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Proteolytic cleavage of epidermal growth factor receptor by caspases

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Abstract Apoptotic proteases cleave and inactivate survival signaling molecules such as Akt/PKB, phospholipase C (PLC)-γ1, and Bcl-2. We have found that treatment of A431 cells with tumor necrosis factor-α in the presence of cycloheximide resulted in the cleavage of epidermal growth factor receptor (EGFR) as well as the activation of caspase-3. Among various caspases, caspase-1, caspase-3 and caspase-7 were most potent in the cleavage of EGFR in vitro. Proteolytic cleavage of EGFR was inhibited by both YVAD-cmk and DEVD-fmk in vitro. We also investigated the effect of caspase-dependent cleavage of EGFR upon the mediation of signals to downstream signaling molecules such as PLC-γ1. Cleavage of EGFR by caspase-3 significantly impaired the tyrosine phosphorylation of PLC-γ1 in vitro. Given these results, we suggest that apoptotic protease specifically cleaves and inactivates EGFR, which plays crucial roles in anti-apoptotic signaling, to abrogate the activation of EGFR-dependent downstream survival signaling molecules.

Key words: Apoptosis; Proteolysis; Receptor

1. Introduction

Besides the growth promoting properties of mitogenic signaling molecules, they can also promote survival signaling by activating anti-apoptotic responses. For example, activation of mitochondrial protein kinase C-α results in the phosphorylation of Bcl-2, and subsequently inhibits apoptosis [1]. Also, Ras has been shown to mediate anti-apoptotic signals through the activation of phosphatidylinositol-3-kinase (PI3K) [2]. It is well known that Akt/PKB plays central roles in anti-apoptotic function. Bad, a pro-apoptotic Bcl-2 family, is phosphorylated by Akt/PKB and dissociates from Bcl-2, strengthening the anti-apoptotic properties of Bcl-2 [3]. Recently, it has been reported that caspase-9 is directly inactivated by Akt/PKB-mediated phosphorylation [4]. Therefore, growth factor receptor downstream signaling molecules participate in the anti-apoptotic signals as well as mitogenic signals.

Apoptosis is a fundamental process for normal development of multicellular organisms and is involved in the regulation of the immune system, embryonic development and maintenance of homeostasis [5]. It is well established that a family of cellular cysteine proteases, collectively known as caspases, act in a cascade to elicit and potentiate a number of apoptotic responses [6]. Upon binding of Fas ligand or tumor necrosis factor-α (TNF-α) to their cognate receptors, caspase-8 is recruited to the death-induced signaling complex through interaction with FADD/MORT-1 [7]. Bid is cleaved by caspase-8, and its C-terminal domain translocates to the mitochondria where it triggers cytochrome *c* release into the cytoplasm [8]. It has been revealed that dATP together with cytochrome *c* associates with Apaf-1, which is a mammalian homolog of CED-4 [9]. Apaf-1 subsequently associates with and activates caspase-9 resulting in the cleavage and activation of caspase-3 [10]. Caspase-3, as an executioner, cleaves several cellular proteins that are involved in morphological and biochemical changes that accompany apoptosis, or in aspects of DNA damage sensing and repair.

Recently, a new paradigm has been established whereby caspases promote apoptosis by not only activating pro-apoptotic parameters but also by inactivating mitogenic or anti-apoptotic molecules. Caspase-3 converts anti-apoptotic Bcl-2 into a Bax-like death effector by cleaving the loop domain [11]. Also, phospholipase C (PLC)-γ1 is cleaved and inactivated by caspases [12]. Widmann et al. have reported that Raf-1 and Akt/PKB are inactivated by caspase-dependent cleavage during the apoptosis [13]. Since the survival signaling molecules inhibit apoptosis, it is reasonable that apoptotic proteases cleave and inactivate survival factors for the efficient progression of apoptosis.

In this study, we have demonstrated that epidermal growth factor receptor (EGFR) is cleaved in a caspase-dependent manner during apoptosis. Cleavage of EGFR by caspases abrogates the activation of PLC-γ1. We suggest here, for the first time, that the gateway of survival signals such as EGFR is a target of apoptotic proteases.

