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A novel firefly luciferase biosensor enhances the detection of apoptosis induced by ESAT-6 family proteins of *Mycobacterium tuberculosis*

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ABSTRACT

The activation of caspase-3 is a key surrogate marker for detecting apoptosis. To quantitate caspase-3 activity, we constructed a biosensor comprising a recombinant firefly luciferase containing a caspase-3 cleavage site. When apoptosis was induced, caspase-3 cleavage of the biosensor activated firefly luciferase by a factor greater than 25. The assay conveniently detected apoptosis in real time, indicating that it will facilitate drug discovery. We screened ESAT-6 family proteins of *Mycobacterium tuberculosis* and found that esxA, esxT and esxL induced apoptosis. Further, activation of nuclear factor-κB (NF-κB) and the NF-κB-regulated genes encoding tumor necrosis factor-α (TNF-α) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) participated in esxT-induced apoptosis. We conclude that this assay is useful for high-throughput screening to identify and characterize proteins and drugs that regulate apoptosis.

1. Introduction

Although apoptosis is a normal and vital process, dysregulated apoptosis contributes to a great variety of diseases, and the level of apoptosis contributes to their diagnosis [1,2]. However, available methods fall short in their capabilities to assess and quantify the efficacy of drug interventions to induce or inhibit apoptotic signaling events, particularly in cell culture screens. Therefore, a fast, accurate, and convenient method for detecting apoptosis is urgently required.

Numerous methods are available for detecting apoptosis that determine caspase activation. Caspase-3 and caspase-7 are critical mediators of apoptosis induced by different factors [3], and methods for determining their activities are widely used to detect apoptosis [4]. Initially, fluorescence resonance energy transfer (FRET) pairs [5] and dark-to-bright fluorescence [6] emitted by peptides containing caspases-3 cleavage sites were used. Although a great improvement, the sensitivities of these assays are very low because they generate only a small change in fluorescence intensity when cells undergo apoptosis. Assays for firefly luciferase activity are more sensitive and convenient compared with assays of fluorescence emission. Firefly luciferase biosensors are widely used to detect apoptosis, enzyme activity, screening for anti-apoptotic drugs and identifying enzyme recognition sequences [7–9]. To increase the sensitivity of luciferase assays for detecting apoptosis, the random mutated thermal stable luciferase construct permuted at 358 or 233 was fused with a caspase-3 cleavage signal sequence-DEVDG. Although the sensitivity of luciferase 358 or 233 reporters for detecting apoptosis is high, background signals are problematic [8,9]. An alternative reporter comprises a genetically encoded switch-on fluorescence-based caspase-3-like protease activity indicator (C3AI) generated by fusion of two Venus domains with an *Npu* DnaE intein [10]. However, the complex imaging process limits its use for high-throughput screening (HTS) assays. Therefore, a simple, quick, and convenient method is urgently required to detect apoptosis.

To address this challenge, we integrated C3AI and firefly luciferase to construct a recombinant luciferase. The recombined firefly luciferase contained the DEVDG sequence inserted at the termini as well as an *Npu* DnaE intein linked to amino acid residues 358 or 233. The recombinant firefly luciferase was circular under the normal state, its luciferase activity was very low, and it emitted lower levels of background signals, compared with other methods. When DEVDG was cleaved by caspase-3/7 during apoptosis the recombinant luciferase linearized and became fully active, which greatly improved the sensitivity.

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Mycobacterium tuberculosis (MTB) causes approximately eight million new cases of tuberculosis (TB) each year and almost two million deaths and therefore represents one of the most infectious causes of death worldwide [11]. Apoptosis of host cells is a common feature of virulent strains of MTB that promotes bacterial colonization and dissemination of bacteria through the host [12,13]. The effects of MTB on apoptosis contribute to the pathogenesis of tuberculosis, which are mediated through the activities of specific proteins encoded by its genome. For example, PE-PGRS33, the 19 kDa lipoprotein (LpqH) and the 38 kDa lipoprotein execute macrophage apoptosis during infection [14]. ESAT-6 (esxA) is located in RD1 encoding ESX-1 closely related to the virulence of MTB, which exists in *M. tuberculosis* but not in Bacillus Calmette–Guérin (BCG) [15,16]. An ESAT-6 mutant induces apoptosis less effectively compared with wild-type MTB [17]. Further, ESAT-6 induces apoptosis through the receptor-mediated extrinsic pathway as well as the endoplasmic reticulum (ER) stress pathway [18,19]. The ESAT-6 family comprises 23 members named *esxA–W* that are all secreted and shows high homologues [20]. Therefore, further research is required to identify ESAT-6 family members other than ESAT-6 that induce apoptosis.

We chose to employ ESAT-6 family members to validate the reliability and feasibility of the firefly luciferase assay described above for detecting apoptosis. Using this reproducible and efficient assay, we show here that esxA, esxT and esxL induced apoptosis, and we investigated the potential molecular mechanism of the apoptotic effects of esxT. Moreover, this is the first report demonstrating that esxT induces apoptosis.

