' serine phosphorylation of a peptide representing a caspase-cleavage site in presenilin-2 (PS-2) could alter its processing, and how P4 serine phosphorylation influences the cleavability of a peptide representing another caspase substrate, Gas-2 (growth arrest-specific protein-2). Finally, we have tested whether substitution of the P1 aspartate with phosphoserine, which is also negatively charged, might turn a non-hydrolysable peptide into a caspase substrate. Our results suggest that, except at position P3, a phosphoserine residue is either poorly or not at all tolerated at these positions for caspase substrates. Therefore phosphorylation at these sites could directly attenuate or block cleavage by caspases. Molecular models for these substrates were constructed and used to interpret the results of kinetic studies.

EXPERIMENTAL

Caspases 3, 7 and 8

Human recombinant purified caspases 3, 7 and 8 were obtained from Biomol Research Laboratories (Plymouth Meeting,

Abbreviations used: AD, Alzheimer's disease; Gas-2, growth arrest-specific protein 2; PARP, poly(ADP-ribose) polymerase; PS-1/2, presenilin-1 and -2 respectively; CTF, C-terminal fragment; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (e-mail tozser@indi.biochem.dote.hu).

Table 1 Substrate sequences of caspases containing determined or potential serine/threonine phosphorylation sites

Arrows indicate the sites of cleavages. Serine or threonine residues that have been proved to be phosphorylation sites (S^P and T^P respectively) are shown in **bold**. The question mark (?) for Gas-2 indicates that the phosphorylation kinase is at present unknown. Erk1/2, extracellular-signal-regulated kinases 1 and 2 respectively; $I\kappa B-\alpha$, inhibitory $\kappa B-\alpha$; MEKK1, mitogen-activated protein kinase/Erk kinase 1; MST1, mammalian STE20-like kinase 1; PKA, protein kinase A; PKC, protein kinase C; SREBP, sterol-regulatory-element-binding protein.

Protein	Sequence	Phosphorylation kinase
PS-1	A-Q-R-D↓ S^P -H-L-G*	PKA
PS-2	M-E-E-D↓ S^P -Y-D↓ S^P -F-G-E-P†	CK1, CK2
Gas-2	S^P -R-V-D↓G-K-T-S‡	?
SREBP-1	S-E-P-D↓ S^P -P-V-F§	Erk1/2
SREBP-2	D-E-P-D↓ S^P -P-P-V§	Erk1/2
$I\kappa B-\alpha$	D-R-H-D↓ S^P -G-L-D	MEKK1
MST1	D-E-M-D↓ S^P -G-T-M¶	MST1
p21(Waf1/Cip1)	S^P -M-T-D↓F-Y-H-S**	PKC
Bid	L-Q- T ^P -D↓G- S ^P -Q-A††	CK1, CK2

* Cleavage occurs after Asp³⁴⁵ by an unidentified caspase [17]. The site contains a PKA and PKC consensus sequence, and PS-1 can be phosphorylated by these kinases [38], although the actual phosphorylation sites have not been determined yet.

† Cleavage occurs at Asp³²⁶ and Asp³²⁹ (the major site) by caspase-3 [16]. Ser³²⁷ and Ser³³⁰ were shown to be phosphorylated by casein kinases [18,39].

‡ Cleavage occurs at Asp²⁷⁹ [29].

§ Cleavage occurs at Asp⁴⁰ and Asp⁴⁶⁸ by caspase-3 in SREBP-1 and SREBP-2 respectively [31]. The cleavage sites are in mitogen-activated protein kinase consensus phosphorylation sites, and these proteins are substrates of Erks 1 and 2 [40].

|| Cleavage occurs after Asp³¹ by caspase-3 [20], whereas Ser³² was shown to be phosphorylated by MEKK1 [21].

¶ Cleavage occurs at Asp³²⁶, and could be mediated by several caspases, and autophosphorylation at Ser³²⁷ attenuated caspase-mediated processing [23].

** Cleavage occurs at Asp¹⁴⁹ by caspase-3 [41], whereas Ser¹⁴⁶ was shown to be phosphorylated by Scott et al. [42].

†† Phosphorylation of Bid with CK1 and CK2 inhibits its cleavage by caspase-8 [37].

PA, U.S.A.). Alternatively, the enzymes were expressed in *Escherichia coli* and then isolated in the fully active form, as described previously [10]. Active site titrations for these enzyme preparations were performed using acetyl-Asp-Glu-Val-Asp-CHO (Calbiochem), a potent inhibitor of these caspases, as described previously [10].