2. Materials and methods

2.1. Materials

Human epidermoid carcinoma A431 cells were purchased from ATCC (Rockville, MD, USA). Tissue culture supplies were purchased from Corning (Corning, NY, USA), and sera were from Hyclone (Logan, UT, USA). Z-DEVD-fmk was purchased from Enzyme Systems Products (Dublin, CA, USA). YVAD-cmk was obtained from Calbiochem (San Diego, CA, USA). Enhanced chemiluminescence detection system was purchased from Amersham (Aylesbury, UK). Monoclonal anti-EGFR antibody that specifically recognizes the

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Abbreviations: PLC, phospholipase C; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol-3-kinase; CHX, cycloheximide; TNF-α, tumor necrosis factor-α

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C-terminal region (amino acids (aa) 996–1022) of human EGFR was obtained from Transduction Laboratories (Lexington, KY, USA). Monoclonal anti-phosphotyrosine antibody (4G10) and monoclonal anti-EGFR antibody that specifically recognizes the extracellular domain of human EGFR were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MA, USA). Rabbit polyclonal antibody raised to *Escherichia coli* expressed caspase-3 was prepared by the immunization of New Zealand white rabbits as previously described [14]. Wheat germ lectin agarose and other chemicals were obtained from Sigma (St. Louis, MO, USA). Caspases were prepared as described previously [15] and caspase activity was measured using specific peptide substrate.

2.2. Cell culture

A431 cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum and were maintained at 5% CO_2/95% air at 37°C. Medium was replaced every 2 days. Cells were maintained in 70% confluence.

2.3. Purification of EGFR

A431 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed with lysis buffer (20 mM Tris–HCl pH 7.4, 1 mM EGTA/EDTA, 1 mM Na_3VO_4, 2 mM sodium pyrophosphate, 10% glycerol and 1% Triton X-100). Cell lysates were applied to wheat germ lectin agarose. Unbound proteins were washed out with lysis buffer. EGFR was eluted with lysis buffer containing 0.3 M of *N*-acetyl-D-glucosamine. The eluted EGFR fraction was applied to a gel filtration column to exchange buffer into cleavage assay buffer (20 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 0.2 mM $CaCl_2$ and 1 mM dithiothreitol).

2.4. Cleavage of EGFR by caspases in vitro

Cleavage of EGFR was performed by active caspases as described previously [16]. Briefly, reactions were initiated by the addition of various caspases to 0.1 μg of purified EGFR in 15 μl of cleavage assay buffer. After 1 h, the reactions were stopped by adding sodium dodecyl sulfate (SDS) sample buffer. Also, the effect of inhibitors such as DEVD-fmk (10 μM) and YVAD-cmk (10 μM) on the cleavage of EGFR by caspase-1, caspase-3 and caspase-7 was determined.

2.5. Cleavage of EGFR in A431 cells

A431 cells were incubated with TNF-α in the presence or absence of cycloheximide (CHX) for 18 h. Both the floating and anchoring cells were harvested followed by washing with PBS twice. Cells were lysed with lysis buffer and lysates were immunoblotted with anti-EGFR antibody recognizing the C-terminal region of EGFR.

2.6. Effect of EGFR cleavage on the tyrosine phosphorylation of PLC-γ1

Purified EGFR (0.1 μg) was cleaved by caspase-3 (0.1 μg) and then reaction mixtures were incubated with DEVD-fmk (20 μM) to irreversibly inactivate caspase-3. 3 h after incubation with PLC-γ1 in a kinase buffer (20 mM HEPES-OH pH 7.4, 25 mM $MgCl_2$, 4 mM $MnCl_2$, 0.1 mM Na_3VO_4), phosphorylated PLC-γ1 was detected by probing with anti-phosphotyrosine antibody. Also, cleavage of EGFR was verified by probing with anti-EGFR antibody that recognizes the extracellular domain of EGFR.

3. Results

3.1. Cleavage of EGFR by caspase-3 in vitro

Apoptotic caspases often cleave anti-apoptotic signaling molecules such as Akt/PKB [13]. Among the anti-apoptotic signaling molecules, the growth factor receptor that initiates anti-apoptotic signaling events could be a possible target molecule of apoptotic proteases such as caspase-3 and caspase-7. To confirm this idea, we incubated EGFR with active caspase-3 in vitro. EGFR was rapidly cleaved by caspase-3 as shown in Fig. 1. However, we could not detect the cleavage product by an anti-EGFR antibody that specifically recognizes the C-terminal domain (aa 996–1022) of human EGFR. This result indicates that caspase-3 may cleave within the C-terminal region of human EGFR.

3.2. Cleavage of EGFR by group II caspases

As other caspase family members have substrate specificity similar to caspase-3, we investigated their ability to proteolyze EGFR. Among those tested, group II caspases such as caspase-3 and caspase-7 were most potent in the cleavage of EGFR (Fig. 2). Interestingly, caspase-1 also potently cleaved EGFR. However, cleavage of EGFR by other caspases such as caspase-4, caspase-6 and caspase-8 was negligible. This result indicates that cleavage of EGFR is specific for group II caspases and caspase-1.

