Anal. Chem. **2006,** *78,* 4945−4951

Assays of Endogenous Caspase Activities: A Comparison of Mass Spectrometry and Fluorescence Formats

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This paper describes a label-free assay for measuring endogenous caspase protease activities in cell lysates. The assay format, termed SAMDI-MS (*s*elf-*a*ssembled *m*onolayers for matrix assisted laser *d*esorption *i*onization time-of-flight *m*ass *s*pectrometry), is based on the enzymatic modification of peptides immobilized to monolayer substrates, followed by direct detection of the products with mass spectrometry. Monolayers presenting peptide substrates for either caspase-3 or -8 were treated with lysates from Jurkat cells that were stimulated with staurosporine and SKW6.4 cells that were stimulated with LzCD95L. In both cases, the SAMDI assays reported on the activation of endogenous caspase enzymes with levels of detection that are similar to those observed using the commonly employed fluorogenic assays. The use of longer peptide substrates, which are not compatible with the fluorogenic assays, provided for a better resolution of the two caspase activities. This work is significant because it demonstrates that the SAMDI assay can be used to measure endogenous enzyme activities and because it avoids the loss of activity and specificity that often accompany label-dependent assay formats.

Biochemical assays that measure enzymatic activities and protein−protein binding interactions are essential in nearly all basic and applied biology programs.[1] The majority of these assays employ a fluorescent or radioisotopic label to transduce a binding event or enzyme activity.[2−5] The introduction of a label can alter the biologically relevant activity of the protein or enzyme to be assayed, and this drawback has motivated significant work to develop label-free assay formats. A common strategy for measuring protease activities, for example, uses peptide substrates that contain a nonnatural fluorogenic residue at the carboxyl- or amino-terminal side of the cleavage site, which can generate a fluorescent signal after it is released from the peptide. The incorporation of a nonnatural residue directly at the cleavage site may perturb the interaction between protease and substrate and does not allow the entire consensus sequence to be represented in the peptide substrate. We have recently described a biochip-based assay that uses MALDI-TOF mass spectrometry to measure enzyme-mediated changes to peptides immobilized to self-assembled monolayers on gold.[6,7] This assay format, which we have named *s*elf-*a*ssembled *m*onolayers for matrix assisted laser *d*esorption *i*onization time-of-flight *m*ass *s*pectrometry (SAMDI-MS), provides a strategy for label-free analysis of enzyme activities.[8] This paper describes the application of SAMDI to measure endogenous caspase activities in mammalian cells. We show that the SAMDI assay compares favorably with the widely used fluorescence assays in terms of sensitivity, but the elimination of the label leads to assays that have improved selectivity in resolving activities of members of the caspase family.

Apoptosis is an evolutionarily conserved and highly regulated process that results in cell death.[9] This process is regulated by a family of proteases known as caspases (cysteine-dependent asp-specific protease).[10] Caspases often function in cascades wherein an upstream caspase (initiator caspase) is activated by its interaction with apoptosis-promoting proteins. Once activated, the initiator caspase processes and activates one or more downstream caspases (effector caspases). The activated effector caspases subsequently cleave specific sets of cellular proteins, leading to apoptotic cell death. The temporal order and degree of activation of individual caspases depend on particular apoptotic signals and vary with different cell types. Understanding the mechanistic features of a death-signaling pathway is important for targeted diagnosis and therapy of diseases due to dysfunction of apoptosis.[11] A critical issue in characterizing the temporal activation of distinct caspases

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10.1021/ac051974i CCC: $33.50
Published on Web 06/20/2006

during apoptosis is the development of specific substrates for individual caspases. The commonly employed fluorogenic tetrapeptide substrates have substantial overlapping activities for different caspases, which ultimately limits the understanding of caspase cascades that operate in cell apoptosis.[12]