2. Materials and methods

2.1. Plasmids and reagents

The construction of 233-DEVDG and 358-DEVDG recombinant firefly luciferases was described previously [8,9]. DNA encoding the Met-(233-544)-GSS-DEVDG-SSG-(2-232)-VAL fusion protein was cloned into the pCAGGS-MCS vector to generate 233-DEVDG. DNA encoding the Met-(358-544)-GSS-DEVDG-SSG-(4-354)-VAL fusion protein was cloned into the pCAGGS-MCS vector to generate 358-DEVDG. DNA encoding the C-fragment, N-fragment and the conserved residues of *Npu* DnaE intein [10] were synthesized and cloned into the pCAGGS-MCS vector to construct pCAGGS-MCS-DnaE with *EcoRI* and *BglII* restriction sites for the insertion of DNA encoding the fusion protein. DNA encoding the Met-(233-544)-GSS-DEVDG-SSG-(2-232)-VAL fusion protein was cloned into the pCAGGS-MCS-DnaE to generate 233-DnaE-DEVDG. DNA encoding the Met-(358-544)-GSS-DEVDG-SSG-(4-354)-VAL fusion protein was cloned into the pCAGGS-MCS-DnaE to generate 358-DnaE-DEVDG. The fusion protein had *EcoRI* and *BamHI* restriction sites at the N and C termini. pCAGGS-MCS and pCAGGS-MCS-DnaE were cleaved by *EcoRI* and *BglII* restriction sites. *BglII* and *BamHI* restriction sites were both destroyed during the ligation step. The polypeptide GSGCG with *NheI* and *BglII* restriction sites was synthesized to replace DEVDG to generate 233, 358, 233-DnaE and 358-DnaE [8,10].

The hemagglutinin (HA) epitope tag was amplified using PCR to generate a pCAGGS-HA-C plasmid with a C-terminal HA-tag. Genes encoding ESAT-6 family members of *M. tuberculosis* H37Rv strain were synthesized and cloned into the pCAGGS-HA-C vector linked to an HA-tag on their C-terminus. All constructs were confirmed using DNA sequencing. The plasmids pNF-κB-Luc as well as pRL-TK that expresses *Renilla reniformis* luciferase were purchased from Stratagene. Cycloheximide (CHX) and nuclear factor-kappaB (NF-κB) inhibitor (BAY11-7082) were purchased from Sigma–Aldrich. TNF-α was purchased from Sino Biological Inc. (China). Antibody against caspase-3 was obtained from Biolegend (USA). Anti-cleaved-caspase-3 antibody was purchased from Cell Signaling Technology. Anti-HA and anti-beta-actin antibodies were purchased from ABclonal Biotechnology. Anti-firefly luciferase antibody was purchased from MBL International Corporation (Japan). Horseradish-peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were purchased from the Beyotime Institute of Biotechnology (Jiangsu, China).

2.2. Cell culture and transfection

HeLa cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco) at 37 °C in an atmosphere containing 5% CO_2. Lipofectamine 2000 (Invitrogen) was used to transfect cells with the plasmids according to the manufacturer's instructions.

2.3. Western blotting

After washing twice with phosphate-buffered saline, cells were lysed in 2× lysis buffer A [65 mM Tris–HCl (pH 6.8)], 4% SDS, 3% DTT, and 40% glycerol), and proteins in the extracts were separated using 12% SDS–PAGE and then electrophoretically transferred onto PVDF membranes (Millipore, Billerica, MA). The membranes were incubated with antibodies and secondary antibodies.

2.4. Reporter assay

HeLa cells were plated into 48-well plates. The luciferase reporter constructs 233-DnaE-DEVDG, 358-DnaE-DEVDG, 233-DEVDG, and 358-DEVDG were used to determine caspase-3 activity. Cells were co-transfected with each luciferase reporter plasmid and pRL-TK using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with either TNF-α/CHX or not for another 24 h. The cells were lysed and assayed for luciferase activity using a luciferase reporter assay system (Promega). All reporter assays were repeated at least three times. Data are presented as the mean ± standard deviations (SD).

2.5. Real-time RT-PCR

Total cellular RNA was extracted from HeLa cells using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to reverse transcribe the RNA. The products were analyzed using a SYBR Green PCR assay (Applied Biosystems) using gene-specific primers. The primes used were supplied in Supplementary Table.

2.6. Apoptosis assay

Twenty-four hours after transfection with the indicated plasmids, HeLa cells in 6-well plates were treated with TNF-α/CHX for another 24 h, harvested, and assayed using an annexin-V-FITC and propidium iodide (PI) apoptosis detection kit (Invitrogen) according to the manufacturer's instructions.

2.7. Statistical analysis

Results are presented as the mean ± SD of at least three experiments. One-way analysis of variance (ANOVA) without terms followed by Dunnett's test was used for multiple comparisons. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$).

3. Results

3.1. Generation of reporters to detect caspase-3 protease activation

To develop a more sensitive reporter to detect caspase-3 activity, we constructed four recombinant luciferases containing the caspase-3 cleavage site as follows: 233-DEVDG, 358-DEVDG, 233-DnaE-DEVDG and 358-DnaE-DEVDG (Fig. 1). Upon caspase-3 activation in response to apoptosis, cleavage of 233-DEVDG or 358-DEVDG at the DEVDG site reconstitutes an active luciferase. However, spontaneous interaction of the two domains, an event that occurs with small probability, would reduce sensitivity. Further, without cleavage, the N-fragment of 233-DEVDG or 358-DEVDG might complement the C-fragment of the 233-DEVDG or 358-DEVDG proteins to form an active firefly luciferase, leading to an increase in the background of the assay (Supplementary Fig. S1A).

