A proteomic strategy for the identification of caspase-associating proteins†

Eunice L. P. Tan,[a] Resmi C. Panicker,[b] Grace Y. J. Chen[ab] and Shao Q. Yao*[ab]

Received (in Cambridge, MA, USA) 6th October 2004, Accepted 29th October 2004
First published as an Advance Article on the web 8th December 2004
DOI: 10.1039/b415506h

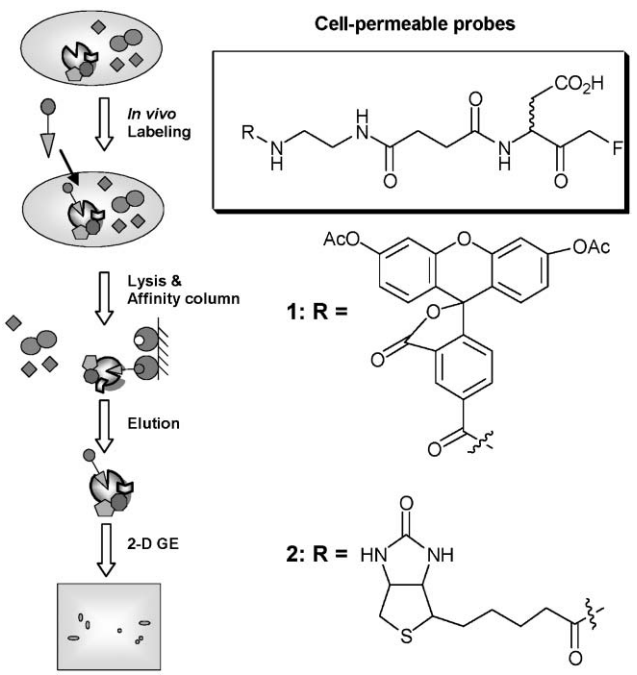
We report the efficient *in vivo* labelling of caspases expressed inside apoptotic HeLa cells using fluoromethyl ketone (fmk)-containing probes; preliminary results indicated that these probes may be used to identify caspase-associating proteins.

Apoptosis is a cellular process essential for animal development and tissue homeostasis. In humans, both excessive and insufficient apoptosis could lead to a variety of diseases. Various stimuli, including cytokines, hormones, viruses, toxins, chemicals and UV have been shown to trigger apoptosis of normal cells, whose initial sign was characterized by morphological changes of cells, followed by a number of intracellular biochemical changes, including the activation of a specific subset of cellular cysteine proteases called caspases.[1] Given the important roles caspases play, a number of methods have been developed to identify new caspases and their interacting proteins (*e.g.* caspase substrates),[2] including cDNA pool expression strategies, two-hybrid approaches and two-dimensional gel electrophoresis (2D-GE), just to name a few. Among them, the 2-D approach,[2c] in which global proteomic profiles of normal and apoptotic cells were compared, often generates a few hundreds of candidate protein spots, the majority of which are proteins unrelated to apoptosis. This thus makes it difficult, as well as impractical, to be used in large-scale studies of proteins interacting/associating with caspases during apoptosis.

We recently reported an activity-based probe that targets caspases *in vitro* in a proteomic experiment.[3] Herein, we extend this approach to the *in vivo* labelling of caspases in apoptotic cells by the use of modified probes which are cell permeable. We also present, for the first time, preliminary findings which show the approach is suitable for large-scale identification of potential caspase-associating proteins. Previously, activity-based profiling strategies had only been developed to target different enzymes.[4] Our findings thus represent the first example into the study of enzyme-associating proteins.