Oligopeptides

Oligopeptides were synthesized by the solid-phase method, using the fluoren-9-ylmethoxycarbonyl ('Fmoc') group for α -amino group protection and acid-labile groups for side chain protection of trifunctional amino acids on a model 430A automated peptide synthesizer (Applied Biosystems, Inc.). Phosphorylated peptides were synthesized as described previously [4]. The crude phosphopeptides were purified by reversed-phase HPLC on a Vydac C₁₈ column (25 cm × 2.5 cm) using a water/acetonitrile gradient. All compounds yielded the correct molecular peak upon ion-spray mass spectrometry. Amino acid composition of the peptides and their proteolytically produced fragments was determined with a Beckman 6300 amino acid analyser. Stock solutions and dilutions were made in distilled water, and the peptide concentrations were determined by amino acid analysis.

Enzyme assay

The proteinase assays were performed in 20 μ l (final vol.) of 50 mM Hepes, pH 7.4, containing 100 mM NaCl, 0.1 % CHAPS,

1 mM EDTA, 10 mM dithiothreitol and 10 % (v/v) glycerol for final substrate concentrations of 0.01–6.0 mM. The substrate concentration range was selected depending on the approximate K_m values. For some peptides, the k_{cat}/K_m values were determined from the linear part of the 'rate-versus-concentration' profile. The reaction mixture was incubated at 37 °C for 1 h, the reaction was stopped by the addition of 180 μ l of 1 % (w/v) trifluoroacetic acid (TFA), and an aliquot was then injected on to a Nova-Pak C₁₈ reversed-phase chromatography column (3.9 mm × 150 mm; Waters Associates, Inc., Milford, MA, U.S.A.) using an automatic injector. Substrates and cleavage products were separated using an increasing water/acetonitrile gradient (0–100 %) in the presence of 0.05 % TFA. The cleavage products of proteinase-catalysed hydrolysis were then identified by amino acid analysis. Kinetic parameters were determined by fitting the data obtained at less than 20 % substrate hydrolysis to the Michaelis–Menten equation (or by linear fitting of k_{cat}/K_m values determined under pseudo-first-order conditions) by using the FigP program (Fig.P Software Corp., Durham, NC, U.S.A.).

Molecular modelling

Caspase-3 with a model of the PS-2 substrate (peptide DSYDS) was built from a human caspase-3–inhibitor crystal structure ([11]; PDB accession code 1CP3) by exchanging residues of the inhibitor with residues of the substrate using the Sybyl 6.7 software package (Tripos Inc., St Louis, MO, U.S.A.). Each of the side chain torsion angles for substituted residues in the peptide substrate was then rotated through 360° in steps of 15° to search for alternate conformations, using a Kollman all-atom force-field and an 8 Å cut-off (1 Å = 0.1 nm). Next, 20 Powell iterations were applied for the whole enzyme–substrate complex after finding the lowest energy conformers of the modified residues. The root-mean-square (RMS) deviation between the C α atoms of the final enzyme model and the C α atoms of the initial crystal structure was found to be 0.13 Å. The structures were built, minimized and examined on Silicon Graphics Indigo2 or O2 computer graphics systems. For a structural comparison, a caspase-7–inhibitor structure ([12]; PDB accession code 1F1J) and a caspase-8–inhibitor structure ([13]; PDB accession code 1F9E) were used. Stereo figures of the enzyme–substrate complex were generated using MolScript version 2.1 [14].

RESULTS AND DISCUSSION

Cleavage of oligopeptides representing a PS-2 cleavage site by caspases 3, 7 and 8

Mutations within the two homologous PS genes (for PS-1 and PS-2) are frequent causes of early-onset familial Alzheimer's disease (AD), leading to an increase in the production of an abnormal amyloid β -peptide, which has a central role in AD pathology [15]. PS proteins are membrane proteins, which are endoproteolytically processed within a large cytoplasmic loop, generating the biologically and pathologically active C-terminal fragment (CTF) and an N-terminal fragment. PS proteins are involved in apoptotic cell death, and are substrates of proteases of the caspase-3 family [16]. Both PS-1 and PS-2 are processed by a caspase downstream of the conventional cleavage site, resulting in shortened CTFs. Whereas PS-1 is cleaved by an unidentified type of caspase after Asp³⁴⁵ [17], during apoptosis PS-2 is cleaved by caspase-3 between Asp³²⁹ and Ser³³⁰ and, to a minor extent, between Asp³²⁶ and Ser³²⁷, *in vivo* ([16] and Table 1). A previous study [18] has demonstrated that both Ser³²⁷ and Ser³³⁰ of PS-2

Table 2 Caspase-3-mediated cleavage of oligopeptides representing non-phosphorylated, phosphorylated or mutated cleavage sites in proteins

For the substrate sequences, phosphorylated serine residues (S^P) and mutated residues are shown in bold. In cases where a linear function was identified for the concentration versus the rate, we were not able to determine the individual K_m and k_{cat} values. These cases are shown by 'N.D.' in the Table.