Assays that employ mass spectrometry (MS) detection, by contrast, are label-free because MS detects the mass-to-charge ratio (m/z) of an analyte, which is an intrinsic property of any chemical structure. Therefore, MS avoids the need to label substrates for enzymes and can be applied to the analysis of nearly all enzyme activities—including posttranslational modifications of substrates, proteolysis, and even binding of proteins to ligands.[13] Advances in soft ionization techniques (matrix-assisted laser desorption/ionization, MALDI;[14] and electrospray, ESI[15]) and detectors (time-of-flight, TOF;[16] and Fourier transform ion cyclotron resonance, FTICR[17]) in the past decade have greatly improved the sensitivity and accuracy of MS analysis for identification of polypeptides, carbohydrates, nucleic acids, and lipids.[18] Yet MS techniques have not emerged as general approaches in routine biochemical research. A principle reason for the slow introduction of MS methods into biochemistry laboratories derives from the time-consuming protocols required for sample preparation, including the isolation and enrichment of an analyte from a complex mixture (i.e., a cell lysate) and removal of salt from the sample.[19] We and others have recently developed strategies to prepare target plates that present immobilized substrates.[6,20] These plates can be exposed to a sample containing the enzyme analyte and only need to be rinsed prior to MS analysis, thereby avoiding the chromatographic steps that make sample preparation difficult. Further, we have shown that plates prepared from self-assembled monolayers of alkanethiolates on gold allow MS to be combined with biochip arrays[7,21] and can be extended to homogeneous format assays.[6c] In the current paper, we report on the development of specific substrates for caspase-3 and caspase-8 and show that the SAMDI assay can be applied to analysis of endogenous enzyme activities in cell lysates and provides for an improved resolution of these caspase activities relative to the commonly used fluorescence assay (Figure 1).

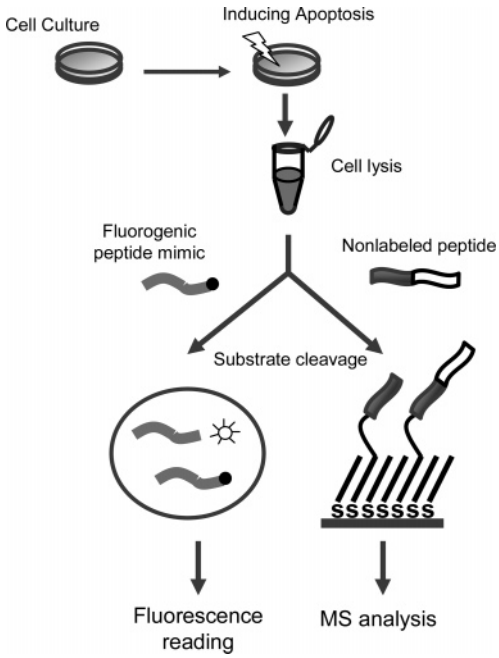

Figure 1. Fluorogenic and SAMDI methods for detection of endogenous caspase activities in apoptotic cells. Cultured cells are stimulated to initiate apoptosis and lysed at different times. Each lysate is split into fractions for analysis by the fluorescence assay and the SAMDI-MS assay.

EXPERIMENTAL SECTION

Materials. Fluorogenic peptide substrates Ac-DEVD-AFC and Z-IETD-AFC were purchased from Calbiochem, Inc. The other peptides were prepared through the standard solid-phase synthetic protocols using Fmoc-Rink amide MBHA resin or 2-ClTrit resin (AnaSpec. Inc., San Jose, CA) with an ABI 430A peptide synthesizer. Recombinant human caspase-3 and caspase-8 (active form) were purchased from Upstate and Calbiochem, respectively. The B lymphoblastoid cell line SKW6.4 (ATCC), T cell lines Jurkat (ATCC), and caspase-8-deficient Jurkat[22] were cultured in RPMI 1640 (Mediatech Inc., Herndon, VA) in a humidified atmosphere, 5% CO_2 at 37 °C, and supplemented with 10% heat-inactivated fetal calf serum (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine (Mediatech Inc., Herndon, VA), 100 U/mL penicillin (Mediatech Inc., Herndon, VA), and 100 μg/mL streptomycin (Mediatech Inc., Herndon, VA). Protease inhibitor cocktail from Roche (Basel, Switzerland) was used in the preparation of cell lysates. Staurosporine was purchased from EMD Biosciences (San Diego, CA). Plasmids to produce the recombinant human leucine zipper-tagged CD95L (LzCD95L) were described elsewhere.[23]

Preparation of Self-Assembled Monolayers (SAMs). Self-assembled monolayers of alkanethiolates on gold were prepared using previously reported procedures.[24] Briefly, gold substrates (10 nm of Ti and 50 nm of Au on a microscope cover glass) were immersed for 8 h in an ethanolic solution containing a symmetric disulfide presenting tri(ethylene glycol) groups and an asymmetric disulfide presenting one maleimide group and one tri(ethylene

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glycol) group at a molar ratio of 19:1. The total concentration of disulfides was 0.1 mM. The monolayers were then rinsed with ethanol and dried under a flow of nitrogen gas. Tris aqueous buffers (10 mM) containing Cys-terminated peptides (1 mM) were applied to SAMs and incubated at 37 °C for 1 h to immobilize peptides on monolayers. The monolayers were rinsed with distilled water and dried under a flow of nitrogen gas and then used in assays.

