Caspase-3-mediated cleavage of Akt: Involvement of non-consensus sites and influence of phosphorylation

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Abstract Here, we show for the first time that Akt1 is cleaved in vitro at the caspase-3 consensus site DQDD 456 ↓ SM. Our data suggest QE£E 116 ↓ E117 ↓ MD, EEMD 119↓L, TPPD 453 ↓ QD and DAKE 498 ↓ IM as novel non-consensus caspase-3 cleavage sites. More importantly, we demonstrate that phosphorylation of Akt1 modulates its cleavage in a site-specific manner: Resistance to cleavage at site DAKE 498 (within the kinase domain) in response to phosphorylation suggests a possible mechanism by which the anti-apoptotic role of Akt1 is regulated. Our result is important in biological models which rely on Akt1 for cell survival.

Keywords: Akt1; Caspase-3; Proteolytic cleavage; Phosphorylation; SELDI-tof mass spectrum

1. Introduction

hAkt1, a human oncogene [23,24], is implicated in a wide variety of cellular processes including adipocyte and muscle differentiation, glycogen synthesis, glucose uptake, apoptosis and cellular proliferation [1,7,9,10,13]. Akt1 phosphorylates p27, impairs the nuclear import of p27 and opposes cytokine-mediated G1 arrest [18]. Over-expression of an activated Akt1, but not of a kinase-deficient mutant, provides protection against apoptosis and results in a malignant phenotype, suggesting that Akt1 activation is necessary and sufficient to inhibit apoptosis and induce transformation [14]. Activation of Akt1 involves phosphorylation on two regulatory sites: threonine 308 and serine 473, both of which are required for maximal kinase activity [6].

We have previously demonstrated that Akt is a physiological substrate of caspase-3 [2], the main executioner of cell death [11,15,16,20]. Caspases are grouped into subfamilies according to their preferred cleavage sites, with caspase-3 as the prototype of DEXD-dependent proteases [17,21]. Phosphorylation renders caspase-3 substrates more resistant to caspase-3-mediated proteolysis [3,8,12]. The exact site(s) of Akt1 cleavage has not been identified, nor is the possible influence of phosphorylation on the cleavage. In this study, we investigated the caspase-3 consensus and non-consensus cleavage sites in Akt1, examined their specificity and compared the extent of cleavage at these sites between phosphorylated and non-phosphorylated Akt1 in vitro. Our data suggest that attenuation of caspase-3-mediated Akt1 cleavage mediated by phosphorylation may be a mechanism by which the activation of this survival pathway is enhanced and that cleavage of the non-consensus site within the kinase domain may be significant in suppressing the function of Akt1.

2. Methods

2.1. Reagents

Anti-Akt1/2 and recombinant (rec)-hAkt1, Akt2, and Akt3 were purchased from Upstate (Lake Placid, NY). Anti-AKT and anti-Akt3 were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Rec-caspase-3 and its substrate Ac-(acetyl) DEVD-MCA was from BD Biosciences Pharmingen (Mississauga, Ont., Canada). The MALDI-mass spectrum plates were purchased from Applied Biosystems (Foster City, CA, USA). Protein sequences of hAkt1 (Genebank #Accession number AAH00479), hAkt2 (Genebank #Accession number AAH453915) and hAkt3 (Genebank #Accession number NP053915) were obtained from NCBI.

2.2. Caspase-3 activity assay

Ac-DEVD-MCA fluorogenic substrate was incubated with active rec-caspase-3 (0–200 ng) at 37°C in 100 μL of PIPES buffer. Free AMC (7-amino 4-methyl coumarin) released was measured spectrophotometrically at excitation and emission wavelengths fixed at 370 and 460 nm, respectively.

2.3. Akt cleavage assay and Western blot

All rec-proteins are of human sequence. Akt1 (125 ng) was incubated with caspase-3 (40 ng for 0.5–4 h or 0–320 ng for 2 h). Akt1 was also incubated with caspase-3 (50 ng) in the absence and presence of its inhibitor (DEVD-CHO, 20 μM) or DMSO (0.09%, control). To compare the cleavage fragments of different isoforms of Akt, Akt1, Akt2 or Akt3 (125 ng each) were incubated with caspase-3 (0–80 ng). To assess the influence of phosphorylation on cleavage, Akt1 and phospho-Akt1 (125 ng each) were incubated with caspase-3 (40 ng; 37°C 0.5–4 h) in 40 μL of PIPES buffer, pH 7.0. We investigated the pH dependence of Akt1 cleavage using a ternary buffer (30 mM sodium acetate, 30 mM 2-[N-morpholino] ethane sulfonic acid and 30 mM N-ethylmorpholine) containing reagents necessary for optimum caspase-3 activity (100 mM NaCl, 10% sucrose, 10 mM DTT, and 0.1% CHAPS) at pH 3, 4, 5, 6, and 7. Akt1 was incubated with caspase-3 at each pH to assess its pH-dependent degradation. (Fig. 3C shows the pH-dependent cleavage of novel band A1/2.) Western blot was performed as described [2].
2.4. Peptide synthesis/purification and amino acid analysis

