WIP is a chaperone for Wiskott–Aldrich syndrome protein (WASP)

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Wiskott–Aldrich syndrome protein (WASP) is in a complex with WASP-interacting protein (WIP). WASP levels, but not mRNA levels, were severely diminished in T cells from WIP–/− mice and were increased by introduction of WIP in these cells. The WASP binding domain of WIP was shown to protect WASP from degradation by calpain in vitro. Treatment with the proteasome inhibitors MG132 and bortezomib increased WASP levels in T cells from WIP–/− mice and in T and B lymphocytes from two WAS patients with missense mutations (R86H and T45M) that disrupt WIP binding. The calpain inhibitor calpeptin increased WASP levels in activated T and B cells and in T and B lymphocytes from two WAS patients with missense mutations (R86H and T45M) that disrupt WIP binding (15). Here we demonstrate that WIP stabilizes WASP and suggest that it may also be important for its function.

WASP, the product of the gene mutated in Wiskott–Aldrich syndrome (WAS), is expressed only in hematopoietic cells and is the first identified member of a family of proteins that include N-WASP and Scar/WAVE (1–3). WASP plays a critical role in T cell activation and actin reorganization. WASP translocates to lipid rafts after T cell receptor (TCR) ligation and localizes at the immune synapse between T cells and antigen-presenting cells (4, 5). T cells from WAS patients and WASP–/− mice are deficient in their ability to increase their F-actin content, secrete IL-2, and proliferate after TCR ligation, are deficient in immune synapse formation, and have a disorganized actin cytoskeleton (13).

Most missense mutations in patients with WAS and X-linked thrombocytopenia are localized to the WIP binding EVH1 domain of WASP (12, 14). In fact, three of these mutations, R86H, Y107C, and A134T, which affect residues that are predicted by NMR studies to be contact points for WIP, were shown to disrupt WIP binding (15). Here we demonstrate that WIP stabilizes WASP from degradation by calpain and the proteasome.

Results

WASP Is Unstable in the Absence of WIP. Expression of WASP in splenic T cells from WIP–/− mice was severely diminished (Fig. 1A). In contrast, expression of Vav1 and actin was unaffected (Fig. 1A). Scanning densitometry revealed that the level of WASP expression in WIP–/− T cells was 84 ± 0.9% of controls (n = 3). Diminished expression of WASP in WIP–/− T cells was confirmed by intracellular FACS analysis by using anti-WASP mAb (Fig. 1B). Northern blot analysis revealed similar levels of WASP mRNA in WIP–/− and WT T cells (Fig. 1C). WASP mRNA levels assessed by quantitative RT-PCR were similar in WT and WIP–/− T cells (mean WASP mRNA level in WIP–/− cells, normalized to GAPDH, 90 ± 8% of control T cells, n = 3; data not shown). Examination of B cells from WIP–/− mice revealed severely decreased WASP levels to 10.0 ± 3.0% of control (n = 3) (Fig. 1D). N-WASP level was similar in cultured lung fibroblasts from WIP–/− and WT controls (mean N-WASP level in WIP–/− fibroblasts, normalized to actin, 111 ± 3% of control fibroblasts) (Fig. 1E). WIP level was not affected by the absence of WASP (mean WASP level in WIP–/− T cells, normalized to actin level, was 94 ± 2% of control WT T cells, n = 3) (Fig. 1F). These results suggest that WIP stabilizes WASP.

Overexpression of WIP Results in Increased WASP Levels. Jurkat cells were stably transfected with myc-tagged constructs that encode C-terminal WIP (C-WIP, amino acids 401–503) or N-terminal WIP (N-WIP, amino acids 1–400), placed under the control of the doxycycline (DOX)-inducible promoter. Myc-tagged C-WIP but not myc-tagged N-WIP was confirmed to bind WASP (Fig. 2A). DOX treatment of C-WIP-transfected cells, but not of N-WIP-transfected cells, for 48 h resulted in an ∼3-fold increase in WASP level (Fig. 2B), with no change in WASP mRNA expression (data not shown). There was no detectable change in the level of endogenous WIP in Jurkat cells transfected with either C-WIP or N-WIP (data not shown). Overexpression of WIP–GFP in T293 cells caused no detectable increase in N-WASP levels (Fig. 2C). These results suggest that WIP stabilizes WASP, but not N-WASP, in cells.