To enhance the efficient complementation of the 233-DEVDG or 358-DEVDG cleavage products, we fused a split *Npu* DnaE intein to the N- and C-termini of 233-DEVDG or 358-DEVDG to generate 233-DnaE-DEVDG or 358-DnaE-DEVDG (Supplementary Fig. S1B). The *Npu* DnaE intein comprising N- and C-fragments would cyclize the firefly luciferase without affecting its activity [10]. Proteolytic cleavage converts the circular 233-DnaE-DEVDG or 358-DnaE-DEVDG proteins to linear form. The background activities of the circular forms of 233-DnaE-DEVDG or 358-DnaE-DEVDG were lower because of steric hindrance. In contrast, their linear forms possessed full enzymatic activity because the *Npu* DnaE intein did not affect activity. Theoretically, assays using 233-DnaE-DEVDG and 358-DnaE-DEVDG should provide higher sensitivity and lower background (Supplementary Fig. S1A). The DEVDG sequence in the 233, 358, 233-DnaE and 358-DnaE proteins was replaced by GSGCG to serve as a control for their cognate proteins [10].

3.2. 233-DnaE-DEVDG faithfully indicates TNF-α-induced apoptosis in cells

HeLa cells were used to test the four recombinant luciferase reporters. TNF-α and CHX triggered significant levels of apoptosis, consistent with previous reports [10,21] (Fig. 2A). Cells were co-transfected with pRL-TK and each of the four reporters or their respective controls. pRL-TK was used to normalize the transfection efficiency. The levels of apoptosis were conveniently determined using the dual-luciferase reporter system. The activities of the four reporters increased significantly as a function of increased apoptosis. However, except for 233-DnaE, the activity of the 233, 358 and 358-DnaE reporters also increased, which led to increased backgrounds and prevented their use for further studies. In contrast, the increase in the activity of 233-DnaE-DEVDG was significant while that of 233-DnaE remained low when apoptosis increased (Fig. 2B). Therefore, only 233-DnaE-DEVDG reproducibly indicated apoptosis with increased sensitivity and decreased background.

To further confirm the correlation between the change of the fold induction and the indicators cleavage in apoptotic process, Western-blot analysis was carried out to analysis the cleavage of the four indicators. Caspase-3 and the four reporters were simultaneously activated during TNF-α/CHX-induced apoptosis (Fig. 2C). Cleaved 233-DnaE-DEVDG and 358-DnaE-DEVDG were identified according to their faster mobility compared with their cyclized forms. Further, 233-DnaE-DEVDG was cleaved and its activity increased during apoptosis, in contrast to the uncleaved control (233-DnaE) with low activity. These data indicate that the increased activity was generated by the cleavage of the DEVDG sequence (Fig. 2B and C). The results of Western blot analysis indicated that the increased activity of the reporters accurately reflected the activation of caspase-3 and caspase-3-dependent apoptosis. Further, the dose-dependent cleavage of 233-DnaE-DEVDG was accompanied by increased apoptosis (Fig. 2D). Therefore, 233-DnaE-DEVDG was used for the studies that follow.

3.3. Analysis of the ability of ESAT-6 family members to induce apoptosis

Considering the fact that *M. Tuberculosis* an intracellular pathogen mainly interacts with the host through its membrane or secreted proteins in the cells [22] and the prokaryotic expression proteins are difficult to entry cells, we transfected HeLa cells with ESAT-6 family members in a mammalian vector, dissecting their capabilities to modulate apoptosis using 233-DnaE-DEVDG. These ESAT-6 expression plasmids were co-transfected with 233-DnaE-DEVDG and pRL-TK and luciferase activities were determined.

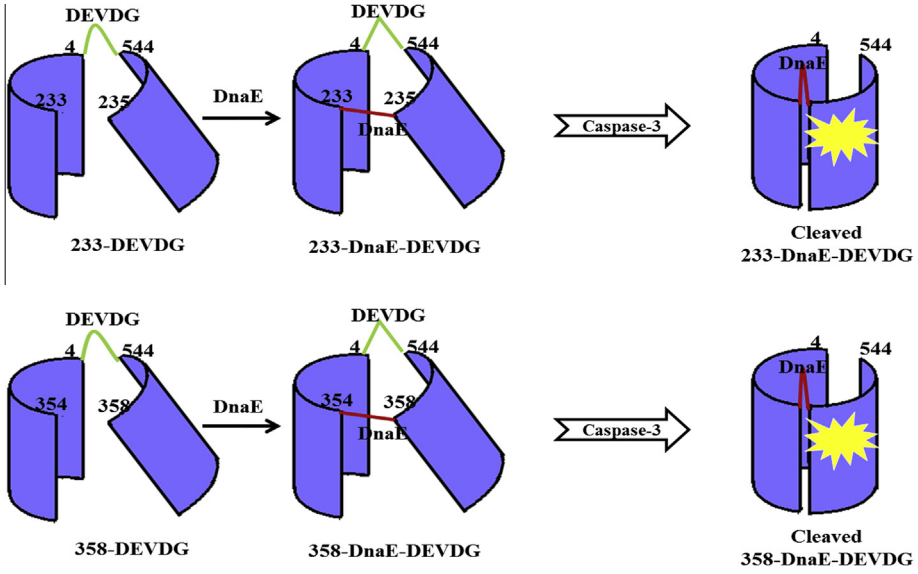

Fig. 1. Scheme for the generation of 233-DEVDG, 233-DnaE-DEVDG, 358-DEVDG and 358-DnaE-DEVDG. The green line presents the peptide sequences of DEVDG and the red line presents the peptide sequences of *Npu* DnaE intein. The blue box present the peptide sequences of recombined firefly luciferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

