Journal of Neurochemistry, 2004, **90**, 231–244 doi:10.1111/j.1471-4159.2004.02486.x

CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70

Makoto Urushitani,*[,1] Junko Kurisu,* Minako Tateno,* Shigetsugu Hatakeyama,† Kei-Ichi Nakayama,† Shinsuke Kato‡ and Ryosuke Takahashi*

*Laboratory for Motor System Neurodegeneration, RIKEN Brain Science Institute, Wako, Saitama, Japan

†Division of Cell Biology, Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

‡Department of Neuropathology, Institute of Neurological Science, Faculty of Medicine, Tottori University, Tottori, Japan

Summary

Over 100 mutants in superoxide dismutase 1 (SOD1) are reported in familial amyotrophic lateral sclerosis (ALS). However, the precise mechanism by which they are degraded through a ubiquitin-proteasomal pathway (UPP) remains unclear. Here, we report that heat-shock protein (Hsp) or heat-shock cognate (Hsc)70, and the carboxyl terminus of the Hsc70-interacting protein (CHIP), are involved in proteasomal degradation of mutant SOD1. Only mutant SOD1 interacted with Hsp/Hsc70 *in vivo*, and *in vitro* experiments revealed that Hsp/Hsc70 preferentially interacted with apo-SOD1 or dithiothreitol (DTT)-treated holo-SOD1, compared with metallated or oxidized forms. CHIP, a binding partner of Hsp/Hsc70, interacted only with mutant SOD1 and promoted its degradation. Both Hsp70 and CHIP promoted polyubiquitination of mutant SOD1-associated molecules, but not of mutant SOD1, indicating that mutant SOD1 is not a substrate of CHIP. Moreover, mutant SOD1-associated Hsp/Hsc70, a known substrate of CHIP, was polyubiquitinated *in vivo*, and polyubiquitinated Hsc70 by CHIP interacted with the S5a subunit of the 26S proteasome *in vitro*. Furthermore, CHIP was predominantly expressed in spinal neurons, and ubiquitinated inclusions in the spinal motor neurons of $hSOD1^{G93A}$ transgenic mice were CHIP-immunoreactive. Taken together, we propose a novel pathway in which ubiquitinated Hsp/Hsc70 might deliver mutant SOD1 to, and facilitate its degradation, at the proteasome.

Keywords: amytrophic lateral sclerosis, CHIP, Hsp70, proteasome degradation, superoxide dismutase 1, ubiquitin.

J. Neurochem. (2004) **90**, 231–244.

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by progressive palsy in limbs and bulbar muscles. A genetic mutation in superoxide dismutase 1 (SOD1) was identified in 1993 as a causal defect shared in 20% of familial ALS patients (Rosen *et al.* 1993). Although SOD1 is an antioxidant enzyme, accumulating lines of evidence strongly support a 'gain of toxic function hypothesis' rather than a 'loss of function hypothesis' (Cleveland and Rothstein 2001; Julien 2001).

Recent lines of evidence indicate that the misfolded nature of mutant SOD1 is a distinct feature common in over 100 mutants (Valentine and Hart 2003). Several misfolded forms taken by mutant SOD1 reportedly include a non-native oligomer, a soluble aggregate, and a detergent-insoluble aggregate (Johnston *et al.* 2000; Shinder *et al.* 2001; Urushitani *et al.* 2002). In addition, most types of mutant SOD1 are more readily monomerized by a reducing agent

Received October 6, 2003; revised manuscript received January 23, 2004; accepted March 3, 2004.

Address correspondence and reprint requests to Ryosuke Takahashi, MD, PhD, Laboratory for Motor System Neurodegeneration, RIKEN Brain Science Institute, 2–1 Hirosawa, Wako, Saitama 351–0198, Japan. E-mail: ryosuke@brain.riken.go.jp

[1]The present address of Makoto Urushitani is Department of Molecular Endocrinology, Research Centre of the CHUL (Centre Hospitalier de l'Université Laval), 2705 boul. Laurier, Québec, Canada, G1V4G2.

Abbreviations used: ALS, amyotrophic lateral sclerosis; CHIP, carboxyl terminus of Hsc70-interacting protein; CHX, cycloheximide; DTT, dithiothrietol; E1, ubiquitin activating enzyme; E2, ubiquitin converting enzyme; E3, ubiquitin ligase; EIP, immunoprecipitation; GST, glutathione-S-transferase; HA, hemagglutinin; Hsc, heat-shock cognate protein; Hsp, heat-shock protein; IB, immunoblotting; LBHIs, Lewy body-like hyaline inclusions; 2ME, 2-mercaptoethanol; SOD1, superoxide dismutase 1; TPR, tetratricopeptide repeat; UPP, ubiquitin-proteasomal pathway.

than the wild-type, thus allowing their intramolecular hydrophobic amino acid residues to be exposed to cytoplasm (Tiwari and Hayward 2003). Other groups have demonstrated that the apo-state enhances the instability of mutant SOD1 (Lindberg *et al.* 2002). These features might be the molecular basis for degradation of mutant SOD1 through the ubiquitin-proteasomal pathway (UPP) (Nakano *et al.* 1996; Johnston *et al.* 2000). We previously demonstrated that mutant, not wild-type, SOD1 is conjugated to a multiubiquitin chain and degraded at the proteasome (Urushitani *et al.* 2002). Although the precise mechanism underlying UPP for handling mutant SOD1 is still an enigma, a mutant SOD1-specific ubiquitin ligase has been proposed (Niwa *et al.* 2002).