Mass Spectrometry. Following an assay, monolayers were rinsed as described above and then treated with an acetone solution containing 2,4,6-trihydroxyacetophenone (THAP, 10 mg mL^{-1}), dried, and loaded in a Voyager DE-PRO Biospectrometry mass spectrometer (Applied Biosystems, Framingham, MA). A 337-nm nitrogen laser was used as the desorption/ionization source with accelerating voltage of 20 kV and extraction delay time of 100 ns. All spectra were acquired using reflector mode for positive ions. Three hundred laser shots were added to obtain each spectrum obtained by moving the laser beam to several different locations.

Quantitative SAMDI MS Analysis. A series of identical monolayers presenting maleimide groups at a constant density were prepared and treated with mixtures of two peptides, Ac-CGGDEVDSG-NH_2 (**c**) and Ac-CGGDEVD-OH (**c′**), to prepare a set of standard substrates that ranged in molar ratio in the two peptides from 10:1 to 1:10. These substrates were analyzed by MALDI-TOF-MS, and the fraction of peptide c was determined by measuring the intensities of the peaks for the two peptides according to the following equation: $I_c/(I_c + I_{c'})$. Experiments were carried out in triplicate.

Preparation of Apoptotic Cell Lysates. SKW6.4 and Jurkat cells (caspase-8+ and deficient) were cultured in RPMI 1640 in a humidified atmosphere, 5% CO_2 at 37 °C, and supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. SKW6.4 cells were stimulated for 0−120 min with 1 μg/mL of LzCD95L. Jurkat cells and caspase-8-deficient cells were stimulated with 1 μM staurosporine for 0−22 h. Cells were harvested and lysed in ice-cold lysis buffer containing 10 mM Tris−HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA and 0.01% Triton X-100, 1 mM phenylmethanesulfonylfluoride (PMSF), 7.5 μg mL^{-1} chymotrypsin, 4 μg mL^{-1} thermolysin, 5 mg mL^{-1} papain, 7.5 μg mL^{-1} Pronase, 7.5 μg mL^{-1} pancreatic extract, and 1.4 μg mL^{-1} trypsin for 10 min on ice. The lysates were centrifuged at 14 000 rpm for 10 min at 4 °C, and the supernatant was used immediately for caspase assays.

Chip-Based Caspase Assays. To compare the relative substrate activities for caspases, SAMs presenting peptide substrates **c**−**h** (Table 1) were prepared, and each was treated with recombinant caspase-3 (50 mU per reaction) or caspase-8 (200 mU per reaction) in 5 μL of buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS, and 10 mM DTT at 37 °C. Reactions were carried out for times ranging from 10 to 90 min and stopped at 10-min intervals by rinsing the monolayers with distilled water to remove the assay reaction mixture. The monolayers were briefly immersed in 0.01 N HCl and then dried under a flow of nitrogen. THAP matrix solution was added to the monolayers for MS analysis. The extent of substrate cleavage by caspases was calculated by measuring the relative intensities (peak heights) of the substrate and product ions $(M + Na)^+$: completion of reaction $= I_{product}/(I_{substrate} + I_{product})$. These kinetic studies provide initial rates of each reaction based on which relative activities of the substrates are derived to compare caspase specificities.

Table 1. Peptide Substrates for Caspase-3 and -8 Used in Fluorescence and SAMDI-MS Assays

peptide	substrate sequence	target caspase
a	Ac-DEVD−AFC[a]	caspase-3
b	Z-IETD−AFC[b]	caspase-8
c	Ac−CGGDEVDSG−NH_2	caspase-3
d	Ac-CGDEVDSGVDEVA−NH_2	caspase-3
e	Ac-CGKRKGDEVDSG−NH_3	caspase-3
f	Ac-CGGIETDSG−NH_2	caspase-8
g	Ac-CDGIETDSGVDDD−NH_2	caspase-8
h	Ac-CGELDSGIETDSG−NH_2	caspase-8

[a] Ac, acetyl; AFC, aminofluoro coumarine. [b] Z, carbobenzoxy.

