Caspases, a unique family of cysteine proteases involved in cytokine activation and in the execution of apoptosis can be sub-grouped according to the length of their prodomain. Long prodomain caspases such as caspase-8 and caspase-9 are believed to act mainly as upstream caspases to cleave downstream short prodomain caspases such as caspases-3 and -7. We report here the identification of caspases as direct substrates of calcium-activated proteases, calpains. Calpains cleave caspase-7 at sites distinct from those of the upstream caspases, generating proteolytically inactive fragments. Caspase-8 and caspase-9 can also be directly cleaved by calpains. Two calpain cleavage sites in caspase-9 have been identified by N-terminal sequencing of the cleaved products. Cleavage of caspase-9 by calpain generates truncated caspase-9 that is unable to activate caspase-3 in cell lysates. Furthermore, direct cleavage of caspase-9 by calpain blocks dATP and cytochrome-c induced caspase-3 activation. Therefore our results suggest that calpains may act as negative regulators of caspase processing and apoptosis by effectively inactivating upstream caspases.

Alterations in intracellular calcium (Ca^{2+}) homeostasis have been implicated both in promoting or inhibiting apoptosis in many cell types. An overload of Ca^{2+} by excitotoxicity induces both necrotic and apoptotic cell death (1). Chelation of extracellular Ca^{2+} protects against apoptosis induced by glucocorticoids in thymocytes (2, 3) but leads to apoptosis in other cell types such as in cultured neuronal cells (4). Ca^{2+} ionophores have been shown to inhibit apoptosis in interleukin-3-dependent hematopoietic cells (5). Caspases are unique cysteine proteases that are synthesized as inactive precursors and activated during apoptosis (6, 7). Caspases can be classified into two subclasses according to the length of their N-terminal prodomains. Caspases-1, -2, -8, -9, and -10, which contain long prodomains, are believed to act as upstream caspases (6, 7). This class of caspases is activated through interaction with specific adapter proteins. In the case of caspase-9, the presence of dATP and cytochrome-c will initiate the interaction between Apaf-1 and caspase-9, which in turn activates caspase-9 by autocatalysis (8–10). The active caspase-9 then acts as an apical caspase to generate active caspase-3. The interaction between caspase-9 and Apaf-1 is mediated by the prodomain of caspase-9 and the CARD domain of Apaf-1 (10). Therefore, the prodomain of caspase-9 plays an important role in its activation and mediation of cytochrome-c and dATP-induced apoptosis.

Calpains are Ca^{2+}-dependent intracellular cysteine proteases (11). Two major calpains have been identified (11–12), μ-calpain (calpain I) and m-calpain (calpain II), which are distinguished by the optimal Ca^{2+} concentration for maximal activity. Activation of calpains is initiated by Ca^{2+} and shows autolysis of both large and small subunits. Unlike caspases that require specific amino acid sequences at the cleavage sites, calpains show no apparent sequence preferences. It is believed that the calpain cleavage site is structure-dependent (11). Several calpain substrates have been identified including p53 (13), IκBα (14), and calpastatin (15). Even though there is evidence that calpains are involved in apoptosis, the direct relationship between calpain and caspase activation has not been demonstrated. Using an in vitro assay, we show here that caspases can be cleaved by a cytosolic factor(s) in the presence of Ca^{2+}. Analysis with a broad spectrum of protease inhibitors suggests that this factor is calpain. Recombinant calpain II generates similar size cleavage products as the cytosolic factor.

Treatment of cells with Ca^{2+} ionophore leads to rapid activation of calpain and cleavage of caspases. These data suggest that caspases can be directly cleaved by calpain. Two specific calpain cleavage sites have been identified in caspase-9. Cleavage of caspase-9 by calpain leads to the inhibition of dATP and cytochrome-c-dependent caspase-3 cleavage. Our data thus suggest an alternative way to modulate apoptosis by intracellular calcium signals.

