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Monitoring of Cleavage Preference for Caspase-3 Using Recombinant Protein Substrates

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The apoptotic caspases have been classified in accordance with their substrate specificities, as the optimal tetrapeptide recognition motifs for a variety of caspases have been determined *via* positional scanning substrate combinatorial library technology. Here, we focused on two proteolytic recognition motifs, DEVD and IETD, owing to their extensive use in cell death assay. Although DEVE and IETD have been generally considered to be selective for caspase-3 and -8, respectively, the proteolytic cleavage of these substrates does not display absolute specificity for a particular caspase. Thus, we attempted to monitor the cleavage preference for caspase-3, particularly using the recombinant protein substrates. For this aim, the chimeric GST:DEVD:EGFP and GST:IETD:EGFP proteins were genetically constructed by linking GST and EGFP with the linkers harboring DEVD and IETD. To our best knowledge, this work constitutes the first application for the monitoring of cleavage preference employing the recombinant protein substrates that simultaneously allow for mass and fluorescence analyses. Consequently, GST:IETD:EGFP was cleaved partially in response to caspase-3, whereas GST:DEVD:EGFP was completely proteolyzed, indicating that GST:DEVD:EGFP is a better substrate than GST:IETD:EGFP for caspase-3. Collectively, using these chimeric protein substrates, we have successfully evaluated the feasibility of the recombinant protein substrate for applicability to the monitoring of cleavage preference for caspase-3.

Keywords: Tetrapeptide, proteolytic indicator, caspase substrate, protease activity

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Apoptosis, or programmed cell death, is a genetically conserved cascade of intracellular proteolytic events mediated by a family of cysteine proteases referred to as caspases (cysteine-dependent aspartyl-specific proteases), which normally cleave their substrates on the C-terminal side of aspartate residues (Asp) [4, 11, 19, 23]. Thus far, approximately 14 mammalian caspases have been identified, 11 of which are known to be present in humans [21]. In particular, the apoptotic caspases have been divided into initiator caspases (caspases-8 and -9) or executioner caspases (caspases-3, -6, and -7) according to the cleavage site specificity for their substrates [22].

Using a positional scanning synthetic combinatorial library of fluorogenic peptides, the optimal tetrapeptide substrate sequences for these caspases have been determined [22]. The primary effector caspases, including caspases-3 and -7, evidence a preference for substrates that harbor the 4-amino-acid recognition sequence of DEVD, a motif that is conserved in human, bovine, and chicken poly(ADP-ribose) polymerase (PARP), which is the first identified substrate for caspase-3 [11, 16, 20]. Recently, caspase-3-dependent DEVD sequences have also been detected within the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [2], the 140 kDa subunit of replication factor C (RFC140) [14], and alpha-fodrin [5]. Along this line, the cleavage of the DEVD motif has become an extensively utilized marker for caspase-3 activation, based on the notion that the peptide recognition sequence corresponding to that detected in endogenous substrates determines the specificity of a particular caspase. Caspase-3 can be directly cleaved and activated by caspase-8 *via* the mitochondria-independent pathway of apoptosis [3, 7, 8]. The corresponding cleavage site within caspase-3 is the IETD sequence [1, 11, 17, 22], which is therefore referred to as the caspase-8

recognition motif. Although both DEVD- and IETD-based substrates have been conventionally considered selective for caspase-3 and -8, respectively, there can be marked overlaps in the substrate preference of these caspases.

Our previous works have pointed out the usefulness of DEVD-based chimeric caspase-3 substrates [12, 13]. In this study, for the comparison of proteolytic cleavage efficiency of caspase-3, an indicator of apoptosis [9], the glutathione S-transferase (GST):DEVD:enhanced green fluorescent protein and GST:IETD:EGFP substrates were evaluated by a variety of assay techniques, including mass [Western blotting and surface plasmon resonance (SPR) imaging] and fluorescence (on-chip visualization and bead assay) measurements. This study reports a new application for the detection of cleavage preference using the chimeric protein substrates that simultaneously allow for mass and fluorescence analyses. Using these proteolytic substrates, we have successfully determined that there can be comparable differences in the substrate preference of caspase-3 between GST:DEVD:EGFP and GST:IETD:EGFP.