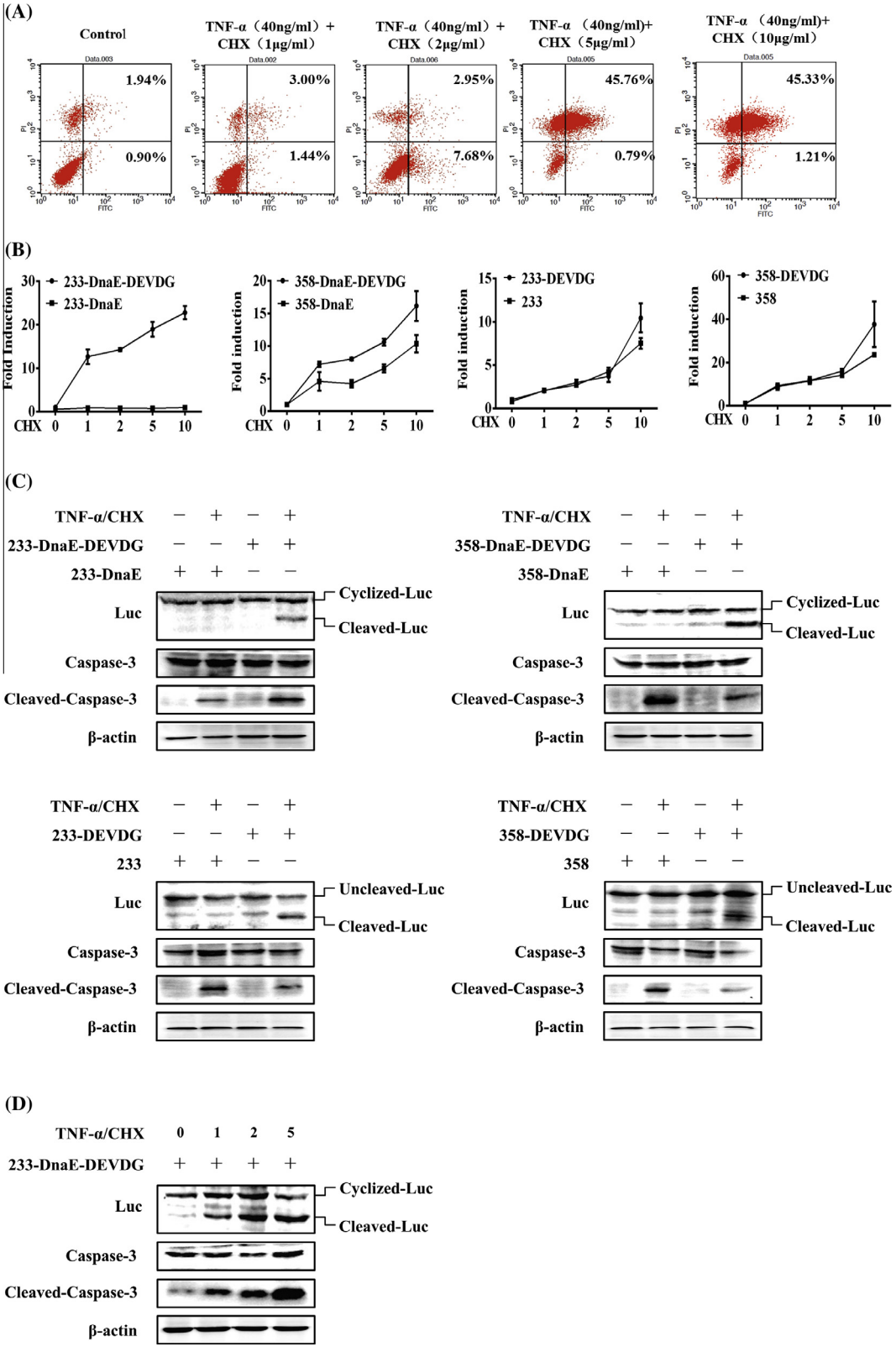

Fig. 2. 233-DnaE-DEVDG faithfully indicates TNF-α-induced apoptosis in cells. (A) HeLa cells were treated with 40 ng/ml of TNF-α/different concentrations of CHX as indicated for 24 h and then detected through flow cytometry. (B) 233-DnaE-DEVDG, 358-DnaE-DEVDG, 233-DEVDG or 358-DEVDG (0.1 μg) was co-transfected with pRL-TK (0.02 μg). At the same time, 233-DnaE, 358-DnaE, 233 or 358 was used as the control. 24 h later, HeLa cells were treated with 40 ng/ml of TNF-α/different concentrations of CHX as indicated for another 24 h. Then HeLa cells were lysed and assayed using the dual-luciferase assay. (C) HeLa cells were transfected with 233-DnaE-DEVDG, 358-DnaE-DEVDG, 233-DEVDG or 358-DEVDG (4 μg). 233-DnaE, 358-DnaE, 233 or 358 was used as the control. 24 h later, HeLa cells were treated with 40 ng/ml of TNF-α and 5 μg/ml of CHX or remained untreated for another 24 h. Western-blot analysis of the levels of the cleavage of the indicators, caspase-3, cleaved-caspase-3 and β-actin. (D) Western-blot analysis of cleavage of 233-DnaE-DEVDG, caspase-3, cleaved-caspase-3 and β-actin in HeLa cells after treatment with 40 ng/ml of TNF-α/different concentrations of CHX as indicated for 24 h.

pRL-TK was used to normalize the transfection efficiency. TNF-α, a key pro-inflammatory mediator elicited during *M. Tuberculosis* infection, was involved in regulating inflammation and apoptosis. Promoting TNF-α induced apoptosis might be an essential mechanism for *M. Tuberculosis* to modulate apoptosis. Therefore, we also screened which protein could promote TNF-α induced apoptosis. EsxA and esxT strongly activated 233-DnaE-DEVDG when cells were treated with TNF-α or remained untreated. EsxL activated 233-DnaE-DEVDG only when the cells were treated with TNF-α (Supplementary Fig. S2). To further confirm the capabilities of the MTB proteins to activate 233-DnaE-DEVDG, HeLa cells were transfected with increasing quantities of the cognate expression plasmids together with 233-DnaE-DEVDG and pRL-TK. The ESAT-6 family members esxA and esxT induced apoptosis as a function of their dose (Fig. 3A and B), and EsxL enhanced TNF-α-induced apoptosis according to its dose as well (Fig. 3C). Activation of 233-DnaE-DEVDG by EsxC was undetectable, which was consistent with the screening result (Fig. 3D).