**MATERIALS AND METHODS**

**Cell Culture and Lysate Preparation**—The caspase inhibitors, Z-VAD-fmk, Z-DEVD-fmk, and Z-YVAD-fmk were purchased from Enzyme Systems Products (Livermore, CA). Calpain inhibitors I and II were obtained from Calbiochem. All other inhibitors and materials were obtained from Sigma unless otherwise stated. For lysate preparation, MCF-7 cells, HeLa cells, and SH-SYSY cells were grown to confluence in RPMI 1640 and Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, respectively. Cells were harvested and pellets were resuspended with an equal volume of cell extraction buffer (50 mM HEPES-KOH, pH 7.5, 0.3 M KCl, 2 mM MgCl2, 1 mM EGTA, and 1 mM EDTA) supplemented with 5 mM DTT and 1 mM PMSF. After 30 min of incubation on ice, cells were lysed by gently passing through a 23 G needle ten times and centrifuged for 30 min on a microcentrifuge. The resultant supernatant (cell lysate) was used for biochemical assay. The total protein concentration was measured with the Bradford assay. For the time course experiment, SH-SYSY cells were plated at 75% confluence overnight. 1 μM A23187 and 1 mM CaCl2 were added into the culture for various times and washed away by serum-free medium, and cell lysate was made as described above.

**Caspase Cleavage Assay**—Caspases-7 and -8 in pET15b (Novagen) and caspase-9 and its mutants in pET21b (Novagen) were translated using an in vitro transcription and translation kit (Promega) according to the instruction of the manufacturer and purified as described by Li et al.

The abbreviations used are: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis.
RESULTS AND DISCUSSION

To identify factors involved in Ca2+-mediated apoptosis, an in vitro biochemical assay was established in which radiolabeled caspase-7 was added to MCF-7 cell lysate in the presence of Ca2+ (Fig. 1A). Cleavage of caspase-7 precursor was found to increase with increasing concentration of Ca2+. The pattern of cleavage products was different from dATP and cytochrome-c-induced cleavage which generated characteristic P10 and P20 fragments (Fig. 1A, lane 2), suggesting that the Ca2+-dependent caspase-7 cleavage might generate proteolytically inactive caspase-7. Cleavage of caspase-7 was strongly inhibited by leupeptin and E64 (Fig. 1B) but not by 20 μM caspase inhibitors (DEVD-fmk, Z-VAD-fmk, and YVAD-fmk (Fig. 1C)). These data suggest that proteases other than caspases are responsible for caspase-7 cleavage in the presence of Ca2+. Preincubation of DEVD at 100 μM, however, resulted in an inhibitory effect on caspase-7 cleavage, consistent with the reported nonspecific inhibition of additional proteases at high concentration (6). We next tested for a direct involvement of calpains in the Ca2+-dependent caspase-7 cleavage based on the fact that calpains can be activated by Ca2+. Indeed preincubation of cell lysates with calpain inhibitors I (20 μM) and II (5 μM) resulted in full inhibition of caspase-7 cleavage (data not shown). Therefore calpain appears to be the primary enzyme responsible for caspase-7 cleavage in the cell lysate upon addition of Ca2+, although we cannot rule out the possibility that other cellular factors may also contribute to the cleavage. Short prodomain caspases-3 and -6 were also cleaved by recombinant calpain II at higher concentration (5×) and did not generate P10 and P20 fragments (data not shown).

Direct cleavage of long prodomain caspases such as caspase-8 and caspase-9 by calpain was also tested. Both caspase-8 and caspase-9 were cleaved by cytosolic proteins and the recombinant calpain II (Fig. 2A) in the presence of Ca2+, generating similar size fragments. Calpain inhibitors I and II, leupeptin, and E64 blocked the cleavage of caspase-8 and caspase-9 by calpain, whereas caspase inhibitors showed no inhibition. The precise cleavage positions in caspase-9 were mapped using recombinant caspase-9 (Cys-287 to Ala). Caspase-9 harboring this mutation is defective in enzymatic activity and thus can be isolated as full-length proteins from...
bacteria. Incubation of recombinant caspase-9 (C287A) with calpain in the presence of Ca\(^{2+}\) yielded four cleaved products (C1, C2, C3, and C4, Fig. 2B) which were analyzed by N-terminal sequencing. Full-length caspase-9 and C1 yielded no sequencing information presumably because of N-terminal modification and blockage of sequencing. Analyses of the size of C1, which is similar to caspase-3 cleavage product (Fig. 2B, left panel, lanes 2 and 5), and peptide sequences of C2, C3, and C4 suggest that calpains cleaved caspase-9 at two positions after residues 115 and 330. Cleavage at amino acid residue 115 would generate truncated caspase-9 without the prodomain (C2). Cleavage at position 330, a well established caspase-3 cleavage site would generate the same product (C1) as that by caspase-3 cleavage. Consistent with the fact that calpain cleavage sites show no sequence conservation, single amino acid substitutions at residues 330 (D\(^{330}\)A), 287 (C\(^{287}\)A), and 315 (D\(^{315}\)A) were used in the reactions. Right panel shows the schematic diagram of the calpain cleavage sites in caspase-9 (arrows on the top) and the corresponding cleavage products. Residue 134 represents the end of the hypothetical prodomain. Cys-287, Asp-315, and Asp-330 represent the enzymatic active sites, caspase-9 self-cleavage site, and caspase-3 cleavage site, respectively. Peptide sequences for C2, C3, and C4 are shown on the left side of the products. N-terminal of C1 is blocked for sequencing. D) calpain cleavage of caspase-9 was not affected by point mutations at residues 287 (C287A), 315 (D315A), and 330 (D330A).