To measure the activities of endogenous caspase-3 and 8 in cell apoptosis, cell extracts were prepared from 8×10^4 cells and were diluted with reaction buffer described above (total volume 5 μL) and spotted onto SAMs. The substrates were incubated in a humidified chamber for 1 h at 37 °C. The monolayers were rinsed with distilled water and 0.01 N HCl and then dried under a flow of nitrogen gas. MS analysis of the SAMs was performed under the same conditions as mentioned above.

All peptide cleavage reactions on the monolayers were carried out in triplicate. In each case, variation of the extent of cleavage was within 10% of the average value that was used to determine the initial rate of peptide cleavage used for preparing Figure 4 and the extent of substrate cleavage used in Figure 6.

Fluorescence Caspase Assays. Fluorescence assays were performed as described previously.[25] Apoptotic cell extracts from 10^6 cells were incubated in reaction buffer (total volume 30 μL) containing 20 μM of caspase-3 substrate Ac-DEVD-AFC or caspase-8 substrate Z-IETD-AFC for 1 h at 37 °C. The fluorescence intensity of the AFC groups released upon substrate cleavage by caspase was detected with a FLX800 fluorescence plate reader (Bio-Tek Instruments, Inc., Winooski, VT) with excitation at 400 nm and emission at 508 nm. Signals from unstimulated cell extracts were taken as background and subtracted from those obtained with stimulated cell extracts. For direct comparison with SAMDI-MS caspase assays, fluorescence intensity (FU) was converted to completion of substrate cleavage: completion of reaction $= FU_t/FU_{max}$ (FU_{max} is the fluorescence intensity of 20 μM AFC in reaction buffer containing unstimulated cell extracts.)

RESULTS AND DISCUSSION

The strategy we employ for measuring caspase activities is illustrated in Figure 2. We prepared the target plates by immobilizing cysteine-terminated peptide substrates for the caspase proteases to SAMs that present maleimide groups against a background of tri(ethylene glycol) groups. The use of SAMs is important for several reasons. First, SAMs are compatible with MALDI-TOF-MS. Irradiation of the monolayer at 337 nm results

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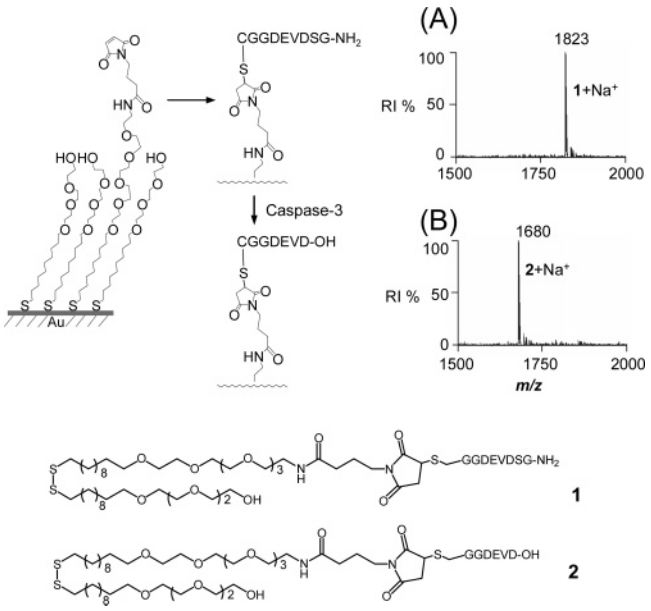

Figure 2. Caspase activity assays by SAMDI-MS. A cysteine-terminated peptide substrate for caspase-3 is immobilized to maleimide-presenting monolayers of alkanethiolates on gold. Treatment of this monolayer with caspase-3 results in enzymatic cleavage of the peptide. Mass spectra of the monolayers before (A) and after caspase treatment (B) reveal the proteolysis of the peptide. Unsymmetrical disulfides **1** and **2** correspond to the substrate peak at *m*/*z* 1823 and product peak at *m*/*z* 1680, respectively, in SAMDI-MS spectra.