No./protein	Substrate sequence	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
1. PS-2	E-D-S-Y-D↓S-F-G-E-P	N.D.	N.D.	$0.41 \pm 0.02^*$
2. PS-2	E-D- S^P -Y-D↓S-F-G-E-P	N.D.	N.D.	$0.41 \pm 0.03^\dagger$
3. PS-2	E-D-S-Y-D↓ S^P -F-G-E-P		Not hydrolysed‡	
4. Gas-2	I-S-R-V-D↓G-K-T-S-P	N.D.	N.D.	$3.7 \pm 0.3§$
5. Gas-2	I- S^P -R-V-D↓G-K-T-S-P	N.D.	N.D.	$0.10 \pm 0.01§$
6. Gas-2	I-D-R-V-D↓G-K-T-S-P	0.221 ± 0.008	40.1 ± 0.5	181.5 ± 7.0
7. PARP	G-D-E-V-D↓G-V-D-E-V	0.056 ± 0.010	2.6 ± 0.1	46.8 ± 8.7
8. PARP	G-D-E-V- S ↓G-V-D-E-V		Not hydrolysed	
9. PARP	G-D-E-V- S^P ↓G-V-D-E-V		Not hydrolysed	

* In the studied concentration range (0–5.0 mM), the [S] versus v curve was linear, and was used to determine the k_{cat}/K_m value. The K_m for this substrate is expected to be higher than 2.0 mM.

† In the 0–0.2 mM concentration range, the [S] versus v curve was linear, and was used to determine the k_{cat}/K_m value. At higher substrate concentrations an inhibitory effect was observed.

‡ Small degree of hydrolysis was observed during a 16 h incubation with the enzyme (50 nM) at a substrate concentration of 0.2 mM.

§ In the studied concentration range (0–2.0 mM), the [S] versus v curve was linear, and was used to determine the k_{cat}/K_m value. The K_m for this substrate is expected to be higher than 2.0 mM.

|| Not hydrolysed when substrate (0.2 mM) was incubated with 50 nM enzyme for 16 h. Based on the detection limit of the assay, it is estimated that the k_{cat} value is $< 0.01 s^{-1}$.

Table 3 Caspase-7- and caspase-8-mediated cleavage of oligopeptides representing non-phosphorylated, phosphorylated or mutated cleavage sites in proteins

For the substrate sequences, mutated and phosphorylated serine residues (S^P) are shown in bold.

Caspase-7			Caspase-8	
No./protein	Substrate sequence	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	Substrate sequence	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
1. PS-2	E-D-S-Y-D↓S-F-G-E-P	$0.06 \pm 0.01^*$	E-L-S-Y-D↓S-F-G-E-P	0.003 ± 0.0006
2. PS-2	E-D- S^P -Y-D↓S-F-G-E-P	$0.06 \pm 0.01^*$	E-L- S^P -Y-D↓S-F-G-E-P	$0.002 \pm 0.0001^*$
3. PS-2	E-D-S-Y-D↓ S^P -F-G-E-P	Not hydrolysed†	E-L-S-Y-D↓ S^P -F-G-E-P	Not hydrolysed†
4. Gas-2	I-S-R-V-D↓G-K-T-S-P	$1.15 \pm 0.07^*$		Not hydrolysed†
5. Gas-2	I- S^P -R-V-D↓G-K-T-S-P	$0.23 \pm 0.01^\ddagger$		Not hydrolysed†
6. Gas-2	I-D-R-V-D↓G-K-T-S-P	6.84 ± 0.95		Not hydrolysed†
7. PARP	G-D-E-V-D↓G-V-D-E-V	$11.1 \pm 0.5^*$	G-L-E-V-D↓G-V-D-E-V	$0.34 \pm 0.05§$
8. PARP	G-D-E-V- S ↓G-V-D-E-V	Not hydrolysed†	G-L-E-V- S ↓G-V-D-E-V	Not hydrolysed†
9. PARP	G-D-E-V- S^P ↓G-V-D-E-V	Not hydrolysed†	G-L-E-V- S^P ↓G-V-D-E-V	Not hydrolysed†

* In the studied concentration range the [S] versus v curve was linear for the hydrolysed substrates, and was used to determine the k_{cat}/K_m value.

† Not hydrolysed when substrate (0.2 mM) was incubated with 1 μ M caspase-7 or 10 μ M caspase-8 for 16 h.

‡ In the 0–0.2 mM concentration range, the [S] versus v curve was linear, and was used to determine the k_{cat}/K_m value. At higher substrate concentrations, an inhibitory effect was observed.