3.4. Validation of the screening results

Because esxA (ESAT-6) and esxT induced significant increases in apoptosis, they were used for further analyses. EsxT and esxA increased the level of cleaved caspase-3 (Fig. 4A and B), which confirmed the results of flow cytometry using Annexin-V-FITC and PI staining of transfected Hela cells (Fig. 4C and D). EsxC was used as a negative control. Caspase-3 activation was undetectable in Western blot analysis of cells transfected with esxC (Fig. 4E), which was consistent with the screening results. Esx-A (ESAT-6) induced apoptosis, which is consistent with previous reports [18]. Further, the present study is the first to report that esxT promoted apoptosis. It was a fine illustration of 233-DnaE-DEVDG's ability to detect apoptosis and suitability for high-throughput screening.

3.5. Activation of NF-κB mediates esxT-induced apoptosis

NF-κB transcription factors are major regulators of apoptosis. Although NF-κB signaling can antagonize apoptosis by promoting the transcription of antiapoptotic genes, under certain conditions, NF-κB activation is associated with apoptosis [23,24]. Proapoptotic genes that are targets for NF-κB, such as TNF-α, TRAIL, P53, Fas, FasL, Bax may be involved in this effect [24–26]. We found that esxT induced a dose-dependent increase in NF-κB promoter activity (Supplementary Fig. S3A). To further explore the role of NF-κB activation in esxT-induced apoptosis, we used 233-DnaE-DEVDG to measure apoptosis after treating HeLa cells with the NF-κB inhibitor BAY11. Increasing concentrations of BAY11 reduced the level of esxT-induced apoptosis (Supplementary Fig. S3B), indicating that activation of NF-κB was involved. To identify the NF-κB proapoptotic target genes that were activated by esxT, we analyzed the expression levels of Bax, TNF-α, TRAIL, Bcl-Xs, and p53 using real-time RT-PCR (Supplementary Fig. S3C). Further, the mRNA and protein level of TNF-α and TRAIL were up-regulated by esxT (Supplementary Fig. S3C and D). Taken together, these results indicate that activation of NF-κB mediated esxT-induced apoptosis.

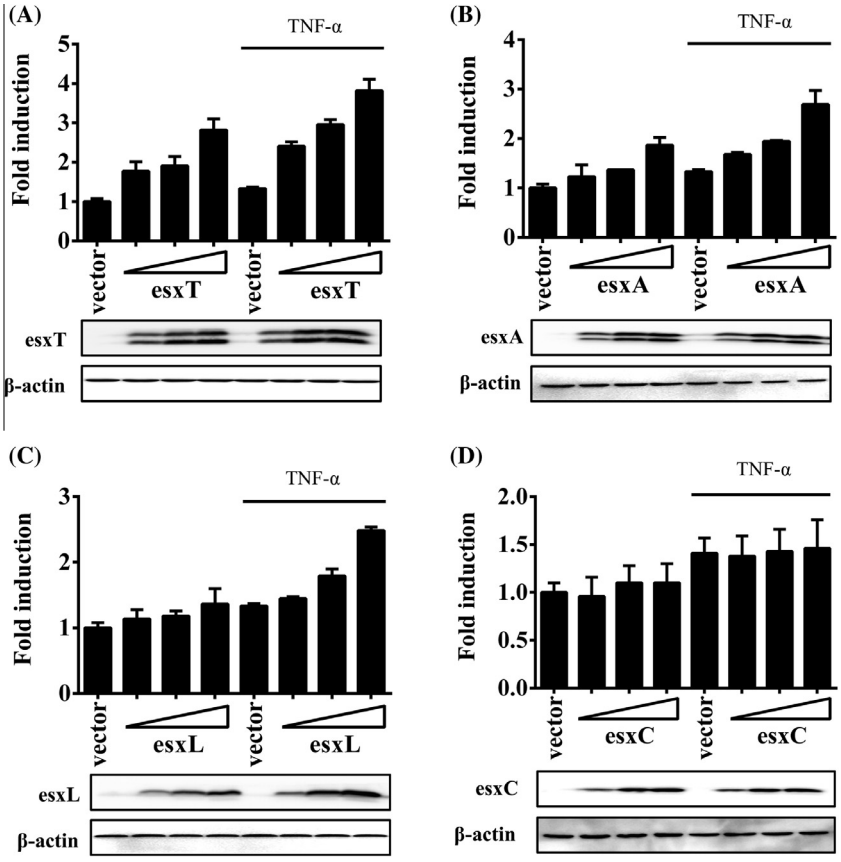

Fig. 3. Analysis of the ability of ESAT-6 family members to induce apoptosis. (A) HeLa cells grown in 48-well plates were transfected with the 233-DnaE-DEVDG reporter plasmid (0.1 μg), pRL-TK plasmid (0.02 μg) and increasing quantities (0, 0.1, 0.2, or 0.4 μg) of plasmid encoding esxT. After 24 h, cells were treated with TNF-α or not for another 24 h. Then HeLa cells were lysed and assayed using the dual-luciferase assay. Mouse anti-HA antibody was used to confirm the expression of esxT. (B) HeLa cells were transfected with esxA expression plasmid as described in (A). (C) HeLa cells were transfected with esxL expression plasmid as described in (A). (D) HeLa cells were transfected with esxC expression plasmid as described in (A).