**Caspases Are Direct Substrates of Calpains**

Because caspase-9 can be cleaved by calpain at position 330, a conserved caspase-3 cleavage site, we checked the possibility whether cleavage by calpain would lead to the activation of caspase-9 by using caspase-3 as a substrate. Because of the low level of endogenous calpain activity and strong induction by dATP and cytochrome-\(c\) in HeLa cell lysates, we performed the calpain cleavage of caspase-9 in HeLa cell lysates by adding exogenous recombinant calpain II. As shown in Fig. 3A, the presence of increasing amounts of calpain did not lead to caspase-3 activation. Caspase-9 was cleaved by calpain in this assay as monitored by Western blot analysis (Fig. 3A, lower panel). Caspase-3 was cleaved in the presence of dATP and cytochrome-\(c\), showing that the pathway for Apaf-1-mediated activation of caspase-9 and subsequent caspase-3 cleavage is intact. These data indicate strongly that cleavage of caspase-9 by calpain does not generate active caspase-9. We hypothesized that calpain cleavage at residue 115 of caspase-9 generates truncated caspase-9 without the prodomain and may be responsible for its inactivation and disruption of dATP and cytochrome-\(c\)-mediated caspase-3 activation. To test this, we incubated the cell lysate with recombinant calpain II in the presence of Ca\(^{2+}\) prior to the addition of dATP and cytochrome-\(c\). No caspase-3 cleavage was observed when cell lysate was pre-incubated with 2 \(\mu\)g of calpain in the presence of Ca\(^{2+}\) (Fig. 3B, upper panel, lane 7). Incubation of calpain or calcium alone prior to the addition of dATP and cytochrome-\(c\) did not inhibit caspase-3 cleavage. The inhibitory effect of calpain on caspase-3 cleavage can be reversed by the inactivation of calpain using calpain inhibitor II (Fig. 3B, upper panel, lane 8). Western blot analysis showed a reduced amount of caspase-9 precursor in the presence of calpain and Ca\(^{2+}\) (Fig. 3B, lower panel, lanes 6 and 7), indicating that caspase-9 is cleaved by calpain in this reaction. Because caspase-3 was suggested to have a positive feedback on caspase-9 activity (6–7), we examined the effect of calpain on caspase-3 enzymatic activity and
observed no inhibition in the presence of calpain (data not shown). Therefore, these data suggest that calpain cleavage of caspase-9 can indeed lead to the inactivation of caspase-9 and inhibition of dATP and cytochrome-c-induced caspase-3 cleavage.