§ The P4 aspartate-containing original peptide was also a substrate of caspase-8, with a k_{cat}/K_m value of $0.16 \pm 0.01 mM^{-1} \cdot s^{-1}$.

at the caspase-cleavage sites are targets of phosphorylation: phosphorylation both inhibited caspase-mediated cleavage and enhanced the anti-apoptotic properties of the protein in cell culture. Interestingly, the cleavage site in PS-1 also contains serine at P1', in a potential phosphorylation consensus sequence (Table 1). Since phosphorylation of PS-2 was mimicked by a serine-to-aspartate mutation [18], no direct correlation between individual-site phosphorylation and cleavability was provided. Furthermore, the effect of phosphorylation at those residues might be a conformational change, instead of a direct specificity change.

To provide direct evidence for the effect of phosphorylation at the PS-2 sites on susceptibility towards proteolysis by caspase-3, oligopeptides with or without phosphorylated serine residues, which represented these cleavage sites in PS-2, were assayed as substrates of caspase-3 (Table 2). The non-phosphorylated peptide was hydrolysed correctly by the enzyme, as determined by amino acid analysis of peptide products collected by reversed-phase

HPLC separation (shown in Figure 1, upper panel). However, this substrate was not cleaved at the other aspartate residue, which is the minor site determined in the protein. The two residues preceding the aspartate in the N-terminal part of the peptide may not provide a sufficient length for cleavage [9]; furthermore, the inherent cleavage rate is also substantially lower at this site of the protein [16]. The kinetic parameters were therefore also determined for this peptide (Table 2). It should be noted that the predicted K_m value for this substrate was substantially higher than the K_m for the peptide representing the poly(ADP-ribose) polymerase (PARP) cleavage site (peptide 7 in Table 2), one of the most studied sites of caspase-3. We also tested the P3 serine- and P1' serine-phosphorylated analogues of this peptide, but although the P3 serine-phosphorylated peptide was cleaved with a very similar specificity constant, the P1' serine-phosphorylated peptide was not cleaved by the proteinase (Table 2). Interestingly, whereas the cleavage rate of the non-phosphorylated substrate exhibited a linear function with the substrate concentration up to 5 mM

(Figure 1, lower panel), an increase in the concentration of the P3-phosphorylated peptide above 0.2 mM resulted in a decrease in the cleavage rate (results not shown). This may be due to an alternative, non-productive binding of the phosphorylated peptide.

The oligopeptides representing the phosphorylated and non-phosphorylated PS-2 cleavage sites were also tested as substrates of caspases 7 and 8. Similar to the results obtained with caspase-3, caspase-7 hydrolysed both the non-phosphorylated and the P3-phosphorylated substrates with the same specificity constant, and did not hydrolyse the P1' serine-phosphorylated one (Table 3). Caspase-8 did not cleave any of these peptides (results not shown). Since caspase-8 prefers branched-chain aliphatic amino acids, especially a leucine residue at P4 instead of an aspartate [19], we also tested P4 leucine-substituted analogues of these peptides to examine the sensitivity of caspase-8 for cleavage-site phosphorylation at positions P3 and P1'. Although the specificity constants for the hydrolysed peptides were very low, the results are in good agreement with those for the two other caspases: the enzyme was sensitive to P1' phosphorylation, but not to P3 phosphorylation (Table 3).

The inability of caspases 3, 7 and 8 to cleave the P1'-phosphorylated substrates is in line with the findings of a previous specificity study [19], which showed that negatively charged residues, such as glutamate or aspartate, at P1' are very unfavourable for caspase-mediated proteolysis. It has been reported that phosphorylation of inhibitory κ B- α (' κ B- α ') inhibited its cleavage by a caspase *in vitro* [20], and the P1' serine of the caspase-cleavage site (see Table 1) was reported to be a phosphorylation site for mitogen-activated protein kinase/extracellular-signal-regulated kinase 1 ('MEKK1') [21], also suggesting that P1' serine phosphorylation might regulate caspase-mediated proteolysis. Recent studies have suggested that cleavage of the transcription factor Max by caspase-5 during apoptosis is also regulated by P1' serine phosphorylation [22], and one of the caspase-mediated cleavage events required for the nuclear localization of the kinase domain of mammalian STE20-like kinase 1 ('MST1') is also blocked by P1' serine phosphorylation [23,24]. The lack of an effect of P3 serine phosphorylation is in line with the results of previous studies, which suggested that various side chains are accepted at P3, with the negatively charged residue glutamate (which is almost isosteric with phosphoserine) at this position being one of the best for caspases 3 and 7 [9]. Although we did not test the effect of phosphorylation of Ser³²⁷ at the minor cleavage site, since it is in position P1' for that cleavage process, it would be expected to completely prevent cleavage at that site.