Materials and Methods

Gene Construction, Recombinant Protein Expression, and Purification

In order to clone the GST:DEVD:EGFP recombinant genes, the full-length gene encoding for EGFP was amplified using the 5' primer (CG GGA TCC GGA GGG GAT GAG GTG GAT GGG GGC ATG GTG AGC AAG GGC GAG) and the 3' primer (CGG AAT TCC TTG TAC AGC TCG TCC ATG) and the 3' primer (CGG AAT TCC TTG TAC AGC TCG TCC ATG), *via* the polymerase chain reaction (PCR). The 5' and 3' termini were designed to harbor the BamHI and EcoRI restriction enzyme cleavage sites, respectively. In order to clone the GST:IETD:EGFP, the EGFP was amplified *via* PCR using the 5' primer (CG GGA TCC GGA GGG ATC GAG ACC GAT GGG GGC ATG GTG AGC AAG GGC GAG) and the 3' primer (CGG AAT TCC TTG TAC AGC TCG TCC ATG) with the BamHI and EcoRI restriction enzyme sites. The PCR products were then purified with a DNA purification kit (Qiagen), and then digested with the BamHI and EcoRI restriction enzyme sites. The resultant DNA fragments were then inserted into the pGEX 4T-1 plasmid in order to generate the in-frame fusion of pGST:DEVD:EGFP and pGST:IETD:EGFP, respectively. Then, the chimeric GST:DEVD:EGFP and GST:IETD:EGFP fusion genes were verified *via* DNA sequencing and transformed into *Escherichia coli* BL21 (DE3) for the expression of the recombinant protein. Recombinant protein expression was performed as described previously [15]. Briefly, the cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), grown for an additional 3 h, and subsequently harvested *via* 10 min of centrifugation at 6,000 ×*g* at 4°C. In order to purify the GST:DEVD:EGFP and GST:IETD:EGFP, 10 ml of the crude cell lysates were loaded onto a GST-miniexcellose affinity column (Bioprogen Co., Korea), and washed three times in 10 ml of equilibration buffer (PBS, pH 7.4). The recombinant proteins were subsequently eluted with 5 ml of 10 mM reduced glutathione in the same buffer (PBS, pH 7.4). After elution, the concentrations of purified GST:DEVD:EGFP and GST:IETD:EGFP were determined *via* the Bradford method using bovine serum albumin as a standard. In order to generate the $pHis_{\times 6}$:caspase-3 fusion gene, the caspase-3 gene (GenBank Accession No. NM_004346) was amplified with the 5' primer (CG GGA TCC ATG GAG AAC ACT GAA AAC) and the 3' primer (CG GAA TTC GTG ATA AAA ATA GAG TTC) using PCR, and then cloned into the pET-23a plasmid using the BamHI/EcoRI sites. The recombinant caspase-3 protein was expressed in *Escherichia coli* and isolated in accordance with the method described by Stennicke and Salvesen [18].

SDS–PAGE and Western Blotting

One hundred μg of the purified recombinant GST:DEVD:EGFP or GST:IETD:EGFP proteins was incubated for 1 h with 300 U/ml of caspase-3 in 20 mM PBS buffer (pH 7.4) at 4°C. The resultant protein solutions were then resolved on 12% SDS gel and transferred onto nitrocellulose membranes. The immunoblotting assay was conducted as described previously [6], using antibody against GST (Santa Cruz, CA, U.S.A.).