All peptides were synthesized using Fmoc-solid phase peptide chemistry [4]. Following amino acid side chain protecting groups were used: t-butyloxy carbonyl (Boc) for Lys; 2,2,4,6,7-pentamethyl dihydrobenzo furan-5-sulphonyl (Pbf) for Arg; t-butyl for Ser, Thr, Asp and Tyr and trityl for His, Asn and Gln. The peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC, Rainin Dynamax) using a semi-preparative column (CSC Exsil, C18, 25 cm × 1.0 cm, Chromatography Specialty Co., St-Laurent, Quebec, Canada) [5]. Each purified peptide was characterized by amino acid analysis and MALDI or SELDI-tof MS. using 4-hydroxycinnamic acid or 1, 2 dihydroxy benzoic acid as matrix [4].

3. Results

3.1. Akt1 Sequence analysis and cleavage at the consensus sequence DQDD

Examination of hAkt1 protein sequence (Genebank® Accession number AAH00479) revealed the presence of a caspase-3-consensus motif DQDD. To examine whether this is indeed a caspase-3 cleavage site, we incubated Akt1 for different durations and with increasing caspase-3 concentrations and the levels of the cleaved fragments were assessed by Western blot and SELDI-tof mass spectrometry analyses. Since rec-Akt1 used in the present study contains an N-terminal His 6-tag (calculated molecular mass ~59 kDa), caspase-3-mediated Akt1 cleavage at this site was expected to result in a 56.3 kDa N-terminus and 2.7 kDa C-terminus fragments. Using N-terminus anti-AKT antibody (epitope: PH domain), we detected a band at 57 kDa (band C) (Fig. 1 A and B). The intensity of band C increased with increasing caspase-3 concentration or period of digestion and was accompanied by a corresponding decrease in Akt1 intensity. The presence of a caspase-3-inhibitor (DEVD-CHO) resulted in complete disappearance of band C (Fig. 1 C). MS analysis of crude digest reveals the presence of a peak around 9 kDa (Fig. 1 D). Considering the size of the intact Akt1 (~59 kDa), the small 9 kDa peak, detected by SELDI MS and the large 51 kDa fragment detected by electrophoresis complement each other to be the fragments generated from cleavage at the same site (site B).

Two additional bands (A1 and A2) were detected in studies in which Akt1 was incubated with active caspase-3 and immunoblotted with a “C-terminus” anti-Akt1 antibody (epitope: 467RPHFPQFSYSASSTA480). Both bands were completely eliminated in the presence of 20 pM of caspase-3 inhibitor (DEVD-CHO, Fig. 3 B), implicating that bands A1 and A2 are Akt1 cleavage fragments possibly at the non-consensus motif. Analysis of the digestion products by SELDI-tof MS reveals presence of two peaks around 16 kDa (Fig. 3 D). Considering the size of the intact Akt1 (~59 kDa), the small 16–16.5 kDa peaks (detected by SELDI-MS) and the large 42–43 kDa fragments (detected by electrophoresis) complement each other to be the fragments generated from cleavage at identical sites (sites A1 and A2). Interestingly, the existence of two distinct cleavage sites in close proximity to each other is

Fig. 1. Akt1 cleavage at the caspase-3 consensus motif DQDD. Akt1 (125 ng) was incubated with increasing concentrations of caspase-3 (Panel A) and for different durations (Panel B) or in the absence and presence of a caspase-3 inhibitor, DEVD-CHO (Panel C). Samples were analyzed by SDS-PAGE. Akt1 was incubated with caspase-3 followed by analysis with SELDI-MS (Panel D). Each panel represents the sum of five replicates.
best supported when comparing the cleavage of non-phosphorylated and phosphorylated Akt1 as described later.

3.3. Influence of phosphorylation on two novel cleavage sites: A and B

When comparing the relative ability of Akt1 and its phosphorylated counterpart (p-Akt1) to serve as substrate for caspase-3, our results indicate that phosphorylation of Akt1 influenced caspase-3-mediated cleavage in a site-specific manner: whereas site A1 was more susceptible to cleavage in the phosphorylated Akt1 (Fig. 3E), the opposite was true for cleavage at sites A2 and B (Figs. 2E and 3E).