It should be noted that the specificity constant for the unmodified PS-2 substrate was much higher for caspase-3 than for caspase-7, which may be, at least partly, due to its much better tolerance of tyrosine at P2 [9]. Furthermore, caspase-3 is a much more efficient enzyme with respect to small synthetic substrates than is caspase-7 [19], which may also contribute substantially to the observed effects. These results are in line with the observation that caspase-3 might be the enzyme cleaving PS-2 *in vivo* [16].

The molecular model of the PS-2 substrate (peptide 1 in Table 2) binding to the caspase-3 was built on the basis of an enzyme-inhibitor crystal structure (Figure 2). Side chains of the P4 aspartate and P1 aspartate residues anchor the substrate to the enzyme via several hydrogen-bonds, in addition to the conserved hydrogen-bond network formed between the main chain atoms of the substrate and the enzyme. Side chains of P3 and P2 residues point approximately outward, explaining the rather tolerant nature of the respective subsites, although a preference for a glutamate residue at P3 and a valine/leucine/proline/threonine residue at P2 has been demonstrated for

Figure 1 Cleavage of an oligopeptide representing the PS-2 cleavage site by caspase-3

Upper panel: reversed-phase HPLC chromatogram of peptide 1 of Table 2 is shown after cleavage by caspase-3. P1, product peak EDSYD; P2, product peak SFGEF; S, substrate peak EDSYDSFGEF. Lower panel: shown is a plot of the velocity measured against the substrate concentration.

caspases [8,19]. To date, there is no P1' amino acid residue in any of the crystal structures deposited into the Protein Data Bank, and so therefore the position of P1' serine of the PS-2 substrate was predicted on the basis of a conformational search. The P1' residue may adopt a β -sheet-like conformation, as seen for the P1' residue of various other proteinases, including serine proteinases [25] and aspartic proteinases [26]; however, the flexibility of the backbone at this position may be crucial for efficient catalysis. A preference for serine and glycine residues at P1' has been demonstrated for caspases [8,19]. Less efficient

Figure 2 Stereo-view of the model of the PS-2 substrate in the active site of caspase-3

The P4–P1' residues of the peptide 1 (Table 2) are modelled into the substrate-binding region of the enzyme shown in blue. The substrate is coloured green, whereas hydrogen-bonds between the enzyme and substrate are indicated by broken red lines.

hydrolysis of substrates with large P1' side chains [19] could be the result of steric hindrance between the P1' side chain and the side chains of Thr¹⁶⁶ and Tyr²⁰⁴ of caspase-3 and the usually hydrophobic P2 side chains. Negatively charged amino acids were also found to be very unfavourable at this position due the electrostatic repulsion of Glu¹²³ in caspase-3 [19]. Caspase-7 also contains a glutamate residue in the equivalent position, whereas caspase-8 contains an aspartate. Phosphorylation of the P1' serine residue leads to an increase in size and an addition of negative charge, and the accumulation of these unfavourable changes could lead to an uncleavable peptide. Alternatively, introduction of negative charge at P1' may also have an unfavourable effect on the catalytic machinery of the enzyme, although the side chain of P1' is pointing away from the residues of the catalytic triad, and is not expected to directly interact with them.

Effect of P4 serine phosphorylation of a caspase substrate on its susceptibility toward cleavage

An important part of the $G_0 \rightarrow G_1$ transition of the cell cycle is the down-regulation of the expression of a set of *gas* (growth-arrest-specific) genes [27]. Gas-2 is a protein of the microfilament system, and is also involved in the regulation of apoptosis [28]. Gas-2 was found to be a substrate of caspase-3 [29]; furthermore, its function is regulated by phosphorylation at serine residues [30]. Although the consensus sequence for caspase-3 cleavage sites is Asp-Xaa-Xaa-Asp↓ (where the arrow denotes the site of cleavage, and 'Xaa' denotes 'any amino acid'), the cleavage site in Gas-2 contains a P4 serine residue, instead of an aspartate, in a potential phosphorylation site. A serine residue in position P4 is also present in the caspase-3 substrate, sterol-regulatory-element-binding protein ('SREBP-1') [31], and in p21-activated kinase 2 ('PAK-2') [32]. We have tested by using oligopeptides the effect of P4 serine phosphorylation on this substrate. Whereas the non-phosphorylated peptide was an acceptable substrate of caspase-3, with a high K_m value, phosphorylation of the P4 serine residue decreased the specificity constant approx. 40-fold, suggesting that, unlike aspartate, phosphoserine (which is also negatively charged) is very unfavourable in this position (even more unfavourable than the non-charged serine residue; Table 2). When the P4 residue was substituted with aspartate, much more

favourable kinetics were obtained. The values of the catalytic constant (k_{cat}) and the specificity constant (k_{cat}/K_m) for this peptide (peptide no. 6 in Table 2) were substantially better than those obtained with the PARP cleavage-site peptide.