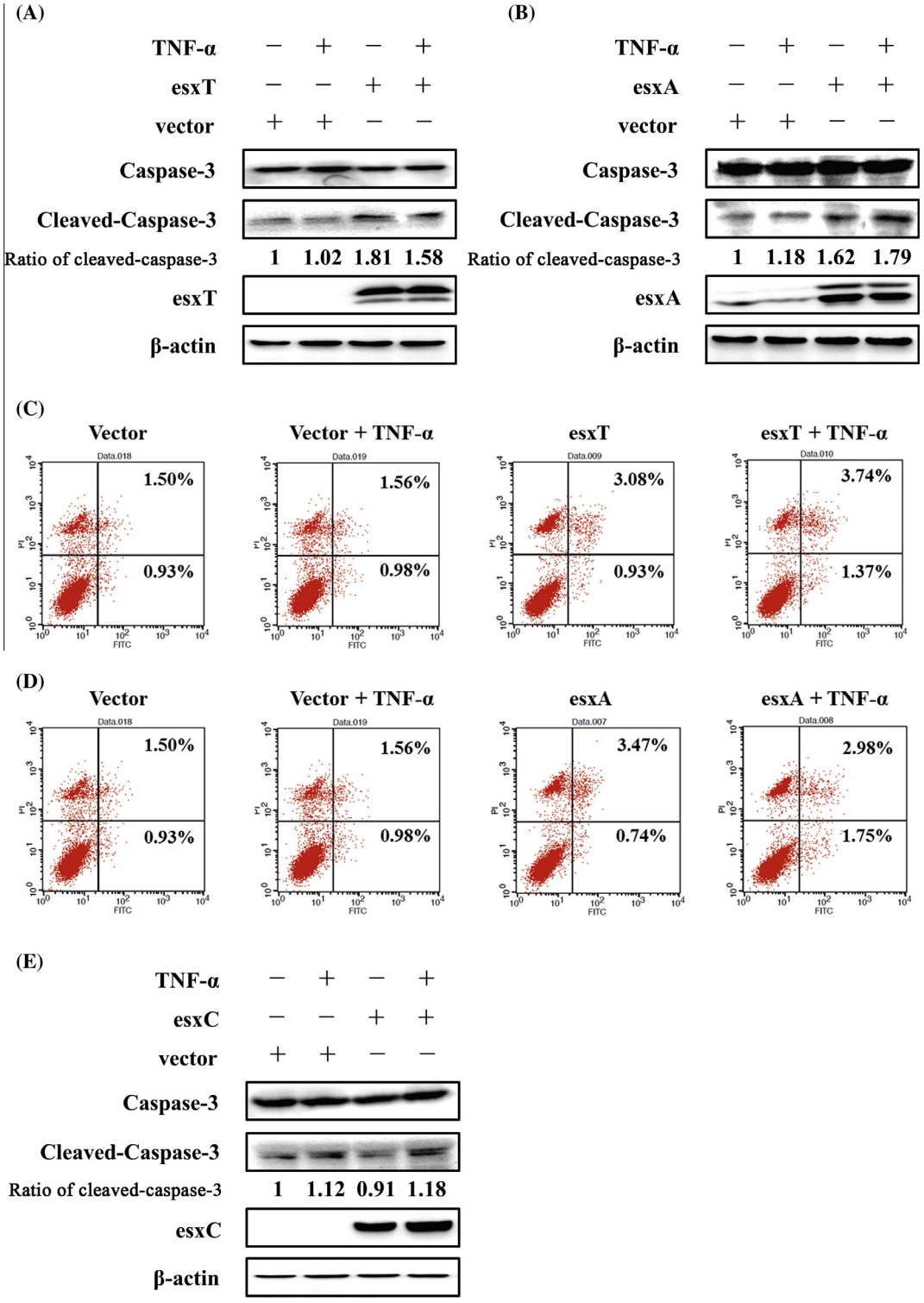

Fig. 4. Validation of the screening results. (A) EsxT enhanced the cleavage of caspase-3. HeLa cells were transfected with the plasmid encoding esxT (4 μg) or empty vector. After 24 h, cells were treated with TNF-α or untreated for another 24 h. Western-blot was performed on whole-cell lysates with the indicated antibodies. Mouse anti-HA antibody was used to confirm the expression of esxT. Quantification of the intensity of each band is listed under each band and total protein of β-actin was used as an internal control. (B) EsxA enhanced the cleavage of caspase-3. HeLa cells were treated as described in (A). (C) EsxT enhanced the level of apoptosis. HeLa cells were transfected with the plasmid encoding esxT or empty vector (4 μg). After 24 h, Cells were treated with TNF-α or not for another 24 h. The level of apoptosis was detected by flow cytometry through annexin-V-TITC and propidium iodide (PI) straining treated cells. (D) EsxA enhanced the level of apoptosis. HeLa cells were treated as described in (C). (E) EsxC could not enhance the cleavage of caspase-3. HeLa cells were treated as described in (A).