The probes are made up of three parts (Scheme 1): (1) a fluoromethyl ketone (FMK)-containing unit which specifically modifies the active site of caspases in an activity-dependent manner, (2) a simple alkyl linker and (3) a report tag (*e.g.* **1** and **2**) for easy visualization and enrichment of target proteins and their associating molecules, respectively. The diacetate form of fluorescein was used in **1** in order to increase the cell permeability of the probe. We envisaged that, with a small size (MW $<$ 800 Da) and high lipophilicity, the two probes may readily cross the membrane barrier of apoptotic cells into their cytosol, where they would react covalently with (and subsequently be trapped by) active caspases. The labelled caspases may be subsequently identified by SDS-PAGE and MS analysis of extracts obtained from the labelled cells. Alternatively, to identify any proteins associating with labelled caspases, apoptotic cells may be lysed to obtain "native" protein extracts, in which caspase-associating proteins are still trapped by the native (but inactive), labelled caspases. Subsequent affinity-based enrichment of the resulting biotinylated caspases together with their associating proteins, once again under "native" conditions, followed by 2-D PAGE and MS would afford the proteomic profiles of caspase-associating proteins. During affinity enrichments, different washing/elution conditions may be explored to obtain either loosely-associating or tight-binding protein/caspase complexes. We chose 2D-GE in our method in anticipation that, during apoptosis, many proteins may be associated with various caspases present in the cell. The high-resolving power of 2D-GE should allow simultaneous and unambiguous identification of multiple proteins.

The probes were synthesized as described in the ESI.† **1** was chosen to assess its cell permeability, which may be readily followed by fluorescence microscopy. Two separate culture plates of HeLa cells were incubated with a control fluorescein dye



Scheme 1 Strategy and probes used in this study.

† Electronic supplementary information (ESI) available: Experimental details. See http://www.rsc.org/suppdata/cc/b4/b415506h/

*chmyaosq@nus.edu.sg

(without the reactive unit) and **1** followed by repeated washes with PBS buffer to remove any excessive free dyes, and imaged (panels *i* and *ii* in Fig. 1(A), respectively). As shown in panel *ii* in Fig. 1(A), *only* cells incubated with **1**, but not with the control dye (panel *i*), were highly fluorescent, indicating the successful entry, as well as retention of the probe, inside the live cells. In a separate experiment, HeLa cells were first induced by UV to become apoptotic, treated with **1** then imaged. As shown in panel *iii*, Fig. 1(A), the apoptotic cells, as characterized by their signature round shape, could be readily labelled by probe **1** and rendered highly fluorescent as well (panel *iv*, Fig. 1(A)). These results thus indicate that **1** is indeed cell-permeable and able to label live cells, both normal and apoptotic. Furthermore, the labelling depends greatly on the structure of the probe (*i.e.* the reactive unit), as the control dye alone did not show any labelling towards the cells. We next assessed whether the probes (*i.e.* **1** and **2**) selectively label caspases in live apoptotic cells. Apoptotic cells were treated with **1** and **2**, respectively. Upon repeated washes, the cells were collected, lysed and the extracts were analyzed by SDS-PAGE followed by visualization with a fluorescence gel scanner and western blotting, respectively. As shown in Fig. 1(B), both probes showed similar labelling profiles; more bands were observed with **1** (panel *i*), probably due to the higher sensitivity of the fluorescence-based detection. A ~ 27 kDa band was observed in both gels as the major band in the labelling reaction, and was subsequently identified, in a pull-down experiment (ESI†), to correspond to Caspase 8 – one of the endogenous caspases known to be activated upon cell apoptosis. We previous showed that, *in vitro*, fluoromethyl ketone (FMK)-containing probes specifically label caspases, as well as other cysteine proteases, *e.g.* granzyme B,