Expression of WIP Increases WASP Levels in WIP–/− T Cells. T cells were transfected by nucleofection with plasmids encoding for WIP, N-WIP, or C-WIP. C-WIP expression increased WASP levels of WIP-deficient Jurkat cells (Fig. 2B). N-WIP expression had no effect on WASP levels (data not shown). WASP levels in WIP–GFP–transfected cells were not affected by the presence of WIP (data not shown). WASP levels in WIP–/− T cells transfected with N-WIP or C-WIP increased to 30.4% (n = 3) (Fig. 2C). These results suggest that WIP stabilizes WASP.


The authors declare no conflict of interest.

Abbreviations: TCR, T cell receptor; WAS, Wiskott–Aldrich syndrome; WASP, WAS protein; WIP, WASP-interacting protein; DOX, doxycycline.

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respectively, in two independent experiments. Fig. 4 shows that virtually no WIP could be detected in the T cell lysates after WIP immunodepletion. Scanning densitometry revealed that the amount of WIP that remained in lysates immunodepleted of WIP was, respectively, 12% and 14% in two independent experiments. Thus, the majority of WASP and WIP are associated with each other in T cells.

**The Majority of WASP and WIP Exist in a Complex in T Cells.** To determine how much of WASP in T cells is associated with WIP we examined the residual amount of WASP in T cell lysates immunodepleted of WIP by five sequential immunoprecipitations using WIP mAb. Fig. 4A shows that virtually no WIP could be detected in the T cell lysates after WIP immunodepletion. Scanning densitometry revealed that the amount of WASP that remained in lysates immunodepleted of WIP was 27% and 20%, respectively, in two independent experiments. Fig. 4B shows that WIP Protects WASP from Degradation by Calpain in Vitro. WASP in platelet lysates is degraded by the calcium-dependent protease calpain (16). We first determined whether WASP is a direct substrate for calpain. *In vitro* transcribed and translated WASP was completely degraded by calpain I at 20 units/ml or more but not by caspase 3 [supporting information (SI) Fig. 7B]. GST-C-WIP (amino acids 401–503), which binds to WASP, but not GST-N-WASP (amino acids 1–108), which does not bind to WIP, inhibited the degradation of WIP by calpain I (SI Fig. 7B). This was not because of interference with calpain enzymatic activity, because GST-C-WIP did not inhibit the degradation of CrkL, a known substrate of calpain (17) (SI Fig. 7B). These results suggest that WIP masks calpain-sensitive sites in WASP.

**Inhibitors of the Proteasome Increase WASP Levels in WIP−/− T Cells.** The finding that WASP is a substrate for calpain and the observation that N-WASP is subject to ubiquitination (18) virtually no N-WASP could be detected in the T cell lysates after N-WASP immunodepletion. Scanning densitometry revealed that the amount of WIP that remained in lysates immunodepleted of N-WASP was, respectively, 12% and 14% in two independent experiments. Thus, the majority of WASP and WIP are associated with each other in T cells.
prompted us to examine whether inhibitors of calpain and/or the proteasome increased WASP expression in WIP−/− T cells. FACS analysis revealed that preincubation for 6 h of WIP−/− T cells with MG132 increased the level of WASP, but not Vav1 protein (Fig. 5A). The mean fluorescence intensity for WASP staining in WIP−/− T cells treated with MG132 was 2.2 ± 0.2-fold that of untreated WIP−/− T cells (n = 3, P < 0.05). The level of WASP in WIP−/− T cells compared with that of untreated WT T cells rose from 0.10 ± 0.02 to 0.22 ± 0.03 after MG132 treatment as determined by mean fluorescence intensity (n = 3, P < 0.05). In contrast to MG132, calpeptin had no detectable effect on WASP levels in WIP−/− T cells and exerted no additional effect to that of MG132 when WIP−/− T cells were treated with both inhibitors. There was no detectable increase in the level of WASP in normal T cells treated with MG132 and/or calpeptin (Fig. 5A).