Surface Modification of a Gold Chip and SPR Imaging Measurement

A bare gold chip (K-MAC, Korea) was cleaned with concentrated "Piranha" solution [70% (v/v) H_2SO_4, 30% (v/v) H_2O_2] and thoroughly rinsed in distilled water and ethanol. The gold chip was then immersed for 16 h in ethanol containing 10 mM 11-mercapto-1-undecanoic acid (MUA). The self-assembled monolayer (SAM) of MUA on the gold surface was activated as a hydroxysuccinimidyl ester, *via* 10 min of incubation with a mixture of 0.1 M *N*-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) in distilled water. After surface activation, a PBS buffer (pH 7.4) harboring amino-dextran (1 mg/ml) was applied to the gold surface and permitted to react for 1 h. The dextran surface, which is hydrophilic, was then activated for 2 h using 0.1 mg/ml *N*-(β-maleimidopropyloxy) succinimide ester (BMPS) in PBS buffer (pH 7.4). The chips were then incubated for 2 h in PBS buffer (pH 7.4) containing 1 mg/ml L-glutathione (reduced form), in order to modify the activated gold surface. The chips were then washed in distilled water. The resultant glutathionylated gold chips were maintained in PBS buffer (pH 7.4) at 4°C. The glutathionylated gold chip was submerged in the GST:IETD:EGFP protein solution (10 mg/ml) in PBS buffer (pH 7.4) for 1 h at 25°C in order to immobilize the recombinant protein onto the chip surface. The chip was washed with distilled water and dried. Caspase-3 protein in PBS buffer with 20% (v/v) ethylene glycol was spotted onto the GST:IETD:EGFP chip with a protein arrayer (Proteogen, Korea) equipped with a Stealth pin (Telechem International, U.S.A.). The diameter of each spot was 330 μm. After spotting, the chip was incubated for 1 h at 80% humidity. The spotted chip was then rinsed three times in PBS buffer (pH 7.4), followed by rinsing in distilled water, and then subsequently analyzed with an SPR imaging system.

On-Chip Visualization for Measuring Caspase-3-Dependent Proteolytic Cleavage

For the on-chip visualization of caspase-3-dependent proteolytic cleavage in GST:DEVD:EGFP and GST:IETD:EGFP, these substrate-immobilized glass chips were constructed as described previously [10]. After the preparation of the substrate-modified glass chips, 300 U/ml of caspase-3 was applied to the GST:DEVD:EGFP- and

GST:IETD:EGFP-coated glass chips for fluorescence-based detection. The fluorescence images were acquired using a GenPix 4200A 488 nm laser and a microarray scanner (Axon Inc., CA, U.S.A.).

Glutathione–Agarose Bead Assay

Ten μl of glutathione–agarose beads coupled to 100 μg of GST: DEVD:EGFP and GST:IETD:EGFP was incubated with 300 U/ml of caspase-3 in 20 mM phosphate-buffered saline (PBS) buffer (pH 7.4) for 1 h at 4°C. After incubation, the beads were washed three times in the same buffer, followed by the fluorescence visualization of caspase-3-dependent proteolytic cleavage in a bead format.

RESULTS AND DISCUSSION

Assay Concept for the Monitoring of Cleavage Preference for Caspase-3

In an effort to evaluate the proteolytic cleavage activity of caspase-3 between DEVD- and IETD-based substrates, the recombinant GST:DEVD:EGFP and GST:IETD:EGFP protein substrates were constructed as described in the Materials and Methods section. The fusion partners utilized in these experiments harbor the DEVD or IETD consensus tetrapeptide sequences for caspase-3 or caspase-8, respectively. The chimeric protein substrate-based caspase assay evidences marked advantages, including simplicity, rapidity, and versatility, all of which will prove useful in the estimation of relative activity measurement. In brief, the presence of the DEVD or IETD motifs between GST and EGFP allowed for caspase-3-induced proteolytic cleavage at the P4 position (aspartate residue) of the cleavage site with DEVD, thus resulting in the removal of the EGFP moiety from the chimeric GST:DEVD:EGFP of GST:IETD:EGFP proteins (Fig. 1). Accordingly, the exclusion of EGFP moiety yielded a corresponding reduction in the molecular mass (see Fig. 2B for Western blotting assay and Fig. 3B for SPR imaging measurement) as well as a substantial decline in EGFP fluorescence after proteolytic cleavage in response to caspase-3 (see Fig. 4A for on-chip visualization and Fig 4B for agarose bead assay). Consequently, the differences in substrate specificity of caspase-3 could be convincingly analyzed using these chimeric protein substrates through mass and fluorescence analyses.