3.3. Effect of caspase inhibitors on the cleavage of EGFR

To further confirm the caspase-dependent cleavage of EGFR, we determined the effect of caspase inhibitors such as DEVD-fmk (specific for group II caspase) and YVAD-cmk (specific for caspase-1). Caspase-1-dependent cleavage of EGFR was inhibited by pretreatment with both DEVD-fmk and YVAD-cmk as shown in Fig. 3. Caspase-3-dependent cleavage of EGFR was also inhibited by pretreatment with both DEVD-fmk and YVAD-cmk. However, DEVD-fmk was more specific than YVAD-cmk in the inhibition of caspase-7-dependent cleavage of EGFR. These results strongly implicate that EGFR is cleaved by apoptotic caspases.

3.4. Cleavage of EGFR in A431 cells

Since EGFR was cleaved by apoptotic caspases in vitro, we next examined the cleavage of EGFR during TNF-α-induced apoptosis in A431 cells. EGFR was not cleaved by treatment of A431 cells with TNF-α or CHX alone. However, treatment of A431 cells with both TNF-α and CHX potently induced cell death (data not shown). Moreover, activation of caspase-3 and cleavage of EGFR was accompanied by co-stimulation of A431 cells with TNF-α and CHX (Fig. 4). This result indicates that EGFR is cleaved by caspases in TNF-α/CHX-induced apoptosis.

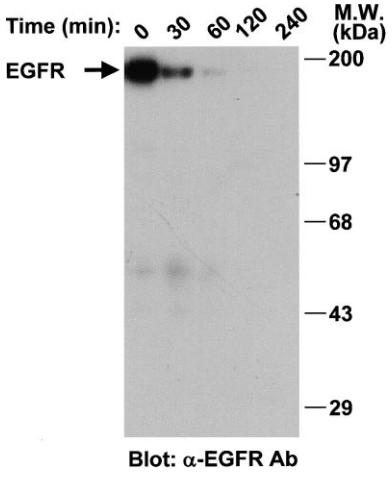

Fig. 1. Proteolytic cleavage of EGFR by caspase-3. EGFR was purified as described in Section 2. 0.1 μg of purified EGFR was incubated with 0.1 μg of active caspase-3 for the indicated times. Reactions were stopped by addition of sample buffer, and Western blot analysis was performed using anti-EGFR antibody that recognizes the C-terminal region (aa 996–1022).

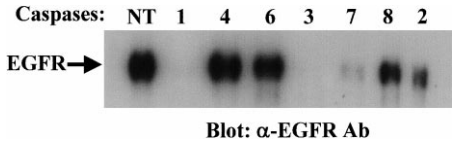

Fig. 2. Cleavage of EGFR by group II caspases. 0.1 μg of purified EGFR was incubated with caspase-1, -4, -6, -3, -7, -8 or -2 for 30 min. Reactions were stopped by addition of sample buffer, and Western blot analysis was performed using anti-EGFR antibody that recognizes the C-terminal region of EGFR.

3.5. *Abrogation of PLC-γl activation by caspase-dependent cleavage of EGFR in vitro*

To dissect the meaning of caspase-dependent cleavage of EGFR, we have examined the effect of cleavage on the tyrosine phosphorylation of a downstream signaling molecule such as PLC-γ1. PLC-γl was clearly tyrosine phosphorylated by intact EGFR. However, tyrosine phosphorylation of PLC-γl by cleaved EGFR was impaired as shown in Fig. 5. PLC-γl was not cleaved by caspase-3 since we inactivated caspase-3 by an irreversible inhibitor (DEVD-fmk) after cleavage of EGFR (data not shown). This result indicates that cleavage of EGFR by caspase results in the loss of a proper signal transduction capability of EGFR.

4. Discussion

The occupation of growth factor receptors by their cognate ligands induces autophosphorylation of tyrosine residues on their receptors [17]. This stimulates recruitment of several cellular proteins that are involved in proliferation, differentiation and survival [18]. Among the downstream signaling molecules, Akt/PKB is known to play a crucial role in anti-apoptotic function through the phosphorylation of regulatory proteins of apoptosis [3,4]. A common end-point in the commitment of apoptosis is the activation of executioner caspases. Inhibition of caspases generally inhibits apoptosis. It seems to be that different caspases differentially contribute to the apoptotic program in different cell types. Caspase-dependent cleavage can inactivate proteins that are involved in repair mechanisms, cell cycle and cellular morphology. Since many proteins in various intracellular signaling pathways are under the control of the apoptotic response, we were interested in determining whether signaling proteins could be cleaved by apoptotic caspases.