Western blot analysis of T cell lysates confirmed the results obtained by FACS (Fig. 5B). Densitometric scanning revealed that the mean WASP levels in WIP−/− T cells treated with MG132 was 2.4 ± 0.2-fold that of untreated WIP−/− T cells (n = 3, P < 0.05). The level of WASP in WIP−/− T cells compared with that of untreated WT T cells rose from 0.09 ± 0.01 to 0.23 ± 0.04 after MG132 treatment (n = 3, P < 0.05). Calpeptin had no detectable effect on WASP levels in WIP−/− T cells. Increasing the concentrations of the inhibitors and/or increasing the incubation time beyond 6 h caused increased cell death and did not result in a detectable increase in WASP levels. These results suggest that degradation by the ubiquitin–proteasome pathway contributes to WASP degradation in the absence of WIP.

We confirmed the above results using additional proteasome

Fig. 4. Effect of immunodepletion of WIP on WASP levels (A) and of WASP on WIP levels (B) in T cells. T cell lysates were immunoprecipitated sequentially five times with anti-WASP or anti-WIP mAbs, and the lysates and immunoprecipitates were run on gels and Western blotted and probed for WASP or WIP. The numbers 0–5 refer to the number of times sequential immunoprecipitation was performed. The numbers below the Western blot of the lysates refer to the percent residual WASP or WIP with 100 being the value in the lysates before immunodepletion.
and calpain inhibitors. Treatment of WIP−/− T cells with the proteasome inhibitor bortezomib caused an increase in WASP levels comparable to that observed with MG132 (SI Fig. 8A). This inhibitor caused negligible cell death after 6 h of treatment but resulted in >90% cell mortality by 24 h. In contrast, treatment of WIP−/− T cells with the calpain inhibitor CS peptide (27-aa peptide encoded by exon 1B of human calpastatin) caused no detectable increase in WASP levels (SI Fig. 8B). Both calpeptin and CS peptide were shown to substantially reduce calpain activity in T cell lysates (SI Fig. 8C).

**Effect of Proteasome and Calpain Inhibitors on WASP Levels in Lymphocytes from WAS Patients with Missense Mutations in WASP That Disrupt WIP Binding.** The majority of WAS patients with missense mutations in WASP have point mutations in the WIP binding domain (19). One of these mutations, R86H, was shown to disrupt WIP binding and to be associated with severely diminished WASP levels with normal mRNA levels (15). Western blot analysis of lysates from freshly isolated T cells from a patient with the R86H mutation (A and B) and in EBV-transformed B cells from a WAS patient with T45M mutation (C).

![Image](Image1)

**Fig. 6.** Effect of calpain and proteasome inhibitors on WASP levels in lymphocytes from WAS patients. Effect of inhibitors on WASP levels in purified peripheral blood T cells and PHA/IL-2 propagated T cells from a WAS patient with R86H mutation (A and B) and in EBV-transformed B cells from a WAS patient with T45M mutation (C).

Examined the effect of calpeptin on WASP levels in PHA- and IL-2-propagated T cell lines derived from the patient with the R86H mutation and two normal controls. The mean WASP content of two independent T cell lines from the patient was 12% that of T cell lines derived from the two normal donors. Preincubation with MG132 increased WASP levels in the patient’s cell lines by 2.3 ± 0.4-fold (n = 3) to 28% that of untreated normal T cell lines. Calpeptin treatment of the patient’s T cell lines caused a 2.1 ± 0.35-fold (n = 3) increase in WASP levels (Fig. 6B). The combination of MG132 and calpeptin caused a 1.8 ± 0.21-fold (n = 3) increase in WASP levels and was associated with increased cell death. This corresponded to a WASP level 25% that of untreated normal T cell lines. MG132 had no detectable effect on Vav1 levels in the patient’s cell lines. MG132 and calpeptin had no detectable effect on WASP or Vav1 levels in normal T cell lines. These results suggest that both calpain and the proteasome participate in the degradation of the mutant WASP in activated T cells.

We examined the effect of calpeptin and MG132 on WASP levels using EBV-transformed B cell lines derived from a WAS patient with a T45M mutation and two healthy controls. The T45M mutation is predicted by NMR studies to be a contact point for WIP binding (12). The patient’s peripheral blood mononuclear cells and B cell line expressed WASP at a level that ranged from 15% to 20% that of control but had normal WASP mRNA levels (25) (Fig. 6C and data not shown). Preincubation of the patient’s B cell line with MG132 or calpeptin increased WASP levels by 2.3 ± 0.27-fold and 2.1 ± 0.22-fold, respectively (mean of three experiments), but had no detectable effect on Vav1 levels (Fig. 6C). The combination of MG132 and calpeptin caused a 1.4 ± 0.21-fold (n = 3) increase in WASP levels and was associated with increased cell death. Addition of both inhibitors resulted in diminished up-regulation of WASP levels compared with either inhibitor alone and was associated with increased cell death. MG132 and calpeptin had no detectable effect on WASP or Vav1 levels in normal B cell lines. These results suggest that both calpain and the proteasome participate in the degradation of the mutant WASP in activated B cells.