3.4. Digestion of Akt-1 peptides with caspase-3

Based on the SDS–PAGE and SELDI-MS analysis, we propose EEEE, EEMD and DAKE as novel cleavage sites giving rise to bands A1, A2 and B, respectively, in the His-tagged fusion recombinant Akt1 protein. To further examine this, we designed three peptides from the sequence of hAkt-1 that encompass these potential caspase-3 cleavage sites.
sites. These are hAkt1\(^{106-130}\) peptide with caspase-3 in the absence and presence of its inhibitor DEVD-CHO. The mass spectrum was performed with 2 \(\mu\)l of sample with CHCA as matrix. Peaks corresponding to the undigested peptide (\(m/z = 2714\)) and one of the major cleaved fragments (\(m/z = 1355\)) are indicated in the spectrum following 3 h incubation (Panels A). The proposed cleavage sites are shown in vertical arrows. \(^{106}\)VADGLKKQEEEEMDFRSGSPSDNSG\(^{130}\) with fragment MDFRSGSPSDNSG representing the peak at 1355. SELDI-tof MS profile of crude digest of hAkt peptide\(^{106-130}\) with excess caspase-3 at various durations is shown in (Panels B) (b1 = full spectrum, b2 and b3 = expanded portion of the spectrum to highlight the cleaved peaks of the fragments). hAkt1\(^{106-130}\), VADGLKKQEEEEMDFRSGSPSDNSG-NH\(_2\) (MW = 2714) VADGLKKQEEEEMDFRSGSPSD-OH (MW 2456, 2478 (+Na)].

**Digestion of hAkt1\(^{385-410}\) + G peptide with caspase-3 led to the formation of two additional peaks at \(m/z = 1470\) and 2505 in the mass spectra due to the presence of the fragments PKQRLLGGGDKEAE and KDQQRLLGGGDKEAE respectively (Fig. 5A). This suggests cleavages at KKDPK and DAEIM and HRFF. The first two cleavages are mediated by caspase-3 whereas the last cleavage is possibly due to a trypsin-like enzyme present as a small contaminant in commercial caspase-3 sample. The extent of cleavage increases with duration of incubation.

**Digestion of hAkt1\(^{441-465}\) peptide with caspase-3 yielded three additional peaks at \(m/z = 1055\), 1257 (major) and 1820 in the mass spectra due to the presence of fragments SMECVDSER, QDSDMECVDS and EFTAQMITITPPDQDD, respectively (Fig. 5B). The peak at \(m/z = 1842\) is attributed to the Na-adduct of fragment peak at \(m/z = 1820\). This suggests cleavages at EFTAQMITITPPDQDD \(\downarrow\) QDD \(\downarrow\) SMECVDSER \(\downarrow\) R by caspase-3. As expected the intensities of cleaved fragments at \(m/z = 1257\) and 1820 increase gradually with time of incubation.

**4. Discussion**

Although analysis of the Akt1 protein sequence reveals presence of a caspase-3 consensus cleavage site DQDD\(^{456}\), the presence of this site does not necessarily suggest its accessibility to caspase-3 and subsequent binding, due to the presence of
flap-like loops projecting over the active site of the enzyme [22]. Phosphorylation could conceivably cause the flap to open or close further, thereby providing a potential regulatory mechanism [19]. Since domain containing DQDD may fail to dock in the catalytic site of caspase-3 due to steric hindrance by the flap, we first investigated possibility of cleavage at this site. Our Western blot and SELDI-MS data support cleavage at the consensus site DQDD in vitro. The significance of this cleavage in the PI3K/AKT pathway remains unknown. Since the consensus sequence DQDD is located at its C-terminus end and cleavage at this site yields a novel Akt1 fragment with only a 2.7 kDa difference in mass from intact Akt1, it is possible that this cleavage may have a minimal influence on the activity and function of the protein.

In addition, we found several non-consensus motifs within Akt1, all of which were confirmed by synthesis of model peptides and their subsequent cleavage with caspase-3. Comparison of the protein sequence of Akt1 with those of Akt2 and Akt3 (gene bank) and the absence of the EEEE and EEMD sites and presence of DAKE site in both Akt2 and Akt3 prompted us to perform a comparative study between these isoforms. Our observation that incubation of Akt2 and Akt3 with caspase-3 resulted in no cleavage fragment similar to A1, and A2, while similar sites to cleavage site B was detected in both Akt2 and Akt3 (Fig. 6) lends support for the proposed cleavage model (Fig. 7).

In conclusion, we tested the hypothesis that attenuation of caspase-3-mediated Akt1 cleavage via phosphorylation may represent a mechanism by which the activation of this survival pathway is enhanced. This was accomplished by comparing the extent of cleavage between Akt1 and phospho-Akt1. We propose that the cleavage at site B, located in the middle of the two Akt1 activation/phosphorylation sites (Thr and Ser) and within the kinase domain of the protein, may be significant in suppressing the function of Akt1. Our data suggest that in addition to regulating its kinase activity, phosphorylation of Akt1 regulates its pattern of cleavage and inactivation by caspase-3. These two outcomes of Akt1 phosphorylation may operate side by side to ensure cell endurance. The biological
Akt1, EEEE 117, EEMD 119, and DAKE 398 are novel sites of cleavage for bands A1, A2, and B, respectively. The pair fragments of 2.7 and 53, 41.7 and 2888 and 13.9, 41.4 and 14.1 and 9.2 and 46.4 kDa are expected cleavage fragments of human Akt1 at site DQDD 456, EEEE117, EEMD119, and DAKE 398, respectively.

References