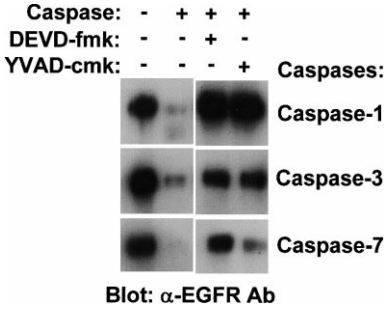

Fig. 3. Effect of caspase inhibitors on the in vitro cleavage of EGFR. Caspase-1, caspase-3 and caspase-7 were incubated with EGFR in the absence or presence of either DEVD-fmk (10 μM) or YVAD-cmk (10 μM) for 30 min. Reactions were stopped and EGFR was probed with anti EGFR antibody that recognizes the C-terminal region of EGFR.

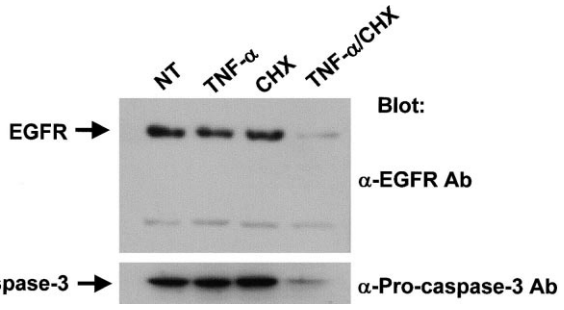

Fig. 4. Cleavage of EGFR during TNF-α-induced apoptosis. A431 cells (1×10^6) were stimulated with TNF-α in the presence or absence of CHX and medium containing 1% fetal bovine serum for 18 h. Floating and anchoring cells were harvested and washed twice with PBS. Lysates were subjected to SDS–PAGE and probed with either anti-EGFR antibody (upper panel) or anti-caspase-3 antibody (lower panel).

We have found that EGFR is one such signaling protein. EGFR was preferentially cleaved by group II caspases, which play a crucial role in the execution phase of apoptosis [19]. It has been reported that pretreatment of cells with a protein synthesis inhibitor such as CHX is required for TNF-α-induced activation of caspases and apoptosis [20]. Cleavage of EGFR by TNF-α treatment was induced in the presence of protein synthesis inhibitor. Therefore, cleavage of EGFR might be induced by apoptotic proteases. Also, our result showed that activation of caspase-3 correlated with the cleavage of EGFR.

Caspases cleave the consensus sequence (DXXD) of their substrate proteins [19]. Recently it has been reported that the P_1 position should be followed by a small amino acid such as alanine or glycine to be a good substrate of caspases [21]. EGFR contains several consensus sequences such as DEED[1006], DMDD[1009] and DYYD[1012] (Fig. 6). The P_1 position of all these candidate sites is followed by a small amino

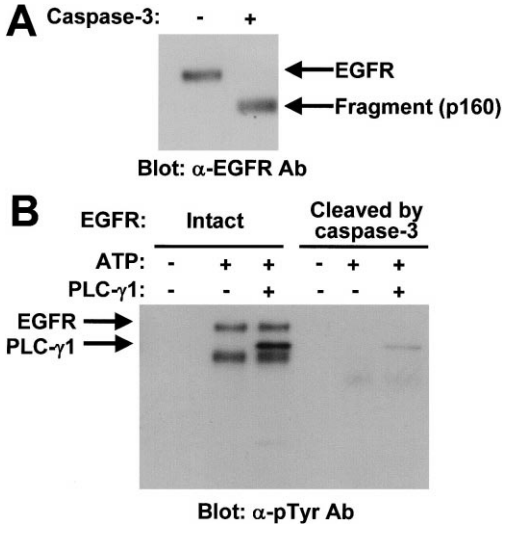

Fig. 5. Effect of cleavage on the activation of PLC-γl. 1 μg of EGFR was incubated with active caspase-3. After 1 h, DEVD-fmk was added to the reaction mixture. Proteolytic cleavage of EGFR was probed with anti-EGFR antibody that recognizes the extracellular domain of EGFR (A). Each intact and cleaved EGFR was incubated with purified PLC-γl (0.1 μg) for 2 h as described in Section 2. Tyrosine phosphorylation of PLC-γl was detected by immunoblotting with anti-phosphotyrosine monoclonal antibody (B).