**Effect of the Proteasome Inhibitor Bortezomib on Actin Content and IL-2 Expression by T Cells with R86H Mutation in WASP.** Primary T cells from patients with WAS have impaired IL-2 gene expression after stimulation by immobilized anti-CD3 mAb (26), and herpes virus saimiri-transformed T cell lines from these patients have a defect in polymerizing actin after TCR ligation (6). Because the proteasome inhibitor bortezomib was not toxic to murine T cells in the first 6 h, we examined its ability to correct the functional defect in primary T cells from the patient with the R86H mutation in WASP. Bortezomib caused negligible death of human T cells at 10 h but substantial death (>90%) at 24 h. Incubation of the patient’s T cells with bortezomib for 10 h resulted in a 2.4 ± 0.5-fold increase in WASP level to 23% of the level in control T cells (n = 3), results similar to those obtained with MG132. However, bortezomib failed to ameliorate the impaired IL-2 gene expression in response to immobilized anti-CD3 mAb (SI Fig. 9A). T cells from the patient had decreased F-actin content, but, in contrast to what was reported for herpes virus saimiri-transformed T cell lines from WAS patients, they were able to polymerize actin after anti-CD3 stimulation. Bortezomib had no effect on the low F-actin content of these cells (SI Fig. 9B).

**Discussion**

Our results show that WIP deficiency or impaired WIP–WIP interaction results in WASP degradation by the proteasome and calpain. WASP levels were markedly diminished in T cells from WIP−/− mice and in lymphocytes of WAS patients with missense mutations in the EVH1 domain of WASP that disrupt WIP
binding. The decrease occurred in the face of normal levels of WASP mRNA (Figs. 1A and 6). In contrast, WIP levels were normal in WASP-deficient T cells (Fig. 1F), indicating that WIP is necessary for the stabilization of WASP, but not vice versa. The finding that N-WASP levels were normal in WIP−/− fibroblasts (Fig. 1E) suggests that WIP may not be needed to stabilize N-WASP. This could be because of an intrinsic property of N-WASP or the expression in nonhematopoietic cells of WIP homologues, such as CR16 and WIRE/WIC (27–29).

Stabilization of WASP by WIP is likely the result of their direct interaction rather than an indirect effect of WIP on WASP degradation. First, overexpression of a WIP fragment that binds WASP, but not of a WIP fragment that does not bind WASP, increased WASP expression in Jurkat cells (Fig. 2). Second, expression of WIP, but not of a WIP deletion mutant that does bind WASP, resulted in an increase in WASP levels in WIP−/− T cells (Fig. 3). Third, a WIP fragment that binds WASP, but not a WIP fragment that does not bind WASP, protected WASP from degradation by calpain in vitro (SI Fig. 7). Finally, WASP levels are decreased in lymphocytes from patients with point mutations that destroy WIP binding (Fig. 6). We were unable to detect WASP fragments in WIP−/− T cells or in lymphocytes from WAS patients with the R86H and T45M mutants. This is possibly because of rapid degradation of these fragments.

Preincubation with the proteasome inhibitor MG132, but not with the calpain inhibitor calpeptin, partially restored WASP levels in primary T cells from WIP−/− mice and from a WAS patient with a missense mutation in the EVH1 domain of WASP, R86H, that disrupts WIP binding (Figs. 5 and 6A). Similar results were obtained in WIP−/− T cells with the proteasome inhibitor bortezomib and the calpain inhibitor CS peptide (SI Fig. 8). This suggests that WIP is a chaperone for WASP and that, in its absence, WASP can be degraded in primary T cells by the ubiquitin–proteasome pathway. The increase in WASP levels was specific because it was not observed in normal T cells and because VaV1 levels were unaffected. The failure to observe a detectable effect of MG132 on WASP levels in normal T cells may be explained by the observation that the majority of WASP is complexed with WIP in T cells (Fig. 4).