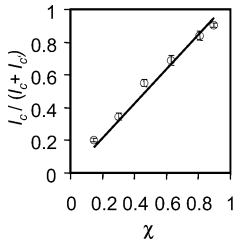

Figure 3. Quantitative detection of immobilized peptides by SAMDI-MS. The peak intensities (*I*) of molecular ions corresponding to peptides **c** (Ac−GGDEVDSG−NH_2, $[M + Na]^+ = 1823$) and **c′** (Ac−CGGDEVD−OH, $[M + Na]^+ = 1680$) were used to calculate the relative density fraction of **c** in the monolayer, $I_c/(I_c + I_{c'})$, which is plotted as a function of the molar fraction of **c** (χ) in the solutions used to immobilize **c** and **c′** onto maleimide-presenting monolayers. The data fit a linear relationship with a slope of 1.06 ($R^2 = 0.98$). Quantitation was carried out in triplicate, and standard deviations are indicated as error bars on each data point.

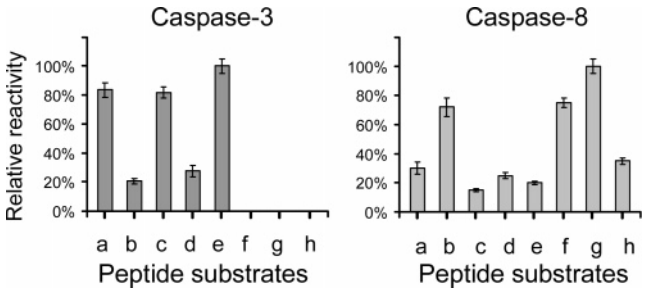

Figure 4. A comparison of the specificities of fluorogenic substrates (**a** and **b**) and label-free substrates (**c**−**h**) listed in Table 1 for caspase-3 and caspase-8. Fluorogenic substrates **a** and **b** have substantial cross-reactivity for the two caspases. Substrates for SAMDI assays showed increases in selectivity and reactivity with their target caspases. In particular, substrates **f**−**h** are cleaved only by caspase-8 and not caspase-3. See text for further explanation.

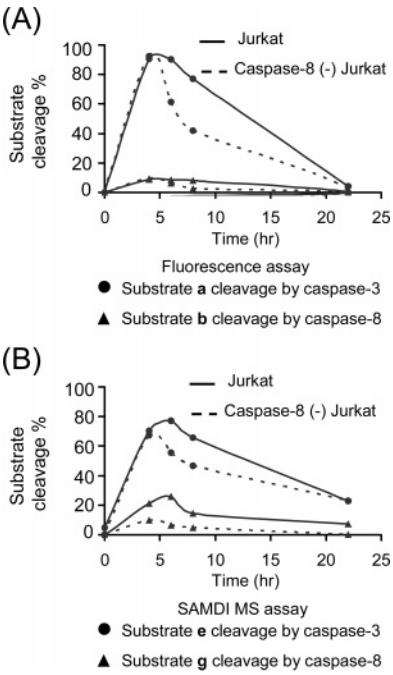

Figure 5. SAMDI-MS and fluorescence detection of time-dependent caspase-3 and -8 activation in staurosporine-induced apoptosis of Jurkat (solid lines) and caspase-8-deficient Jurkat cells (dotted lines). The SAMDI-MS assays show a significant decrease of caspase-8 activity in the caspase-8-deficient cells, whereas the fluorescence assays do not have the selectivity to reveal this loss of activity. Structures of peptide substrates **a**, **b**, **e**, and **g** are listed in Table 1.

in cleavage of the thiolates from the gold surface, and therefore, the full alkanethiol (and the corresponding disulfide) can be detected with little fragmentation of the molecule.[26] Second, the densities of the peptides can be adjusted by varying the density of the maleimide-terminated alkanethiolate in the monolayer, which can be realized by adjusting the molar ratio of the disulfides in the solution from which the monolayers are prepared. Therefore, the amount of peptide on the surface can be optimized (and, hence, the signal in the MS spectra) while ensuring that the peptides remain accessible for interaction with the enzyme (higher densities of substrates can result in "crowding" and loss of activity.)[27] The use of glycol-terminated alkanethiolates is critical to our approach, because these groups render the monolayer inert to nonspecific adsorption of protein. Previous work has shown that the monolayers are among the most effective "inert" surfaces and are effective in cell lysates and other complex samples.[28]

We demonstrate the assay using a monolayer that presents a peptide substrate for caspase-3 (peptide **c** in Table 1). Figure 2 shows the mass spectrum of this monolayer, with a peak at *m*/*z* 1823 that corresponds to the sodium adduct of the unsymmetrical