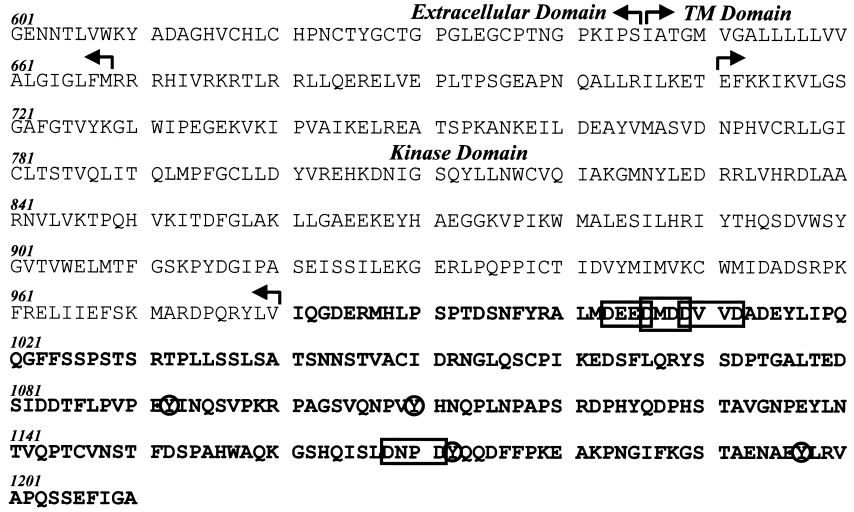

Fig. 6. Schematic representation of candidate cleavage sites on EGFR sequence. Candidate cleavage sites containing DXXD motif are boxed. Extracellular domain, transmembrane domain (TM domain), and kinase domain are denoted. Tyrosine phosphorylation sites are indicated by circles.

acid. Since we have detected a 160 kDa cleavage product with an anti-EGFR antibody that recognizes the extracellular domain of EGFR, it is reasonable that apoptotic caspases may cleave the sequence around aa 1006–1012. However, we could not detect other small cleavage products with an antibody that recognizes the C-terminal region (aa 996–1022). It has been reported that the major phosphorylation site of EGFR is Tyr^{1197} [22]. Because anti-pTyr antibody could not detect any cleavage product of EGFR, caspases may cleave a 22 kDa cleavage product into a smaller fragment, which is undetectable by Western blot analysis. Therefore, $DNPD^{1171}$ might be another possible cleavage site of caspases though the P_1 position is followed by a bulky amino acid. Collectively, EGFR may be cleaved at multiple sites of the C-terminal region that acts as a docking site for signaling molecules. The mutational studies are required for the identification of cleavage sites.

Many signaling molecules are activated by EGFR through its tyrosine phosphorylation at specific sites. It has been reported that PLC-γ1 is activated by EGFR through tyrosine phosphorylation at Tyr^{771}, Tyr^{783} and Tyr^{1254} [23]. Especially, tyrosine phosphorylation at Tyr^{783} takes precedence over all other sites for the activation of PLC-γ1. Based on our results, EGFR cleavage by caspase-3 results in defective tyrosine phosphorylation of PLC-γ1. Since candidate cleavage sites reside in the docking site of signaling molecules including PLC-γ1, cleavage of EGFR by caspase-3 may affect the recruiting of signaling molecules to EGFR. Also, it has been reported that the C-terminal region deleted mutant of EGFR is defective in the recruitment of PLC-γ1 and GAP [24]. Therefore, activation of apoptotic caspases leads to the inactivation of a signaling complex between EGFR and PLC-γ1.

Stimulation of the growth factor receptor results in the suppression of apoptosis in various cells [25]. Many signaling molecules such as PI3K, PLC-γ1, Ras, ERK and Akt are involved in the suppressive mechanism of apoptosis. Also, there are other anti-apoptotic proteins such as Bcl-2 and $Bcl\text{-}X_L$ that control the activation of caspases. All of these proteins are involved in the inhibition of apoptosis. It is well established that apoptotic caspases specifically cleave signaling molecules playing negative roles in apoptosis. Since activation of anti-apoptotic signaling molecules is initiated by stimulation of the growth factor receptor, inactivation of the growth factor receptor itself by apoptotic caspases may be the most efficient way to propagate death signals. However, we could not exclude the possibility that different sets of proteins should be cleaved at various stages of the apoptotic responses in a temporally ordered manner to cause cell death. The full understanding of the interplay between EGFR and caspases and their signaling mechanisms still requires a lot of study.

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