Mass-Based Analyses for Detecting Caspase-3 Activation

In order to compare the caspase-3-induced proteolytic cleavage efficiency, the recombinant GST:DEVD:EGFP and GST:IETD: EGFP proteins were purified using an in-house purification system (Fig. 2A), and then treated with caspase-3 at the concentration observed to elicit the total proteolytic cleavage of the synthetic tetrapeptide fluorogenic substrate DEVD-AFC (R&D Systems). Recombinant caspase-3 activity was determined to be approximately proportional to its concentration, and the increase in caspase-3 activity was sustained to a level of 15 U (50 μl of 300 U/ml), at

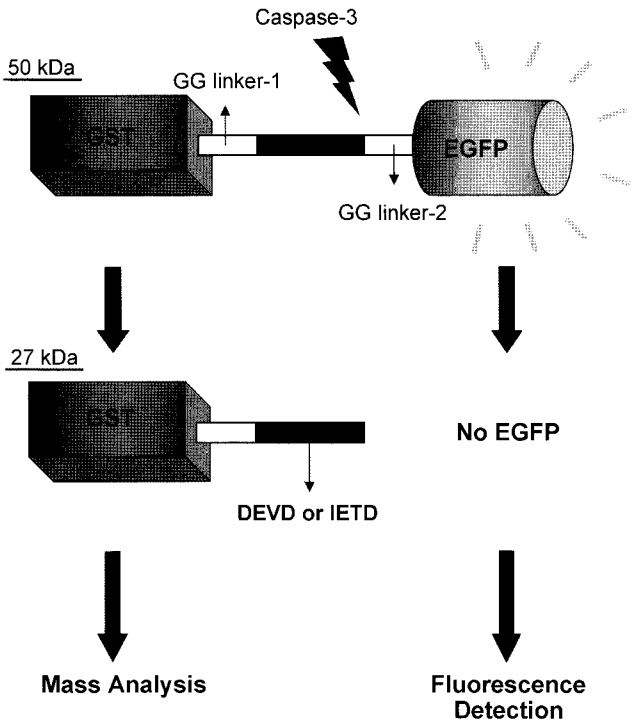

Fig. 1. Experimental concept for evaluating the cleavage preference for caspase-3 between GST:DEVD:EGFP and GST:IETD:EGFP. Cleavages of GST:DEVD:EGFP or GST:IETD:EGFP occur in response to caspase-3. The GST:DEVD:EGFP and GST:IETD:EGFP proteins harbored flanking linkers on both sides of the DEVD and IETD motifs. The flanking linkers at the N-terminus or C-terminus of DEVD or IETD were artificial sequences consisting of two glycine residues, respectively.

which concentration the proteolytic activity of caspase-3 may be saturated (data not shown). Thus, 15 U of caspase-3 was exploited for further experiments with the *in vitro* monitoring of cleavage preference for caspase-3 using the chimeric substrates. Western blot analysis with anti-GST antibody was conducted in order to detect the cleavage activity of these proteolytic substrates in response to caspase-3. As shown in Fig. 2B, approximately 49% of the GST:IETD:EGFP fractions were partially cleaved in comparison with that observed with GST:DEVD:EGFP, thereby suggesting that caspase-3 could proteolyze GST: DEVD:EGFP as well as GST:IETD:EGFP, even though the DEVD-based substrate is more efficient than the IETD-based substrate.

Besides Western blotting, SPR imaging technology also provides a good example for mass detection. This technology can be employed for the detection of events on a surface, because the addition/removal of biomolecules to/from the surface alters the resonance, thereby changing the percent of light reflected, which is proportional to the change in mass on the surface. Using this optical detection system, we evaluated the substrate preference for caspase-3 between GST:DEVD:EGFP and GST:IETD:EGFP on a gold chip surface. Prior to the immobilization of GST:DEVD:EGFP