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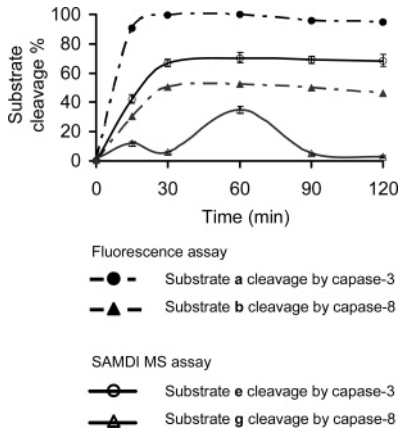

Figure 6. A comparison of the SAMDI-MS assay and the fluorescence assay in characterizing endogenous caspase-3 and caspase-8 activation during apoptosis in SKW6.4 cells induced by LzCD95L. The SAMDI-MS assay reveals that activation of caspase-8 slightly precedes that of caspase-3 activation, and there is a positive feedback on caspase-8 activation from active caspase-3. Fluorescence assays could not reveal these temporal features in activity. Structures of peptide substrates **a**, **b**, **e**, and **g** are listed in Table 1.

disulfide derived from a peptide-terminated alkanethiol and a tri(ethylene glycol)-terminated alkanethiol. Treatment of this monolayer with recombinant active caspase-3 (25 mU) for 1 h at 37 °C resulted in the loss of a C-terminal dipeptide segment from the immobilized peptide, giving a mass spectrum with a new peak at *m/z* 1680. This peak corresponds to the expected product that results from cleavage of the peptide by caspase-3. The mass spectrum also shows that this enzymatic reaction is complete, because the original peak at *m/z* 1823 is absent. Treatment of an identical monolayer presenting peptide **c** with the reaction buffer that did not contain caspase-3 did not give the proteolytic product. This example demonstrates that peptide substrates, when immobilized to monolayers, remain active for processing by the caspase proteases.

Quantitative Analysis of Peptide Cleavage by Caspases. Solid phase format assays, that is, assays wherein one of the reagents is immobilized to a substrate, are often less effective at providing quantitative data than are the corresponding homogeneous phase assays. This limitation often derives from a nonuniform presentation of immobilized ligands due to, for example, different orientations, conformations, and environments, and therefore, a range of activities. The monolayer substrates present ligands in a regular and uniform environment and, therefore, avoid this limitation and have been shown to be quite effective at providing quantitative measurement of biological activities.[5,7] But the use of mass spectrometry may lead to a nonlinear response in signal intensity with density of peptide on the monolayer.[29] To determine whether SAMDI can quantitatively measure the extent of an enzyme reaction on the surface, we prepared a series of monolayers that present a mixture of peptides **c** and **c′** (Ac-GGDEVD-OH, the product of caspase-3 action on peptide **c**) at ratios ranging from 1:10 to 10:1. MS analysis of these SAMs gave spectra showing peaks corresponding to both immobilized peptides. Peak intensities of the immobilized peptides were used to calculate a relative density fraction of **c** on the monolayers, $I_c/(I_c + I_c')$. A plot of this relative density against the molar fraction of **c** in the monolayers shows a good linear relation between relative surface density of immobilized peptides and the molar fraction in the solution (Figure 3). The variation of relative peak intensities for both peptides is within 5% of the average value from triplicate experiments. This result suggests that the SAMDI assay provides data that is quantitatively sufficient for many biochemical applications.

Specificity of Peptide Substrates toward Caspases. In studies of endogenous caspase activations, the ability to quantitate the activities of discreet caspase enzymes is often limited by the specificity of the peptide substrate for the given protease, since many of the proteases act on the same substrates. Thornberry and colleagues established tetrapeptide consensus sequences for most of the known caspases.[30] Based on their results, tetrapeptide substrates containing chromogenic groups at the carboxyl side of cleavage site Asp have been developed and widely applied in numerous studies involving caspase activations. Upon cleavage by caspases, these substrates release free amine-containing species that display a higher fluorescence signal than the original amides and, therefore, can be directly detected in the assay cocktail. Yet the substantial overlap of substrate specificity of these short peptides is widely recognized.[12,31] Previous reports have also revealed that the catalytic efficiency of proteases for hydrolyzing the amide bond is altered by incorporation of the unnatural chromogenic group into the substrate and alters the substrate specificities that are observed with natural peptide structures.[32] Talanian and co-workers used HPLC analysis to show that residues at both the N-termini and C-termini of the tetrapeptide sequence have a significant influence on the peptide reactivity toward a recombinant human caspase.[30] Further, the requirement for the chromogenic groups directly at the cleavage site of the peptide prevents the use of expanded sequences that provide for a better substrate specificity.

