Because these quantities represent estimates of the average cellular content (based on ensemble averaging of a large number of cells), it is possible that many cells within a population could have substantially higher levels. Quantitative CE analysis based on MPE intrinsic fluorescence of neuropeptides may offer a useful strategy for identifying such heterogeneities.

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Isoelectric Focusing and Enzyme Overlay Membrane Analysis of Caspase 3 Activation

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The proteolytic conversion of procaspase-3 to active caspase 3 appears to be a committed step in caspase-dependent forms of apoptosis (1, 2). Consequently, the measurement of caspase 3 activation has become a useful indicator for determining the efficacy of agents that promote or inhibit apoptotic pathways. There are a number of sensitive methods for measuring cellular caspase 3 activity including spectrofluorometric assays of cell extracts using substrates such as Asp-Glu-Val-Asp-7-amino-4-trifluromethylcoumarin (DEVD-AFC[2]) or Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-

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[2] Abbreviations used: DEVD-AFC, Asp-Glu-Val-Asp-7-amino-4-trifluromethylcoumarin; DTT, dithiothreitol; EOM, enzyme overlay membrane; IEF, isoelectric focusing; Triton X-100, *t*-octylphenoxypolyethoxyethanol.

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AMC) (3, 4) and Western blot analysis of the conversion of procaspase-3 to caspase 3 subunits (1, 2). In addition, recently developed antibodies specific for epitopes of activated caspase 3 have enabled *in situ* immunohistological (5) and flow cytometric (6) analyses.

Each of these analytical approaches has strengths and weaknesses. The fluorometric assays, while quick and sensitive, may not yield uncomplicated caspase 3 activity, especially when analyzing cell extracts that contain other caspases with similar peptide specificity. On the other hand, while Western blot analyses and the *in situ* methods are sensitive and more specific for activated caspase 3, they are usually more time-consuming and expensive than simple fluorometric assays.

Here we present a sensitive, straightforward, and inexpensive protocol to unambiguously resolve and quantify caspase 3 activities in multiple samples. The methodology combines minigel isoelectric focusing (IEF) with enzyme overlay membranes (EOM) impregnated with DEVD-AFC. The technique is sensitive to femtomolar concentrations of caspase 3 and is particularly useful for analyzing caspase 3 activity in cell extracts. The utility of this method is illustrated by contrasting the activation of caspase 3 upon γ-irradiation of apoptosis-sensitive and apoptosis-resistant mouse B cell lymphomas.

Materials and Methods

Induction of apoptosis and preparation of whole cell extracts. All reagents were purchased from Sigma Chemical Co. unless otherwise indicated. The B cell lymphoma clonal sublines, apoptosis-sensitive LYas, and apoptosis-resistant bcl-2-transfected LYas (LYas: bcl2) were maintained in culture as described previously (7). Each subline (10^6 cells/ml) was exposed to 5 Gray of γ-irradiation from a ^{60}Cobalt source. Cell suspensions (10 ml) were removed at various times and centrifuged at 200*g* for 10 min. The pellets ($\sim 10^7$ cells) were suspended in isotonic phosphate-buffered saline, pH 7.2, at room temperature and centrifuged as before. These pellets were extracted with 75 μl of ice-cold Tris-buffered saline, pH 7.5, containing Complete protease inhibitors (Boehringer Mannheim) and 1% oleyldecaoxyethylene ether (Brij 97). Suspensions were kept on ice for a total of 10 min with intermittent vortexing. Subsequent extraction steps were performed at 4°C. Suspensions were centrifuged for 10 min at 15,000*g* and 30-μl aliquots of supernatant were centrifuged through Micro Bio-spin P-6 columns (Bio-Rad) equilibrated with 20% glycerol and 2% Triton X-100. This step removed buffer salts and low-molecular-weight components that interfere with isoelectric focusing.

Native gel isoelectric focusing. The IEF protocol utilized Ready Gel cassettes (Bio-Rad) that have a lower "peg" and bottom ridge that hold the gel in place during electrofocusing. Ten-well slab gels (1-mm thickness) consisted of 6.5% polyacrylamide (29:1 acrylamide:bisacrylamide), 12.5% glycerol, 2% Triton X-100, and 3% ampholytes with three parts pH 5/7 to one part 3/10 ampholytes. After overnight polymerization with ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamide (TEMED), gel cassettes were inserted into a Mini-Protean III apparatus (Bio-Rad). The bottom anode chamber was filled with ice-cold 0.1% H_3PO_4, and subsequent IEF steps were performed at 4°C in a cold room.

Protein extracts containing 10–20 μg of protein/μl were mixed with one-third volume of 3× IEF sample buffer (3% Triton X-100, 6% pH 3/10 ampholytes, and 30% glycerol). Recombinant caspase 3 (PharMingen) was diluted in caspase buffer containing 100 mM NaCl, 10 mM DTT (fresh), 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), 10% glycerol, and 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (Hepes), pH 7.2 (8), and incubated 1 h on ice before adding 3× IEF sample buffer. Prereduction of recombinant enzyme with DTT was necessary for the generation of closely spaced bright bands upon IEF and EOM. Prereduction of cellular extracts was not necessary. For sample loading, 15 μl of 0.5× IEF sample buffer was added to each well, and then 15- to 20-μl aliquots of recombinant caspase 3 or cell extracts (containing equal amounts of protein) were applied to the well bottom. The remaining well volume and the cathode chamber were filled with ice-cold 0.1% NaOH. IEF was initiated at 250 V for 45 min and then 500 V for 3 h.