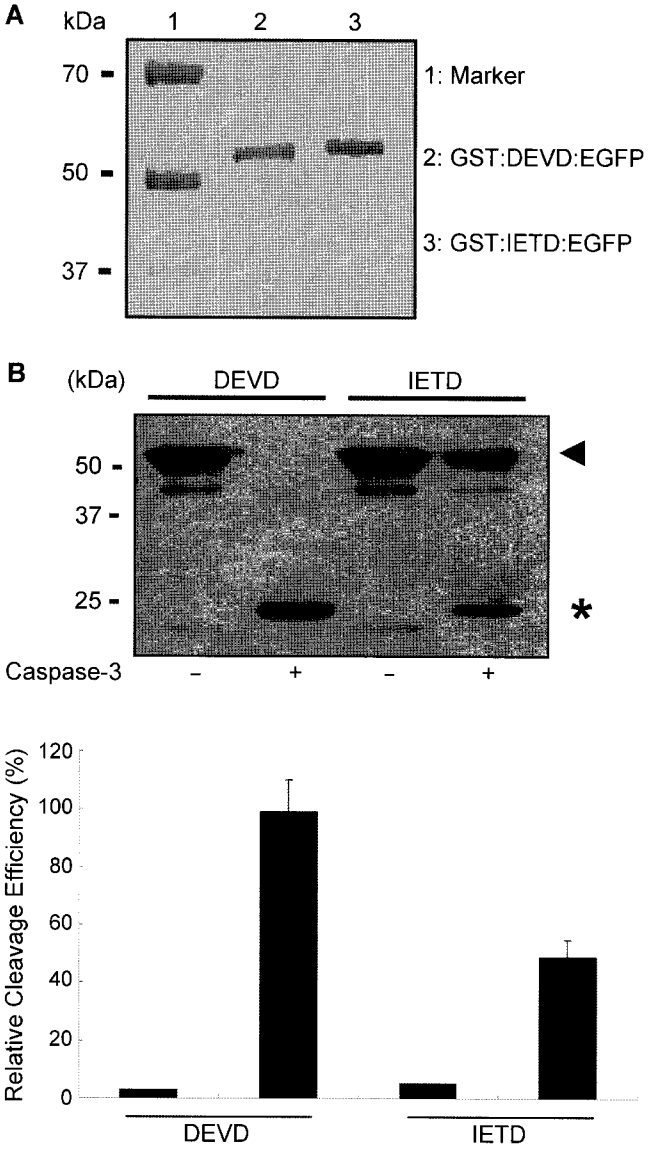

Fig. 2. Immunoblotting analysis for studying caspase-3-dependent substrate cleavage preference.
A. Purification of GST:DEVD:EGFP (52 kDa) and GST:IETD:EGFP (52 kDa) for use in the measurement of caspase-3-mediated proteolytic cleavage. The purified GST:DEVD:EGFP and GST:IETD:EGFP proteins were visualized *via* Coomassie staining. **B.** Western blotting showing the cleavage of GST:DEVD:EGFP and GST:IETD:EGFP by caspase-3. Western blotting was achieved with anti-GST antibody to detect the GST-fused proteins. The migration positions of the intact substrates (arrowhead; GST:DEVD:EGFP or GST:IETD:EGFP) and cleaved fragments (asterisk; GST:DEVD or GST:IETD) were indicated. The blots were analyzed using a densitometer; Fuji Film (Tokyo, Japan).