Our findings implicate calpain in WASP degradation in activated lymphocytes of patients with WASP mutations that disrupt WIP binding. In contrast to primary T cells, calpain inhibition increased WASP levels in activated T cells of the R86H patients and in EBV-transformed cells from another WAS patient with a missense mutation, T45M, that disrupts WIP binding (Fig. 6 B and C). Calpain is known to be activated after TCR and B cell receptor cross-linking (22, 30). Furthermore, calpain mRNA and expression levels are increased after activation of T and B cells (31).

WASP has seven potential calpain-sensitive sites based on the highly stringent criterion of P1 residue being Y, M, or R and P2 residue being a hydrophobic amino acid (32). Four of these sites are in the EVH1 domain. Using less stringent criteria, there are several additional potential calpain-sensitive sites in WASP. Our finding that the C-terminal WASP binding domain of WIP specifically protected WASP from degradation by calpain (SI Fig. 7) suggests that calpain attacks sites in the EVH1 domain of WASP and that WIP masks these sites. The minimal WIP peptide that binds WASP spans amino acids 461–485. We found that this peptide does not protect WASP from degradation by calpain in vitro (data not shown), suggesting that other regions of WIP contribute to its ability to protect WASP from degradation.

There are many similarities in the phenotype of WAS knock- out mice and WIP knockout mice. They include defective proliferation and IL-2 secretion in response to immobilized anti-CD3 and defective immune synapse formation and F-actin reorganization (8, 13, 33). The finding that WASP levels are severely diminished in T cells from WIP−/− mice suggests that WASP deficiency contributes to the phenotype of these mice. However, WIP−/− T cells, but not WASP−/− T cells, have a disrupted subcortical actin cytoskeleton (13) and show severe defects in chemotaxis in response to stromal cell-derived factor 1α (34). Furthermore, the phenotype of WIP and WASP double deficient mice is markedly more severe than that of either WIP or WASP single deficient mice, with reduced numbers of thymocytes and peripheral CD4+ T cells and early development of colitis, pneumonitis, and iritis (our unpublished observations). These observations strongly suggest that WIP, which interacts independent of WASP with a number of proteins that include Nck, CrkL, Syk, actin, and profilin (5, 11, 35, 36), has distinct functions, which are not dependent on WASP.

We were unable to observe amelioration of the impaired IL-2 gene expression in response to immobilized anti-CD3 and of the decreased F-actin content of T cells with R86H mutation in WASP after incubation with bortezomib for 10 h, a time at which cell viability was intact (SI Fig. 9). It is possible that the WASP levels achieved may have not been sufficient to correct these defects. Alternatively, the ability of WASP to correct these defects may depend on its capacity to interact and function with WIP as a complex. A genetic approach in which a minimal WIP fragment that is sufficient to normalize WASP level is expressed in WIP−/− T cells is needed to distinguish between these possibilities.

**Experimental Procedures**

**Cells, Mice, Reagents, and Antibodies.** Jurkat E6–1 T cells were obtained from American Type Culture Collection (Manassas, VA). Generation and characterization of WIP−/− and WASP−/− mice have been reported previously (8, 13). Mouse thymocytes were purified as described previously (13). Blood was obtained from WAS patients and controls with informed consent, and T cells were purified from peripheral blood mononuclear cells by using a human MageCleCt Cell isolation kit (R & D Systems, Minneapolis, MN). T cell lines were derived by stimulation with PHA (10 μg/ml) for 3 days, and after 3 days the cells were expanded by addition of 100 units/ml human rIL-2 (R & D Systems). EBV-transformed B cell lines were established by using a standard protocol. Cells were cultured in RPMI medium 1640 with 10% FCS. WIP−/− fibroblasts derived from lung were cultured in Iscove’s modified Dulbecco’s medium with 10% FCS (13).