On the basis of the enzyme–inhibitor structures, the P4 aspartate forms strong hydrogen-bond interactions with both the side chain of Trp²¹⁴ and the amide nitrogen of Phe²⁵⁰, and a weaker interaction is also possible with the side chain of Asn²⁰⁸ (Figure 2 and [11]). These interactions appear to anchor the substrate, and cannot be formed with smaller (serine) or bigger (phosphoserine) residues. In a detailed specificity study of caspase-3, preference for aspartate at P4 was found to be almost absolute: even peptides containing charge-conserving (glutamate) and isosteric (asparagine) substitutions resulted in poor substrates [9]. Our results suggest that a phosphoserine residue is also very unfavourable for caspase-3 at this position. As an alternative of the direct effect of the S4–P4 interaction, the unfavourable nature of the negatively charged residues at P4 might result from altered internal substrate side chain interactions. Such substrate side chain co-operativity resulting in a substrate sequence context-dependent specificity has been observed for various proteases, including papain [33] and HIV protease [34].

The oligopeptide representing the non-phosphorylated Gas-2 substrate was also cleaved by caspase-7, whereas the P4 phosphorylated analogue was hydrolysed with a substantially lower rate, indicating that caspase-7 is also sensitive to P4 substrate phosphorylation, like caspase-3. None of these peptides were cleaved by caspase-8 (Table 3).

Substitution of the P1 aspartate residue with phosphoserine prevents cleavage by caspases 3, 7 and 8

Since all caspases cleave substrates at the negatively charged aspartate residue, we tested whether substitution of P1 aspartate in a consensus caspase-3 cleavage site sequence representing the cleavage site in PARP could provide a substrate for the enzyme. The unmodified substrate was hydrolysed efficiently by caspase-3 (Table 2) and caspase-7 (Table 3), whereas, in contrast, it was a poor substrate for caspase-8, although a substitution by leucine at P4 in this sequence appreciably improved the specificity constant (Table 3). The peptides with the P1 phosphoserine residue were

not hydrolysed by the caspases, an observation similar to that of peptides with P1 serine (Table 2 and Table 3). The P1 aspartate residue of the substrate is anchored by interactions with the side chain guanidino groups of Arg⁶⁴ and Arg²⁰⁷, as well as with the side chain of Gln¹⁶¹ in caspase-3 (Figure 2 and [11]). These residues are conserved in other caspases, including caspases 7 and 8 [12,13]. Owing to this complex hydrogen-bond/salt-bridge network, neither smaller nor larger charged residues than aspartate can form similarly strong interactions.

Conclusions

Caspases are important mediators of cell death and inflammation [8]. Several of the caspase cleavage-site substrates contain potential or verified phosphorylation sites (Table 1). Since phosphorylation at these sites may prevent or promote proteolysis, this may provide an additional regulatory tool. In several studies, introduction of aspartate residues is used to mimic phosphorylated serine residues for the functional analysis of phosphoproteins [18,35,36]. In terms of caspase specificity, our studies demonstrate that aspartate and phosphoserine residues are non-equivalent: whereas aspartate is exclusively required at the P1 position of the substrates for caspases 3, 7 and 8, and is also important at the P4 position of the substrates for caspases 3 and 7, phosphoserine at the same positions diminished or completely prevented the susceptibility towards cleavage. Therefore phosphorylation at these sites could provide a regulatory mechanism to protect substrates from caspase-mediated degradation. Phosphorylation at other sites, such as P2 and P3', may also protect from caspase-mediated cleavage, as found for the cleavage of Bid by caspase-8 [37], and therefore direct cleavage-site phosphorylation at proper positions may provide a general regulatory system to control caspase-mediated proteolysis.

We thank Professor Guy Salvesen (The Burnham Institute, La Jolla, CA, U.S.A.) for providing us with the caspase clones, János Kádas (of our Department) for help in caspase-3 purification, Szilvia Peto (also of our Department) for technical assistance, and Dr Terry Sumpter and Dr Tad Guszczynski (at NCI at Frederick, Frederick, MD, U.S.A.) for MS analyses. Research was sponsored in part by the Hungarian Science and Research Fund (T 30092) and also by the National Cancer Institute and the Department of Health and Human Services (DHHS), under contract with ABL. The contents of this publication do not necessarily reflect the views or policies of the DHHS, nor does the mention of trade names, commercial products or organizations imply an endorsement by the U.S.A. Government.