4. Discussion

To advance the development of new therapeutics and the evaluation of combination therapies for cancer, detection methods suitable for HTS are urgently required [4]. Because of its sensitivity and convenience, the dual-luciferase reporter system meets the requirements for screening [27]. Moreover, the caspase-3-based dual-luciferase reporter system specifically detected caspase-3-

related apoptosis, which provides a convenient assay that distinguishes between the different mechanisms of cell death.

Fusing the *Npu* DnaE intein renders C3AI a reliable indicator of apoptosis based on the emission of fluorescence by green fluorescent protein [10]. The *Npu* DnaE intein is commonly used to cyclize proteins without affecting activity [10,28,29]. Similarly, we employed the *Npu* DnaE intein here to cyclize 233-DEVDG to generate the new and improved reporter 233-DnaE-DEVDG. Using 233-DnaE-DEVDG, we detected apoptosis reproducibly, quantitatively and sensitively. Moreover, the background activity of 233-DnaE-DEVDG was lower before cleavage. Therefore, 233-DnaE-DEVDG has the potential to detect apoptosis in living animals. 233-DnaE-DEVDG was sensitive, because its active form with firefly luciferase is significantly distinct from the inactive state, which renders 233-DnaE-DEVDG as a very convenient and reliable indicator of apoptosis based on the emerging of firefly luciferase activity. The firefly luciferase activity of 233-DnaE-DEVDG and 358-DnaE-DEVDG increased significantly upon the induction of apoptosis. However, unlike the significant increase in the activity of 358-DnaE, that of 233-DnaE remained at a very low level. We hypothesized that the luciferase activity of 233-DnaE is higher than 358-DnaE [8] and is therefore not readily affected by apoptosis caused decrease in the activity of pRL-TK. The control 233 and 358 had high levels of bioluminescent signal, which was caused by the fact that the N-fragment of 233 or 358 might complement the C-fragment of the 233 or 358 proteins to form an active firefly luciferase as described in Fig. 1B. In summary, using the *Npu* DnaE intein to cyclize firefly luciferase significantly enhances the detection of apoptosis.

As ESAT-6 is one of the most extensively investigated proteins secreted by MTB and induces apoptosis and inflammation, ESAT-6 family members attract more attention. Therefore, we chose to determine the ESAT-6 family members that induced apoptosis and show here that esxA, esxL, and esxT exhibited this activity. Our findings that activation of NF-κB mediated esxT-induced apoptosis provide further evidence that ESAT-6 family members play a key role in apoptosis and inflammation. Further experiments are required to determine exactly how esxL induces apoptosis.

In summary, the 233-DnaE-DEVDG-luciferase reporter represents an improved biosensor that is sensitive and yields reproducible data. Therefore, it shows promise for screening for proteins and drugs that regulate apoptosis and for quantitating apoptosis. Among MTB ESAT-6 family proteins, esxA, esxT, and esxL induced apoptosis of HeLa cells. Moreover, activation of NF-κB was involved in esxT-induced apoptosis. These novel findings further illuminate the relationship between proteins secreted by MTB and MTB-induced apoptosis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.09.047.