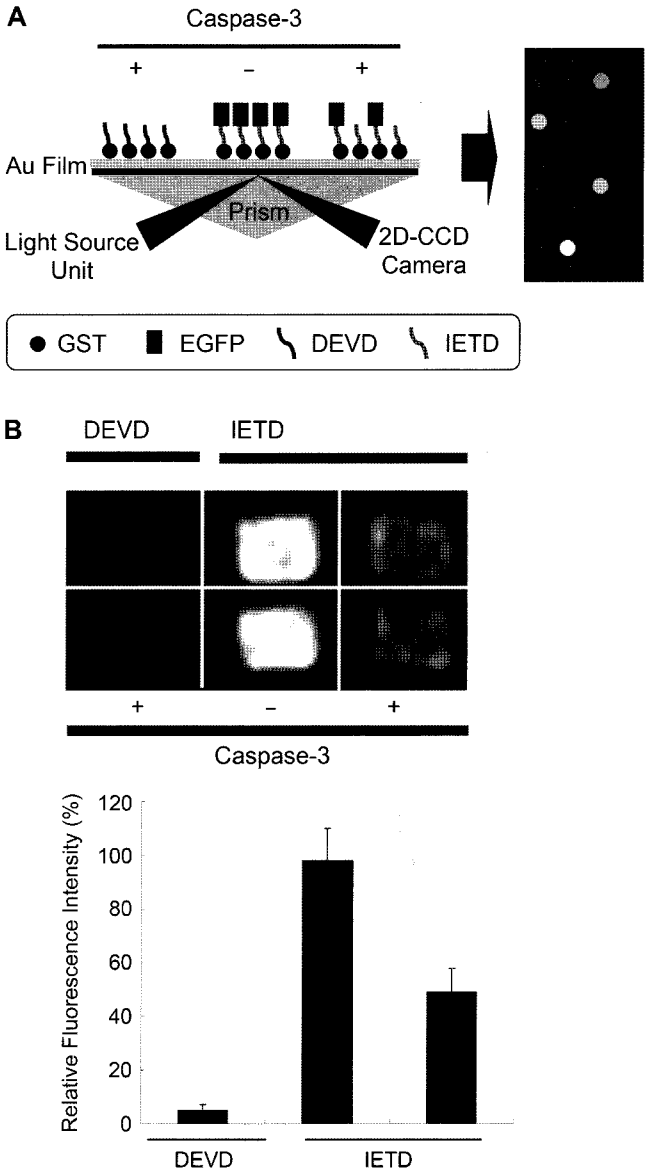

Fig. 3. SPR imaging analysis.
A. Schematic diagram of SPR imaging measurement. The purified GST:DEVD:EGFP, and GST:IETD:EGFP were immobilized onto a glutathionylated gold chip surface, and subsequently analyzed using SPR imaging under caspase-3 treatment conditions. The caspase-3-induced cleavages of proteolytic substrates resulted in the loss of EGFP from GST:DEVD:EGFP or GST:IETD:EGFP attached onto the chip surface, thus allowing for alterations in the SPR images. **B.** The SPR image intensity of the GST:DEVD:EGFP as a positive control was set at 0%, and the imaging intensity relative to the control was provided. The lower panel shows the relative intensity of substrate cleavage in response to caspase-3.

and GST:IETD:EGFP on a glutathionylated gold chip surface, the surface modification of the gold chip for the specific binding of these GST fusion proteins was conducted as described in the Materials and Methods section. Following the immobilization of the chimeric protein substrates, the GST:DEVD:EGFP- or GST:IETD:EGFP-spotted gold surfaces were incubated with 15 U of caspase-3 for 1 h at 25°C for the analysis of proteolytic cleavage by caspase-3 using the SPR imaging system. This experimental concept will be illustrated by the notion that the loss of EGFP moiety elicits a subsequent reduction in the molecular mass of the GST:DEVD:EGFP or GST:IETD:EGFP substrates, thereby