MG132, calpain I, calpeptin, caspase inhibitor Z-VAD-FMK, and human calpastatin exon 1B encoded peptide (CS peptide) were purchased from Calbiochem (San Diego, CA). Bortezomib (Velcade) was obtained from Millennium Pharmaceuticals (Cambridge, MA). FuGene transfection agent was from Roche (Indianapolis, IN). Generation of rabbit anti-WASP antisemum C14, anti-WASP antisemum K-374, and anti-WASP mAb 5A5 was described previously (5, 6, 37). Anti-WASP mAb B-9, anti-Vav, anti-CrkL, mAb, and rabbit anti-NWASP antibody (H-100) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin mAb was purchased from Chemicon (Temecula, CA). Anti-Vav1 was purchased from Abcam (Cambridge, MA). Anti-c-myc mAb 9E10 was from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-WASP mAb 3D10 was raised against the N terminus of WIP. Calpain inhibition was quantified by using a calpain assay kit (Biovision, Mountain View, CA) as suggested by the manufacturer except that the cell lysates were incubated with the substrate overnight, protected from light.

**Transfection of T Cells with WIP–GFP and GFP–WASP Constructs.** WT WIP and WIP Δ461–488 were cloned in phase with GFP at the C terminus of WIP in the expression vector pCDNA3. Five micrograms of each construct was used to transfect 1 × 10⁵ T cells by using a mouse T cell nucleofector kit and an Amaza Biosystem nucleofector. The program X-01 was used, and opti-
minal expression of the fusion proteins was observed 18 h after transfection.

For the analysis of WASP expression level, transfected cells were permeabilized and stained with mAb SA5 or mAb B-9 by using goat anti-mouse phycoerythrin (BD Biosciences, San Jose, CA) as the secondary antibody. WASP fluorescence in gated GFP+ cells was analyzed by FACS. In parallel, 1 × 10^6 transfected cells were lysed in lysis buffer [50 mM Tris, pH 7.4/100 mM NaCl/1% Triton X-100/protease inhibitors (Complete, Roche)], immunoprecipitated with anti-GFP polyclonal antibody (BD Biosciences), Western blotted, and probed with anti-WASP mAb B-9. The blots were stripped and reprobed with anti-GFP mAb (BD Biosciences).

**Stable Transfection and Inducible Expression of WIP.** Human N-terminal WIP (1–400) and C-terminal WIP (401–503) were cloned in frame into pTRE2hyg-2-myc vector (Clontech, Mountain View, CA) and sequence-verified. A total of 10 μg of plasmids was transfected by electroporation into 1 × 10^6 Jurkat cell clones stably transfected with pTet-on vector (Clontech) by using a Bio-Rad Genepulser II set at 250 V and 975 g. Cells were lysed in lysis buffer containing 1% Triton X-100, and lysates were clarified at 16,000 g for 15 min. The cell lysates were analyzed by immunoblotting.

**Cell Stimulation, Immunoprecipitation, Immunoblotting, and Northern Blotting.** Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, and lysates were clarified at 16,000 × g for 15 min at 4°C and preclared for 1 h with protein G-Sepharose (Amersham Pharmacia Biotech, Fairfield, CT). Immunoprecipitation was performed overnight at 4°C with the indicated antibody (4 μg) or antiserum (4 μl) preadsorbed onto protein G-Sepharose. Beads were washed five times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on SDS-PAGE 4–15% gradient gels, and analyzed by Western blotting with indicated antibodies followed by goat anti-mouse or anti-rabbit antibodies conjugated to HRP and ECL detection (PerkinElmer, Boston, MA) as previously described (5).

**Protein Preparation and in Vitro Calpain Cleavage.** Linearized human WASP cDNA (1 μg) was transcribed and translated in vitro with 50 μl of TNT-coupled reticulocyte lysate system (Promega, Madison, WI). GST-CrKL, GST-C-WIP (401–503), and GST protein were purified as described previously (5). In vitro translated WAP or GST-CrKL (~25 ng) was preincubated with 40 ng of GST-WIP or GST alone for 1 h on ice. In vitro cleavage assay with calpain was performed by incubating WASP at the indicated concentrations of calpain I in reaction buffer containing 100 mM imidazole (pH 7.4), 10 mM 2-mercaptoethanol, and 5 mM CaCl2, with or without 100 μM calpeptin or 5 mM EDTA for 10 min at 30°C. Cleavage assay with caspase-3 was performed by incubating the same substrates at the indicated concentrations of caspase-3 in a reaction buffer containing 50 mM Heps (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10% glycerol, and 10 mM DTT with or without 50 μM Z-DEVd-FMK for 30 min at 30°C.

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