DEVD-AFC enzyme overlay membranes. EOMs (Enzyme System Products) were cut to appropriate size, wetted with caspase buffer, and then gently blotted to remove excess liquid. For convenience, the IEF gel was left in the cassette with the glass plate removed. The EOM was centered on the gel surface and incubated at 37°C in a humidified plastic container. The progress of the caspase 3 reactions was monitored by translumination at 365 nm. When the EOM displayed sufficient fluorescence (usually within 15 min), the membrane was carefully removed from the gel, immersed for 5 min in 10% glycerol, and then allowed to air dry. The fluorescent EOM patterns were captured and analyzed with a UVP Inc. Data Acquisition system using ImageStore 7500 software. The IEF gels were subsequently silver stained (Bio-Rad).

Results and Discussion

The IEF protocol in combination with EOM was used to effectively resolve and quantitate caspase 3 activity of both purified recombinant enzyme and crude cellular extracts. Similar analyses of proteolytic enzymes using IEF and EOM have been reported previously (9–11).

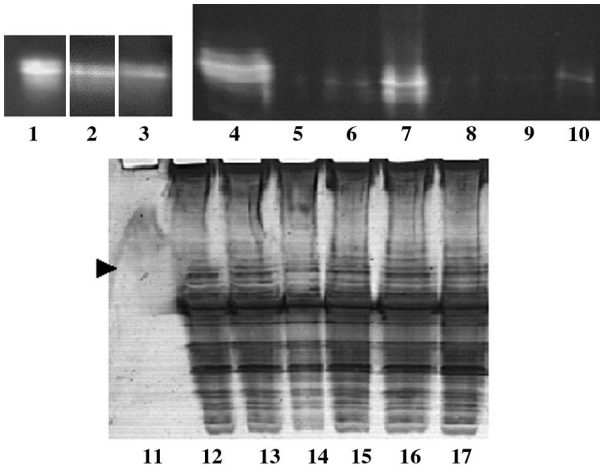

FIG. 1. IEF, EOM, and silver staining were performed as described under Materials and Methods. Lanes 1, 2, and 3 show 18, 3.6, and 1.8 ng of DTT-reduced caspase 3, respectively. Lane 4 is a control sample of recombinant caspase 3 (30 ng). Lanes 5–10 represent a time course of γ-irradiated LYas and LYas:bcl2 sublines, where equal amounts of cell extract (100 μg) were added to each sample well. Lanes 5–7 are 0-, 1.5-, and 3-h postirradiation samples from LYas cells, while lanes 8–10 are corresponding samples from LYas:bcl2 cells. This same gel was silver stained to visualize protein bands; lanes 11–17 correspond to lanes 4–10. The arrowhead indicates the region of the IEF gel that contained caspase 3 activity as determined by EOM.

Typically, these methods employed specialized flatbed IEF systems with horizontal slab gels, a format that required meticulous manual application of enzyme samples to the gel surface. High-resolution electrofocusing could be obtained within 4 h but required very high voltages (up to 2000 V). The present protocol offers significant improvements over these previous methods. The IEF utilizes common minigel electrophoresis equipment; the vertical gel format allows rapid yet precise loading of relatively large concentrations of protein into preformed sample wells; and high-resolution electrofocusing is obtained within 4 h using a maximum of 500 V.

The sensitivity and resolution of this method are illustrated in Fig. 1, where lanes 1, 2, and 3 show 18, 3.6, and 1.8 ng of DTT-reduced caspase 3, respectively. The lowest quantity, approximately 30 fmol, was detected as a bright, well-defined band of enzymatic activity. As discussed below, this level of sensitivity was more than adequate to visualize the activation of caspase 3 during apoptosis.

A time course characterizing the activation of caspase 3 in LYas and LYas:bcl2 cells is shown in Fig. 1, lanes 4–10. Lane 4 is a control sample of prereduced recombinant caspase 3 (30 ng). At the time of γ-irradiation, both sublines had essentially undetectable caspase 3 activities (lanes 5 and 8). At 1.5 h after γ-irradiation, the LYas cells had two faint caspase 3 bands (lane 6), while at 3 h an intense major band and two slightly more acidic minor bands were apparent (lane 7). This time course for activation of caspase 3 corresponds closely to our previous reports regarding other apoptotic events in LYas cells (7, 12). As expected for an apoptosis-resistant cell line, caspase 3 band intensities for the γ-irradiated LYas:bcl2 cells were much less (<5%) than for LYas cells at both 1.5 h (lane 9) and 3 h (lane 10). Western blot analyses indicated that both sublines have comparable levels of procaspase 3 (data not shown). The mobility and banding pattern of recombinant caspase 3 (lane 4) matched closely those bands that appeared during induction of apoptosis.

After EOM analysis, the gel was silver stained to reveal the protein patterns of the cellular extracts (lanes 11–17). Even with the high protein loads used in this study (~100 μg/sample), we observed sharp silver-stained bands throughout the gel. The arrowhead in Fig. 1 at an apparent isoelectric point of 6.6 to 6.8 indicates the region of the silver-stained gel that contained caspase 3 activity as determined by EOM. Although the fluorescent bands for recombinant caspase 3 were quite bright, we were unable to detect corresponding silver-stained bands in that region of the gel.

In summary, we present a sensitive, straightforward, and inexpensive method for detecting caspase 3 activity in multiple samples of purified enzyme and complex cellular extracts. Since substrate-specific EOMs are available for other apoptotic proteases, it would be simple to sequentially analyze the same IEF gel for multiple caspase activities. In addition, this protocol in combination with Western blotting techniques would be equally useful in the analysis of other apoptotic factors, thus yielding a wealth of information from a single IEF gel.

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