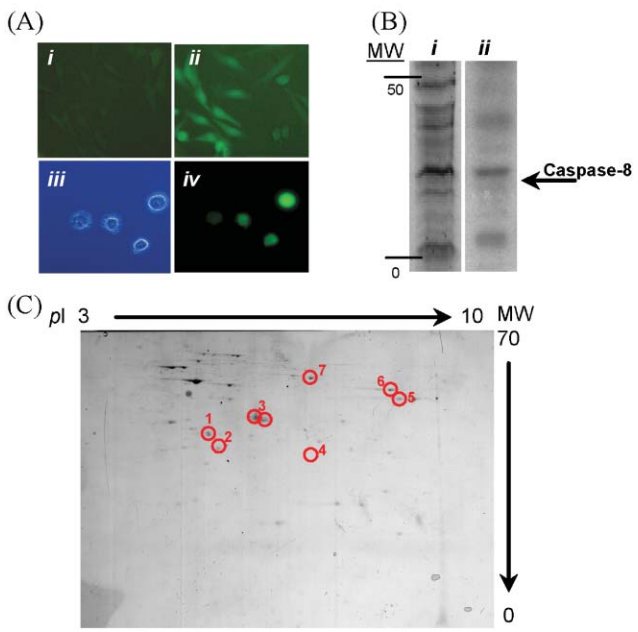


Fig. 1 (A) Fluorescence microscopes of normal HeLa cells labelled with (*i*) control dye and (*ii*) probe **1**, together with (*iii*) phase contrast and (*iv*) fluorescence image of apoptotic cells labelled with **1**. (B) SDS-PAGE of apoptotic cells labelled with (*i*) **1** followed by fluorescence gel scanning and (*ii*) **2** followed by western blotting with anti-biotin. (C) 2D-GE of proteins bound to streptavidin particles upon affinity purification; circled were the ones identified by MS.

which prefer substrates containing a P_1 Asp residue, on the basis of their enzymatic activity. It should be noted that, although only Caspase 8 was identified in the present study, it is likely that other overexpressed caspases (and possibly granzyme B, *etc*) were also labelled, but escaped our detection due to low expression level. Additional control experiments were done, in which apoptotic cells preincubated with an unlabeled fluoromethyl ketone inhibitor were treated with **1** and **2** then analyzed by SDS-PAGE: all bands as shown in Fig. 1(B) were abolished, indicating they originally arose from specific labelling by the two probes, respectively.

We next assessed whether the activity-based profiling approach could be used to pull-down caspase-associating proteins from live cells. UV-induced apoptotic cells, prepared as described above, were incubated with the biotin-containing probe, **2**. Following extensive washings, the cells were collected, lysed under native conditions, and the resulting extract was purified with Streptavidin MagneSphere Paramagnetic Particles (Promega, USA). Control experiments were done simultaneously in which cell extracts without probe treatment was subjected to the same affinity purification procedures. To ensure only high-affinity caspase-binding proteins are enriched, and at the same time minimize any background binding, the particles were washed with 1% SDS before boiling to elute the bound proteins, which were subsequently separated by 2D-GE, followed by identification with MS (Fig. 1(C) and Table 1). Despite stringent washing conditions employed in our purifications, a number of spots still showed up in both the positive (*i.e.* pull-down experiment with **2**) and control (without **2**) gels, indicating they were non-specific protein binders. Consequently, only spots uniquely present in the positive gel were further characterized and identified (circled in Fig. 1(C)). As shown in Table 1, out of a total of seven proteins identified, they could be broadly classified into three groups: (1) known caspase substrates, (2) proteins with shared homology/associated with caspase substrates and (3) proteins with no known link to caspases. In the first group, actin, a highly abundant cytoskeleton protein, is a previously known caspase substrate, directly linked to the collapse of the cell morphology during apoptosis.[5a] Similarly, proteasome activator 28 subunit γ (PA28γ) was previously identified as an effector substrate of caspases from two-hybrid experiments.[5b] It should be pointed out that, our method described herein should be particularly useful for the identification of caspase substrates which bind caspases at their exo-sites. The second group comprises proteins that share homology or are associated to known caspase substrates, comprising T-cell receptor beta chain C region and GTP-binding nuclear protein RAN.[5c,d] Surprisingly, the last group of proteins, which has no known link to caspases or their substrates, was all made up of proteins involved in glycolysis. They include glyceraldehyde 3-phosphate dehydrogenase,