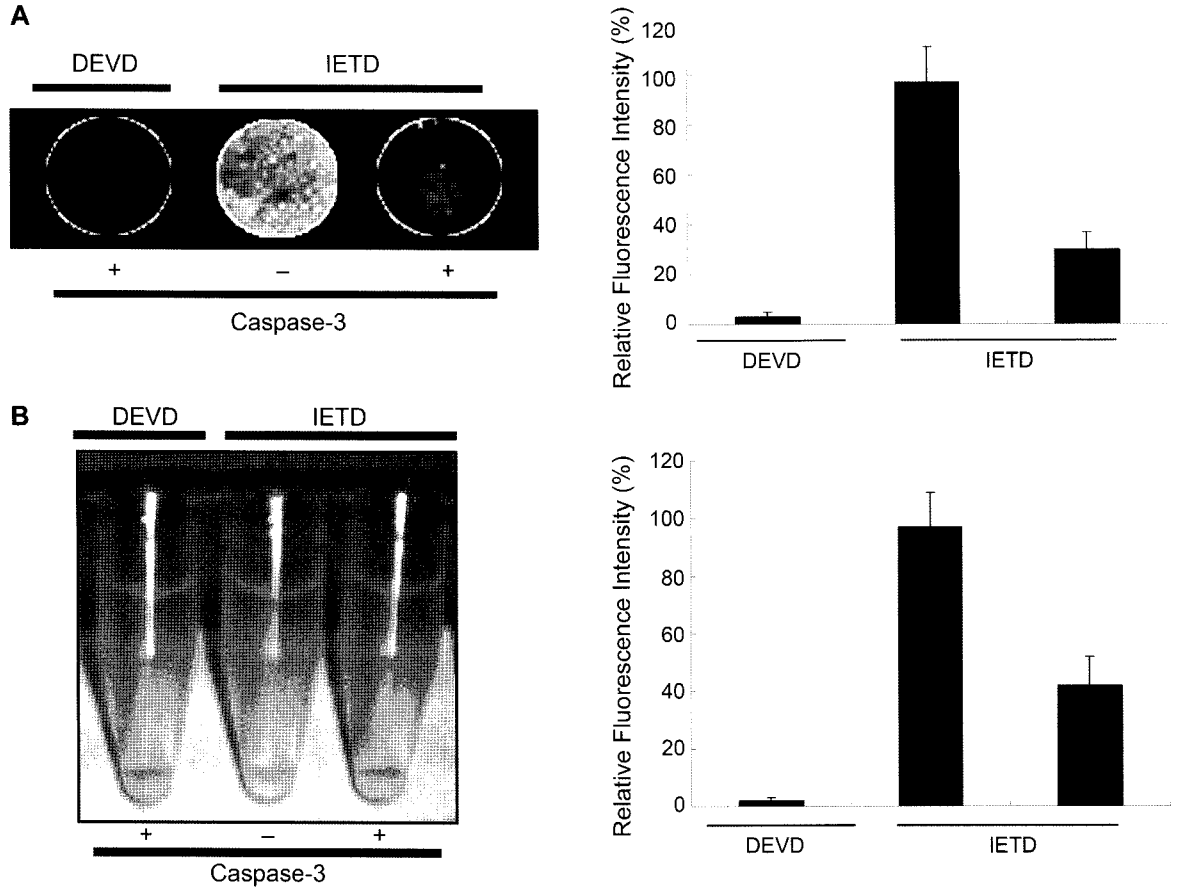

Fig. 4. Fluorescence-based analyses.
A. On-chip visualization assay. Green fluorescent signals indicate EGFP. Fluorescent signals of GST:DEVD:EGFP and GST:IETD:EGFP on a glass chip were obtained with a GenePix 4200A 488 nm laser. **B.** Glutathione–agarose bead assay. Stimulation of GST:DEVD:EGFP with caspase-3 exhibited complete decline of EGFP (compare 3rd tube with 1st tube). The graphs show the relative intensity of substrate cleavage in response to caspase-3.

resulting in the observed alterations in the SPR imaging signal intensity (Fig. 3A).

The SPR images were captured at a slightly smaller incident angle than the SPR angle of the chip surface matching the bare surface, where the difference of the intensity among SPR images was at a maximum. Accordingly, the brighter areas in the SPR images are reflective of the existence of the innate GST:DEVD:EGFP or GST:IETD:EGFP proteins on the gold chip surface. As shown in Fig. 3B, the brighter SPR image with GST:IETD:EGFP on a gold surface was observed without caspase-3 addition, implying no proteolytic cleavage. By contrast, a substantial reduction in the SPR image signal intensity was detected with GST:IETD:EGFP in response to caspase-3. The GST:DEVD:EGFP substrate exhibited even more significant weakness in the SPR image signal intensity than GST:IETD:EGFP under caspase-3 treatment (compare lane 1 with lane 3 in Fig. 3B). This finding also indicated that GST:DEVD:EGFP is more specific for caspase-3 than GST:IETD:EGFP, which corresponds to the result obtained by Western blotting assay in Fig. 2B.