References

[1] T. Yasuda, H. Mochizuki, The regulatory role of alpha-synuclein and parkin in neuronal cell apoptosis; possible implications for the pathogenesis of Parkinson's disease, Apoptosis 15 (2010) 1312–1321.
[2] Y.Y. Wang, D.Y. Ouyang, H. Huang, H. Chan, S.C. Tam, Y.T. Zheng, Enhanced apoptotic action of trichosanthin in HIV-1 infected cells, Biochem. Biophys. Res. Commun. 331 (2005) 1075–1080.
[3] S.A. Lakhani, A. Masud, K. Kuida, G.A. Porter Jr., C.J. Booth, W.Z. Mehal, I. Inayat, R.A. Flavell, Caspases 3 and 7: key mediators of mitochondrial events of apoptosis, Science 311 (2006) 847–851.
[4] O. Kepp, L. Galluzzi, M. Lipinski, J. Yuan, G. Kroemer, Cell death assays for drug discovery, Nat. Rev. Drug Discov. 10 (2011) 221–237.
[5] M. Rehm, H. Dussmann, R.U. Janicke, J.M. Tavare, D. Kogel, J.H. Prehn, Single-cell fluorescence resonance energy transfer analysis demonstrates that caspase activation during apoptosis is a rapid process. Role of caspase-3, J. Biol. Chem. 277 (2002) 24506–24514.
[6] S.B. Nicholls, J. Chu, G. Abbruzzese, K.D. Tremblay, J.A. Hardy, Mechanism of a genetically encoded dark-to-bright reporter for caspase activity, J. Biol. Chem. 286 (2011) 24977–24986.
[7] A. Kilianski, A.M. Mielech, X. Deng, S.C. Baker, Assessing activity and inhibition of Middle East respiratory syndrome coronavirus papain-like and 3C-like proteases using luciferase-based biosensors, J. Virol. 87 (2013) 11955–11962.
[8] S.S. Wigdal, J.L. Anderson, G.J. Vidugiris, J. Shultz, K.V. Wood, F. Fan, A novel bioluminescent protease assay using engineered firefly luciferase, Curr. Chem. Genomics 2 (2008) 16–28.
[9] S. Galban, Y.H. Jeon, B.M. Bowman, J. Stevenson, K.A. Sebolt, L.M. Sharkey, M. Lafferty, B.A. Hoff, B.L. Butler, S.S. Wigdal, B.F. Binkowski, P. Otto, K. Zimmerman, G. Vidugiris, L.P. Encell, F. Fan, K.V. Wood, C.J. Galban, B.D. Ross, A. Rehemtulla, Imaging proteolytic activity in live cells and animal models, PLoS One 8 (2013) e66248.
[10] J. Zhang, X. Wang, W. Cui, W. Wang, H. Zhang, L. Liu, Z. Zhang, Z. Li, G. Ying, N. Zhang, B. Li, Visualization of caspase-3-like activity in cells using a genetically encoded fluorescent biosensor activated by protein cleavage, Nat. Commun. 4 (2013) 2157.
[11] E. Vynnycky, P.E. Fine, Lifetime risks, incubation period, and serial interval of tuberculosis, Am. J. Epidemiol. 152 (2000) 247–263.
[12] J. Lee, T. Repasy, K. Papavinasasundaram, C. Sassetti, H. Kornfeld, *Mycobacterium tuberculosis* induces an atypical cell death mode to escape from infected macrophages, PLoS One 6 (2011) e18367.
[13] M. Rojas, L.F. Barrera, L.F. Garcia, Induction of apoptosis in murine macrophages by *Mycobacterium tuberculosis* is reactive oxygen intermediates-independent, Biochem. Biophys. Res. Commun. 247 (1998) 436–442.
[14] V. Briken, *Mycobacterium tuberculosis* genes involved in regulation of host cell death, Adv. Exp. Med. Biol. 783 (2013) 93–102.
[15] S.A. Stanley, S. Raghavan, W.W. Hwang, J.S. Cox, Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system, Proc. Natl. Acad. Sci. USA 100 (2003) 13001–13006.
[16] S. Chandra, M. Kaur, S. Midha, R. Bhatnagar, N. Banerjee-Bhatnagar, Evaluation of the ability of N-terminal fragment of lethal factor of *Bacillus anthracis* for delivery of Mycobacterium T cell antigen ESAT-6 into cytosol of antigen presenting cells to elicit effective cytotoxic T lymphocyte response, Biochem. Biophys. Res. Commun. 351 (2006) 702–707.
[17] A. Welin, D. Eklund, O. Stendahl, M. Lerm, Human macrophages infected with a high burden of ESAT-6-expressing *M. tuberculosis* undergo caspase-1- and cathepsin B-independent necrosis, PLoS One 6 (2011) e20302.
[18] S.C. Derrick, S.L. Morris, The ESAT6 protein of *Mycobacterium tuberculosis* induces apoptosis of macrophages by activating caspase expression, Cell. Microbiol. 9 (2007) 1547–1555.
[19] H.H. Choi, D.M. Shin, G. Kang, K.H. Kim, J.B. Park, G.M. Hur, H.M. Lee, Y.J. Lim, J.K. Park, E.K. Jo, C.H. Song, Endoplasmic reticulum stress response is involved in *Mycobacterium tuberculosis* protein ESAT-6-mediated apoptosis, FEBS Lett. 584 (2010) 2445–2454.
[20] S. Uplekar, B. Heym, V. Friocourt, J. Rougemont, S.T. Cole, Comparative genomics of Esx genes from clinical isolates of *Mycobacterium tuberculosis* provides evidence for gene conversion and epitope variation, Infect. Immun. 79 (2011) 4042–4049.
[21] M. Miura, R.M. Friedlander, J. Yuan, Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway, Proc. Natl. Acad. Sci. USA 92 (1995) 8318–8322.
[22] H.A. Rapanoel, G.K. Mazandu, N.J. Mulder, Predicting and analyzing interactions between *Mycobacterium tuberculosis* and its human host, PLoS One 8 (2013) e67472.
[23] A. Mangolini, M. Bogo, C. Durante, M. Borgatti, R. Gambari, P.C. Harris, R. Rizzuto, P. Pinton, G. Aguiari, L. del Senno, NF-kappaB activation is required for apoptosis in fibrocystin/polyductin-depleted kidney epithelial cells, Apoptosis 15 (2010) 94–104.
[24] J. Dutta, Y. Fan, N. Gupta, G. Fan, C. Gelinas, Current insights into the regulation of programmed cell death by NF-kappaB, Oncogene 25 (2006) 6800–6816.
[25] Y. Tao, D. Yan, Q. Yang, R. Zeng, Y. Wang, Low K+ promotes NF-kappaB/DNA binding in neuronal apoptosis induced by K+ loss, Mol. Cell. Biol. 26 (2006) 1038–1050.
[26] T.L. Liu, H. Shimada, T. Ochiai, T. Shiratori, S.E. Lin, M. Kitagawa, K. Harigaya, M. Maki, M. Oka, T. Abe, M. Takiguchi, T. Hiwasa, Enhancement of

chemosensitivity toward peplomycin by calpastatin-stabilized NF-kappaB p65 in esophageal carcinoma cells: possible involvement of Fas/Fas-L synergism, Apoptosis 11 (2006) 1025–1037.
[27] Y. Xie, J.M. Rosser, T.L. Thompson, J.D. Boeke, W. An, Characterization of L1 retrotransposition with high-throughput dual-luciferase assays, Nucleic Acids Res. 39 (2011) e16.
[28] H. Iwai, A. Pluckthun, Circular beta-lactamase: stability enhancement by cyclizing the backbone, FEBS Lett. 459 (1999) 166–172.
[29] Y.J. Shyu, H. Liu, X. Deng, C.D. Hu, Identification of new fluorescent protein fragments for bimolecular fluorescence complementation analysis under physiological conditions, Biotechniques 40 (2006) 61–66.