Table 1 Proteins identified by MS

Spot	Protein	MW	*p*I
1[a]	Actin, beta	41	5.5
2	Proteasome activator complex subunit 3	31	5.8
3	T-cell receptor beta chain C region	34	6.1
4	GTP-binding nuclear protein RAN	24	7
5	Glyceraldehyde 3-phosphate dehydrogenase	36	8.5
6	Fructose-bisphosphate aldolase A	39	8.5
7	Alpha enolase	50	7

[a] Numbering same as in Fig. 1(C).

fructose-bisphosphate aldolase A and alpha enolase. Glycolysis and apoptosis have long been viewed as two separate pathways. Recently, new studies have shown that BAD protein, a key apoptosis inducer protein, is a component of an enzyme complex that maintains glucose homeostasis in mouse liver mitochondria and connects cellular metabolism and apoptosis.[5e] Hence, we speculate that this group of proteins may in fact be caspase-associating, although further experiments need to be carried out to confirm this.

In conclusion, we have developed a proteomic method that allows identification of caspases and their associating proteins from live apoptotic cells. We anticipate that, given the increasing availability of activity-based probes that target other enzymes,[4,6] our approach may provide a viable tool for future high-throughput studies of enzyme-associating proteins.

Funding support was provided by the Agency for Science, Technology and Research (A*STAR) of Singapore.

Eunice L. P. Tan,[a] Resmi C. Panicker,[b] Grace Y. J. Chen[ab] and Shao Q. Yao*[ab]
[a]*Department of Biological Sciences, National University of Singapore, 3 Science Drive 3, Singapore 117543, Republic of Singapore*
[b]*Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543, Republic of Singapore.*
E-mail: chmyaosq@nus.edu.sg; Fax: 65 6779 1691; Tel: 65 6874 1683

Notes and references

1 E. S. Alnemri, *Cell*, 1996, **87**, 171.
2 (*a*) V. L. Cryns, Y. Byun, A. Rana, H. Mellor, K. D. Lustig, L. Ghanem, P. J. Parker, M. W. Kirschner and J. Y. Yuan, *J. Biol. Chem.*, 1997, **272**, 29449; (*b*) Y. H. Wu, S. F. Shih and J. Y. Lin, *J. Biol. Chem.*, 2004, **279**, 19264; (*c*) J. H. Wan, J. L. Wang, H. P. Cheng, Y. T. Yu, G. C. Xing, Z. Y. Qiu, X. H. Qian and F. C. He, *Electrophoresis*, 2001, **22**, 3026.
3 M. L. Liau, R. C. Panicker and S. Q. Yao, *Tetrahedron Lett.*, 2003, **44**, 1043.
4 N. Jessani and B. F. Cravatt, *Curr. Opin. Chem. Biol.*, 2004, **8**, 54.
5 (*a*) C. Kayalar, T. Ord, M. P. Testa, L. T. Zhong and D. E. Bredesen, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 2234; (*b*) R. Araya, R. Takahashi and Y. Nomura, *Cell Death Differ.*, 2002, **9**, 322; (*c*) B. R. Gastman, D. E. Johnson, T. L. Whiteside and H. Rabinowich, *Cancer Res.*, 1999, **59**, 1422; (*d*) L. Faleiro and Y. Lazebnik, *J. Cell Biol.*, 2000, **151**, 951; (*e*) N. N. Danial, *Nature*, 2003, **424**, 952.
6 (*a*) Q. Zhu, A. Girish, S. Chattopadhaya and S. Q. Yao, *Chem. Commun.*, 2004, 1512; (*b*) E. W. S. Chan, S. Chattopadhaya, R. C. Panicker, X. Huang and S. Q. Yao, *J. Am. Chem. Soc.*, 2004, **126**, 14435–14446.