FRET-based assays for measuring caspase activities have recently been demonstrated.[33] This technique utilizes substrates containing two fluorescent groups attached at opposite ends of the peptide substrate. Energy transfer between the two labels is disrupted on cleavage of the peptide, giving a wavelength-specific measurement of the protease activity. Although this method allows more flexibility in adjusting the amino acid sequence at both sides of the cleavage site, attaching these acceptor−donor pairs to substrates is synthetically demanding and can introduce nonspecific interactions with proteins.[34,35] The restricted substrate length

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and conformation required for efficient FRET[36] and the need to customize donor−acceptor pair for each specific system of interest limit the application of this technique for general caspase studies.[33c,34] In addition, a potential problem in applying these FRET substrates is the interference from these relatively large donor/acceptor groups with caspase−substrate interactions.[33a]

In caspase assays with SAMDI-MS detection, peptide substrates are not labeled with nonnatural groups and, therefore, can be prepared from the natural sequences without restrictions on length or conformation of the peptides. We based the design of peptide substrates for SAMDI assays on the sequences of natural substrates for endogenous caspases. Substrates for caspase-3 almost always contain a DXXD segment. A DEVD cleavage site is found in poly ADP-ribose polymerase (PARP), one of the most widely assayed caspase-3 substrates. Substrates for caspase-8 were taken from the structure of procaspase-3, a substrate for caspase-8 to form activate caspase-3. Substrates **c** and **f** are based on tetrapeptide consensus sequences but with natural peptide residues adjacent to the Asp cleavage sites. A comparison of the caspase specificities of **c** and **f** with those of fluorogenic substrates **a** and **b** addresses the consequence of installing the nonnatural fluorogenic groups in the commonly used assays. To investigate whether amino acid residues flanking the tetrapeptide consensus sequences in caspase substrates can influence the substrate specificity, we designed peptide substrates with extended sequences at either C- or N-terminal sides of the tetrapeptide segments: **d** and **e** for caspase-3 and **g** and **h** for caspase-8, respectively.

We tested the relative activities of the designed substrates for recombinant active human caspases 3 and 8 using the SAMDI assay, and we compared these activities with those that use fluorescence detection of substrates **a** and **b**. Figure 4 shows that the fluorogenic substrates have a substantial cross-reactivity for the two caspases, whereas the label-free substrates **c** and **f** show an increased specificity toward their target caspases. This result demonstrates that the incorporation of the synthetic fluorophore into the peptide sequence compromises the specificity of the peptides for the caspases. We also found that the extended substrates **e** and **g** are both more selective and more reactive with their caspase targets. In particular, peptide **g** is cleaved only by caspase-8, not by caspase-3. This selectivity is significant because many apoptotic cells have an abundance of effector caspase-3, making it more difficult to measure caspase-8 activity. In practice, cleavage of caspase-8 substrates is often dominated by endogenous caspase-3 and not caspase-8. Our data also indicate that the C-terminal sequence in the caspase-8 cleavage site IETD in procaspase-3 favors the specific interaction between caspase-8 and procaspase-3 because substrate **g** shows higher activity for caspase-8 than **f**, whereas peptide **h** with an extension at the N-terminal to IETD site has a much lower reactivity.

Detecting Caspase Activation during Cell Apoptosis. Caspases are present in all cells as inactive proforms. Upon apoptotic signals, these procaspases are processed through proteolytic cleavages and converted to active caspases. Therefore, caspase activations during apoptosis can be characterized by either characterizing individual procaspase processing or by measuring individual caspase activities with specific substrates. Immunoblotting techniques, in which tedious experimental protocols are required,[37] are currently used to measure the conversion of procaspases to caspases. Furthermore, because the amount of enzyme is not always linearly related to the catalytic activity, a more effective approach is to characterize caspase activation by measuring the proteolytic activity with enzyme substrates.