REFERENCES

- Kaspari, A., Diefenthal, T., Grosche, G., Schierhorn, A. and Demuth, H. U. (1996) Substrates containing phosphorylated residues adjacent to proline decrease the cleavage by proline-specific peptidases. *Biochim. Biophys. Acta* **1293**, 147–153.
- Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. I. Papain. *Biochem. Biophys. Res. Commun.* **27**, 157–162.
- Gechtman, Z. and Shaltiel, S. (1997) Phosphorylation of vitronectin on Ser362 by protein kinase C attenuates its cleavage by plasmin. *Eur. J. Biochem.* **243**, 493–501.
- Tözsér, J., Bagossi, P., Boross, P., Louis, J. M., Majerova, E., Oroszlan, S. and Copeland, T. D. (1999) Effect of serine and tyrosine phosphorylation on retroviral proteinase substrates. *Eur. J. Biochem.* **265**, 423–429.
- Thornberry, N. A. and Lazebnik, Y. (1998) Caspases: enemies within. *Science* **281**, 1312–1316.
- Thornberry, N. A. (1999) Caspases: a decade of death research. *Cell Death Differ.* **6**, 1023–1027.
- Slee, E. A., Adrain, C. and Martin, S. J. (1999) Serial killers: ordering caspase activation events in apoptosis. *Cell Death Differ.* **6**, 1067–1074.
- Nicholson, D. W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ.* **6**, 1028–1042.
- Talanian, R. V., Quinlan, C., Trautz, S., Hackett, M. C., Mankovich, J. A., Banach, D., Ghayur, T., Brady, K. D. and Wong, W. W. (1997) Substrate specificities of caspase family proteases. *J. Biol. Chem.* **272**, 9677–9682.
- Stennicke, H. R. and Salvesen, G. S. (1999) Caspases: preparation and characterization. *Methods* **17**, 313–319.
- Mittl, P. R., Di Marco, S., Krebs, J. F., Bai, X., Karanewsky, D. S., Priestle, J. P., Tomaselli, K. J. and Grütter, M. G. (1997) Structure of recombinant human CPP32 in complex with the tetrapeptide acetyl-Asp-Val-Ala-Asp fluoromethyl ketone. *J. Biol. Chem.* **272**, 6539–6547.
- Wei, Y., Fox, T., Chambers, S. P., Sintchak, J., Coll, J. T., Golec, J. M., Swenson, L., Wilson, K. P. and Charifson, P. S. (2000) The structures of caspases-1, -3, -7 and -8 reveal the basis for substrate and inhibitor selectivity. *Chem. Biol.* **7**, 423–432.
- Blanchard, H., Donepudi, M., Tschopp, M., Kodandapani, L., Wu, J. C. and Grütter, M. G. (2000) Caspase-8 specificity probed at subsite S(4): crystal structure of the caspase-8-Z-DEVD-cho complex. *J. Mol. Biol.* **302**, 9–16.
- Kraulis, P. J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* **24**, 946–950.
- Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. D., Hardy, J., Hutton, M., Kull, W. et al. (1996) Secreted amyloid β -protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. *Nat. Med.* **2**, 864–870.
- Kim, T. W., Pettingell, W. H., Jung, Y. K., Kovacs, D. M. and Tanzi, R. E. (1997) Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. *Science* **277**, 373–376.
- Grünberg, J., Walter, J., Loetscher, H., Deuschle, U., Jacobsen, H. and Haass, C. (1998) Alzheimer's disease associated presenilin-1 holoprotein and its 18–20 kDa C-terminal fragment are death substrates for proteases of the caspase family. *Biochemistry* **37**, 2263–2270.
- Walter, J., Schindzielorz, A., Grünberg, J. and Haass, C. (1999) Phosphorylation of presenilin-2 regulates its cleavage by caspases and retards progression of apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1391–1396.
- Stennicke, H. R., Renatus, M., Meldal, M. and Salvesen, G. S. (2000) Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8. *Biochem. J.* **350**, 563–578.
- Barkett, M., Xue, D., Horvitz, H. R. and Gilmore, T. D. (1997) Phosphorylation of I κ B- α inhibits its cleavage by caspase CPP32 *in vitro*. *J. Biol. Chem.* **272**, 29419–29422.
- Lee, F. S., Peters, R. T., Dang, L. C. and Maniatis, T. (1998) MEK1 activates both I κ B kinase α and I κ B kinase β . *Proc. Natl. Acad. Sci. U.S.A.* **95**, 9319–9324.
- Krippner-Heidenreich, A., Talanian, R. V., Sekul, R., Kraft, R., Thole, H., Ottleben, H. and Lüscher, B. (2001) Targeting of the transcription factor Max during apoptosis: phosphorylation-regulated cleavage by caspase-5 at an unusual glutamic residue in position P1. *Biochem. J.* **358**, 705–715.
- Graves, J. D., Draves, K. E., Gotoh, Y., Krebs, E. G. and Clark, E. A. (2001) Both phosphorylation and caspase-mediated cleavage contribute to regulation of the Ste20-like protein kinase Mst1 during CD95/Fas-induced apoptosis. *J. Biol. Chem.* **276**, 14909–14915.
- Ura, S., Masuyama, N., Graves, J. D. and Gotoh, Y. (2001) Caspase cleavage of MST1 promotes nuclear translocation and chromatin condensation. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10148–10153.
- Bone, R., Sampson, N. S., Bartlett, P. A. and Agard, D. A. (1991) Crystal structures of α -lytic protease complexes with irreversibly bound phosphonate esters. *Biochemistry* **30**, 2263–2272.
- Davies, D. R. (1990) The structure and function of the aspartic proteinases. *Annu. Rev. Biophys. Biophys. Chem.* **19**, 189–215.
- Schneider, C., King, R. M. and Philipson, L. (1988) Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**, 787–793.
- Lee, K. K., Tang, M. K., Yew, D. T., Chow, P. H., Yee, S. P., Schneider, C. and Brancolini, C. (1999) Gas2 is a multifunctional gene involved in the regulation of apoptosis and chondrogenesis in the developing mouse limb. *Dev. Biol.* **207**, 14–25.
- Brancolini, C., Benedetti, M. and Schneider, C. (1995) Microfilament reorganization during apoptosis: the role of Gas2, a possible substrate for ICE-like proteases. *EMBO J.* **14**, 5179–5190.
- Brancolini, C. and Schneider, C. (1994) Phosphorylation of the growth arrest-specific protein Gas2 is coupled to actin rearrangements during G0–G1 transition in NIH 3T3 cells. *J. Cell Biol.* **124**, 743–756.
- Wang, X., Pai, J. T., Wiedenfeld, E. A., Medina, J. C., Slaughter, C. A., Goldstein, J. L. and Brown, M. S. (1995) Purification of an interleukin-1 β -converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. *J. Biol. Chem.* **270**, 18044–18050.
- Walter, B. N., Huang, Z., Jakobi, R., Tuazon, P. T., Alnemri, E. S., Litwack, G. and Traugh, J. A. (1998) Cleavage and activation of p21-activated protein kinase γ -PAK by CPP32 (caspase 3). Effect of phosphorylation on activity. *J. Biol. Chem.* **273**, 28733–28739.