The direct cleavage of caspase-9 by calpain was further demonstrated in vivo in SH-SY5Y human neuroblastoma cells after Ca\textsuperscript{2+} ionophore (A23187) treatment. The time course of the treatment showed that activation of calpain in these cells is rapid (Fig. 4A). Within 5 min after treatment, calpain was activated (Fig. 4A, lane 4). Cleavage of both caspase-7 and caspase-9 correlated very well with calpain activation, with the appearance of cleaved products at 5 min (Fig. 4, B and C, lane 4). We have also shown here that calpain II inhibitor (5 μM) was able to block calpain activation and caspase cleavage (Fig. 4, lanes 7) suggesting that calpain II is responsible for the caspases-7 and 9 cleavage. Because autolysis of small subunit (common for calpain I and calpain II) was used as a marker for calpain activation, we cannot rule out the possibility that calpain I is also involved in caspase cleavage as both calpains demonstrated similar substrate specificity and a lower amount of calcium was required for the activation of calpain I. The specificity of calpain I and calpain II on the cleavage of caspases will be further demonstrated by using specific antibodies against large subunits of calpain I and calpain II which were shown to be autolysed during activation. Activation of calpain II and cleavage of caspases were not because of the activation of caspases as caspase inhibitor (Z-VAD) did not block calpain activation and caspase cleavage (Fig. 4, lane 8). Correlation of caspase cleavage and calpain activation was also observed in other cell types such as in HeLa cells and in MCF-7 cells (data not shown). These results are consistent with our in vitro data and strongly suggest that caspases are direct calpain substrates in vivo. We noticed that calpain activation by A23187 and caspase cleavage in vivo was not very efficient. However, this amount of caspase cleavage is physiologically relevant as pre-incubation of SH-SY5Y cells with A23187 for 30 min protected cells from etoposide and H7-induced apoptosis (data not shown). We also observed that the efficiency of caspase cleavage by calpain was affected by physiological states of the cells such as cell density and differentiation state (data not shown). Signals other than Ca\textsuperscript{2+} ionophores may be required to activate calpain more efficiently in vivo.

We have shown here that caspases are direct substrates of calpains. Cleavage of caspase-7 by calpain II did not generate the characteristic P10 and P20 fragments which represent its active forms, but yielded inactive fragments. Upstream caspases such as caspase-8 and caspase-9 were also cleaved by calpain II. Two calpain cleavage sites in caspase-9 were identified and the C-terminal calpain cleavage site coincides with the caspase-3 cleavage site (D330). Cleavage at this position by calpain generates a similar fragment to that of caspase-3 cleavage (C1). However, calpain cleavage of caspase-9 did not result in its activation. These data suggest that cleavage of caspase-9 at the appropriate position (adventitious proteolysis) may not lead to the activation of caspase-9, in agreement with a previous observation (16). Unlike other caspases such as caspase-3 which are activated by proteolysis of precursor, cytosolic factors (such as Apaf-1) are required for generating active caspase-9 presumably by binding to caspase-9 (in the presence of dATP and cytochrome-c in the case of Apaf-1). Elevation of Ca\textsuperscript{2+} concentration in the cell lysate cannot initiate interaction between the cytosolic factors and caspase-9, thus caspase-9 activation and subsequent caspase-3 cleavage are not observed.

We also demonstrate that calpain cleavage generates inactive caspase-9 and blocks cytochrome-c and dATP-induced caspase-3 activation in vivo. The inhibitory effect of calpain is rather intriguing as it represents a novel mechanism to regulate apoptosis by the elevation of intracellular Ca\textsuperscript{2+} concentration and activation of calpains. This effect may be because of the cleavage of the prodomain of caspase-9 by calpain because it is crucial for caspase-9 self-activation and enzymatic activity by interacting with Apaf-1 (10, 16). The inhibitory role of cal-
Pain in apoptosis has been shown in limb-girdle muscular dystrophy type 2A disease by perturbation of the IκBα/NF-κB pathway (17). This observation is contrary to the previous observation that inhibition of calpain blocks apoptosis in thymocytes (2). Therefore inhibition of apoptosis by calpain is likely to be cell type-specific. Elevation of cytosolic Ca\(^{2+}\) concentration is an important signal for regulating apoptosis, such as the dephosphorylation of BAD by the Ca\(^{2+}\)-dependent activation of calcineurin (18). We demonstrate here that Ca\(^{2+}\) ionophore treatment in SH-SY5Y cells induced rapid calpain activation and cleavage of caspase-9, preceding other biological or biochemical responses such as caspase activation. Calpain-dependent inhibition of apoptosis by cleavage of upstream caspases may be more typically associated with the transient increase in intracellular Ca\(^{2+}\) concentration that occurs during excitation in neuronal cells or muscle cells. Our data thus suggest that regulation of apoptosis by elevation of cytosolic Ca\(^{2+}\) concentration is in part through modulation of calpain activity and direct cleavage and subsequent inactivation of caspases.

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