We compared the sensitivity and resolution of the conventional fluorescence assay with our SAMDI assay for detecting endogenous caspase activities during cell apoptosis. We first explored the activation of caspase-3 and 8 during staurosporine-induced apoptosis of normal Jurkat and caspase-8-deficient Jurkat cells. Staurosporine-induced apoptosis of Jurkat cells goes through the mitochondrial pathway during which cytochrome *c* is released from mitochondria and participates in the formation of the apoptosome together with procaspase-9 and Apaf-1. In the apoptosome, procaspase-9 is activated and cleaves procaspase-3 to form active effector caspase-3. In this pathway, caspase-8 is activated downstream of the mitochondria by active caspase-3.[38] Here, we treated both normal and caspase-8-deficient Jurkat cells with staurosporine at 1 μM in culture medium for 0−22 h at 37 °C. Cells were then lysed, and extracts were divided into a fraction for fluorescence assays and a fraction for SAMDI assays. Our data from SAMDI assays shows a significant decrease of caspase-8 activity in Jurkat cells that are deficient in caspase-8 as compared to the normal Jurkat cells, but caspase-3 activities were at similar levels in both cell lines (Figure 5 B). These results are consistent with previous studies that used immunoblotting to analyze procaspase processing.[37] When this study is performed using the fluorescence assay (Figure 5A), however, there is little change in caspase-8 activity. This misleading result stems from the lack of specificity of the tetrapeptide substrate **b**, which is also processed by caspase-3 (see Figure 4). The cleavage of **b**, therefore, reflects a combination of caspase-3 and -8 activities, and with the higher levels of caspase-3, it is difficult to isolate caspase-8 activity. This experiment further verifies that peptide **g** in the SAMDI assay accurately reports the endogenous caspase-8 activity.

We next investigated the activation of endogenous caspase-3 and caspase-8 in SKW6.4 cells when apoptosis was induced byLzCD95L. The stimulator binds to the Fas receptor on the cell surface and initiates the Fas pathway of apoptosis in which formation of the death-inducing signaling complex (DISC) first results in activation of caspase-8.[39] Caspase-8 subsequently activates downstream caspase-3 that cleaves various cellular substrates leading to irreversible cell death. SKW6.4 cells were stimulated with 1 μg/mL LzCD95L for periods ranging from 0 to 120 min at 37°C. Extracts from lysed cells were again split into fractions for SAMDI assays and fluorescence assays.

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Figure 6 shows the time-dependent caspase-3 and -8 activations during Fas-stimulated apoptosis by both fluorescence and SAMDI assays. The SAMDI assay shows that the two proteases have distinctive activity profiles. Caspase-3 activity increased rapidly after stimulation with Fas and reached a plateau after 30 min. The activity of caspase-8 reached a maximum at 15 min after stimulation with LzCD95L and then a second maximum at 60 min. Our data indicated that caspase-8 activation preceded caspase-3 activation in this apoptotic system, which is consistent with the initiator role of caspase-8 in this pathway. The later increase of caspase-8 activity at 60 min possibly reveals the caspase-8 activation downstream of effector caspase-3 later in the pathway. It has been reported that effector caspase-3 can cleave initiator procaspases in the cytosol and, thus, leave a potential positive feedback to the caspase cascade.[38,40] In comparison, the fluorescence assays gave a similar time-dependent activity profile of both caspase-3 and caspase-8. This misleading data again derives from the cross-activity of the two fluorogenic tetrapeptide substrates for both caspase-3 and caspase-8. Therefore, the assay does not reveal the temporal order of the caspase activations as shown in the SAMDI MS analysis. These results demonstrate that the label-free SAMDI MS assay enables the use of highly specific substrates to temporally resolve the activities of individual caspase activation steps during apoptosis, and represents a significant advance over conventional fluorescence assays.

CONCLUSIONS

This work describes a label-free caspase activity assay that allows greater flexibility in designing peptide substrates and results in substrates that are more selective for individual caspases. This example adds to previous examples that have shown the SAMDI-MS technique to be well-suited to analyzing a broad range of biochemical activities. This example is particularly significant because it shows that the SAMDI assay can be applied to the measurement of endogenous enzyme activities and that it offers significant advantages over the current assays that are in widespread use. The combination of biochips based on monolayer chemistries and MS analysis enables the assays to be performed in a simple, specific, sensitive, and quantitative mode. Most importantly, this approach avoids the use of labels that can alter the activities of the substrates. The comparison between caspase assays using the fluorogenic tetrapeptide substrates and the label-free substrates shows that the SAMDI assay format performs better at resolving caspase activities and, therefore, may provide a new opportunity to understand caspase activation in several biological contexts.

ACKNOWLEDGMENT

This work was supported by the National Science Foundation. J.S. is supported by the Burroughs Wellcome Fund Interfaces Program.

Received for review November 4, 2005. Accepted March 10, 2006.

AC051974I