- 33 Berti, P. J., Faerman, C. H. and Storer, A. C. (1991) Cooperativity of papain-substrate interaction energies in the S2 to S2' subsites. *Biochemistry* **30**, 1394–1402
- 34 Tózsér, J., Bagossi, P., Weber, I. T., Louis, J. M., Copeland, T. D. and Oroszlan, S. (1997) Studies on the symmetry and sequence context dependence of the HIV-1 proteinase specificity. *J. Biol. Chem.* **272**, 16807–16814
- 35 Egelhoff, T. T., Lee, R. J. and Spudich, J. A. (1993) *Dictyostelium* myosin heavy chain phosphorylation sites regulate myosin filament assembly and localization *in vivo*. *Cell* **75**, 363–371
- 36 Huang, W. and Erikson, R. L. (1994) Constitutive activation of Mek1 by mutation of serine phosphorylation sites. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8960–8963
- 37 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 kinases I and II regulates its cleavage by caspase 8. *Mol. Cell* **8**, 601–611
- 38 Walter, J., Grünberg, J., Capell, A., Pesold, B., Schindzielorz, A., Citron, M., Menda, K., George-Hyslop, P. S., Multhaup, G., Selkoe, D. J. and Haass, C. (1997) Proteolytic processing of the Alzheimer disease-associated presenilin-1 generates an *in vivo* substrate for protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5349–5354
- 39 Walter, J., Grünberg, J., Schindzielorz, A. and Haass, C. (1998) Proteolytic fragments of the Alzheimer's disease associated presenilins-1 and -2 are phosphorylated *in vivo* by distinct cellular mechanisms. *Biochemistry* **37**, 5961–5967
- 40 Davis, R. J. (1993) The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **268**, 14553–14556
- 41 Park, J. A., Kim, K. W., Kim, S. I. and Lee, S. K. (1998) Caspase 3 specifically cleaves p21^{WAF1/CIP1} in the earlier stage of apoptosis in SK-HEP-1 human hepatoma cells. *Eur. J. Biochem.* **257**, 242–248
- 42 Scott, M. T., Morrice, N. and Ball, K. L. (2000) Reversible phosphorylation at the C-terminal regulatory domain of p21^{Waf1/Cip1} modulates proliferating cell nuclear antigen binding. *J. Biol. Chem.* **275**, 11529–11537

Received 9 December 2002/14 February 2003; accepted 17 February 2003

Published as BJ Immediate Publication 17 February 2003, DOI 10.1042/BJ20021901