Fluorescence-Based Analyses of the Proteolytic Cleavage for Caspase-3

Furthermore, in order to more stringently evaluate the caspase-3-dependent cleavage efficiency between GST:DEVD:EGFP and GST:IETD:EGFP, we conducted fluorescence analysis. This assay concept will be illustrated *via* the use of an experimental procedure referred to as on-chip visualization. First, a glass chip surface was selectively modified for the bioaffinity-based oriented immobilization of these substrates on the surface, which was identical to that of the SPR imaging protein chip. Following the on-chip immobilization of these protein substrates, the GST:DEVD:EGFP- or GST:IETD:EGFP-spotted glass chip surface was incubated with 15 U of caspase-3 for 1 h at 25°C. As shown in Fig. 4A, a very distinct green fluorescence image on a glass chip surface was observed with GST:IETD:EGFP in the absence of caspase-3 as a fluorescence image saturation. However, a considerably reduced green fluorescence image was obtained with GST:IETD:EGFP in response to caspase-3, indicating the caspase-3-induced cleavage of GST:IETD:EGFP substrate. Meanwhile, no

visible green fluorescence image was detected with GST:DEVD:EGFP, the preferred substrate for caspase-3, in the presence of caspase-3. The results clearly indicated the complete elimination of the EGFP moiety from the GST:DEVD:EGFP protein immobilized onto the glass chip surface. Fig. 4A revealed that the cleavage efficiency of GST:DEVD:EGFP in response to caspase-3 is approximately 3.2 times higher than that observed with GST:IETD:EGFP under caspase-3 treatment, thereby demonstrating that GST:DEVD:EGFP is a better substrate for caspase-3 than GST:IETD:EGFP.

In order to confirm the substrate preference of caspase-3 in a bead format consisting of the GST:DEVD- or GST:IETD-tagged EGFP bound to glutathione-coated agarose beads, GST:DEVD:EGFP and GST:IETD:EGFP were attached to glutathione–agarose beads. The beads conjugated with these chimeric protein substrates were green-fluorescent for ease of visualization, and were used for the measurement of caspase-3-dependent proteolytic cleavage. The EGFP moiety can be liberated from the GST:DEVD:EGFP or GST:IETD:EGFP substrates immobilized to the glutathione–agarose beads under caspase-3 exposure (Fig. 4B). The GST:DEVD:EGFP- or GST:IETD:EGFP-modified agarose beads were incubated with 15 U of caspase-3 for 1 h at 25°C. In order to verifiably remove the cleaved EGFP, these resultant beads were washed thoroughly in PBS buffer. As shown in Fig. 4B, a complete decline in the EGFP fluorescence image was observed with GST:DEVD:EGFP in response to caspase-3, revealing the complete cleavage of GST:DEVD:EGFP by caspase-3. In sharp contrast, after caspase-3 treatment, a partial reduction in the green fluorescence signal was detected with GST:IETD:EGFP, implying the incomplete cleavage of IETD-based substrate in the presence of caspase-3 (compare 3rd tube with 1st tube in Fig. 4B). A distinctly visible EGFP signal was detected with caspase-3-untreated GST:IETD:EGFP as a negative control. The efficiency of caspase-3-induced proteolytic cleavage with GST:DEVD:EGFP was shown to be approximately 3.8 times higher than that obtained with GST:IETD:EGFP in the glutathione–agarose bead assay, thus showing that the GST:IETD:EGFP substrate could be partially, but not completely, cleaved by caspase-3. These results obtained from the fluorescence measurements also give independent confirmation of the mass analyses (Figs. 2 and 3).

In conclusion, we have described here another application of recombinant protein substrates for evaluating the preference of substrate cleavage for caspase-3, which simultaneously allows for mass and fluorescence measurements. For this aim, two kinds of chimeric substrates, GST:DEVD:EGFP and GST:IETD:EGFP harboring amino acid linkers that possessed DEVE and IETD sequences, were employed and simply produced by the biochemical laboratory protein purification system. With these potent protein substrates, the proteolytic cleavage preference for caspase-3 could be successfully monitored by using a variety of bioanalytical techniques.

Acknowledgments

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