However, since over 100 mutants of FALS-related SOD1 have been identified, its recognition mechanism may rely upon a common mediator. In this context, a molecular chaperone, especially heat shock protein 70 (Hsp70), is a very attractive candidate. Overexpression of Hsp70 in cultured motor neurons rescues them from mutant SOD1-induced apoptosis (Bruening *et al.* 1999). This group also reported that detergent-insoluble, but not detergent-soluble, mutant SOD1 associates with Hsp70 in spinal cord lysates from G93A transgenic mice (Shinder *et al.* 2001). Moreover, mutant SOD1 interacts with Hsp70 under heat-shock conditions (Okado-Matsumoto and Fridovich 2002). Pathologically, hSOD1-immunoreactive inclusions in spinal cord of the patients or transgenic mice expressing mutant SOD1 are frequently stained with antibody against heat-shock cognate (Hsc)70, a constitutively expressed form of Hsp70 (Watanabe *et al.* 2001). However, the role of Hsp/Hsc70 in mutant SOD1 metabolism remains unclear. Interestingly, Hsc70 facilitates the degradation of several proteins at the proteasome in a ubiquitination-dependent manner (Bercovich *et al.* 1997), suggesting a role for Hsp/Hsc70 in handling various types of misfolding proteins for proteasomal degradation. For instance, the rate of degradation of androgen receptors containing expanded polyglutamine in bulbospinal muscular atrophy was accelerated by Hsp70 both in cells (Bailey *et al.* 2002) and transgenic mice (Adachi *et al.* 2003).

The carboxyl terminus of Hsc70-interacting protein (CHIP) contains a tetratricopeptide repeat (TPR) domain with which Hsp/Hsc 70 interacts, and inhibits chaperoning by Hsp/Hsc70 through a decrease in ATPase activity (Ballinger *et al.* 1999; Aravind and Koonin 2000). To date, various molecules have been identified as CHIP substrates, including glucocorticoid receptors (Connell *et al.* 2001), misfolded cystic fibrosis transmembrane-conductance regulator (CFTR) (Meacham *et al.* 2001), heat-denatured luciferase (Murata *et al.* 2001) and transmembrane receptor tyrosine kinase ErbB2 (Xu *et al.* 2002). The wild-type androgen receptor containing a normal polyglutamine repeat is also ubiquitinated by CHIP (Cardozo *et al.* 2003).

This study investigated the role of Hsp/Hsc70 and CHIP in the degradation of mutant SOD1 in UPP. Evidence is provided that CHIP promotes the formation of a ubiquitin-mutant SOD1 complex and proteasomal degradation. Surprisingly, however, this effect was not caused by ubiquitination of mutant SOD1, but of Hsp/Hsc70. This result indicates that ubiquitinated Hsp/Hsc70 recruits mutant SOD1 toward the proteasome. This is a novel pathway that explains the degradation mechanism of more than 100 mutant SOD1, as well as the pathology of ALS.

Experimental procedures

Materials

Antisera specific for His-CHIP were prepared in rabbits (Imai *et al.* 2002). Bovine Hsc70 was purchased from Medical and Biological Laboratories (Nagoya, Japan). Purified human E1, ubiquitin converting enzyme (E2; ubcH5a) and ubiquitn aldehyde were purchased from Boston Biochem (Cambridge, MA, USA). Rabbit polyclonal anti-hSOD1 and monoclonal antibody recognizing both Hsc70 and Hsp70 (C92F3 A-5) were purchased from StressGen (Victoria, BC, Canada). Rat monoclonal anti-HA (3F10) antibodies was purchased from Roche (Basel, Switzerland). Mouse monoclonal anti-V5 tag antibody was purchased from Invitrogen (Carlsbad, CA, USA).

Plasmid construction

Human SOD1 cDNA (hSOD1), with or without the FLAG-tag (wild-type, G85R or G93A mutant), was produced as previously described (Urushitani *et al.* 2002). pcDNA3.1-human Hsp70-V5 was a generous gift from N. Nukina (RIKEN Brain Science Institute). The preparation of hemagglutinin (HA)-tagged ubiquitin (HA-Ub) and CHIP cDNAs is described elsewhere (Hatakeyama *et al.* 2001). cDNA construction of mutant CHIP, devoid of tetratricopeptide repeat or U-box (ΔTPR or ΔU-box), was conducted by inverted PCR, using the primer pairs: 5′-ACTCGGGCTC TTATCAGGGCTGCC-3′ and 5′-CAGCGACTCAACTTTGGGGA TGATA-3′ for ΔTPR, and 5′-GTCAGGGATATCTCGCTTCTT TCTT-3′ and 5′-ATTGACGCTTTCATCTCTGAGAACG-3′ for ΔU-box. PCR products were ligated into pcDNA3 containing either the HA or Myc tag sequence at the 5′ end. 6 × His-tagged CHIP recombinant protein was produced by subcloning CHIP cDNA into pET28a(+) vector at the EcoRI/XhoI sites (Novagen, Madison, WI, USA). 6 × His-tagged Hsp70 (His-Hsp70) of full length and deletion mutant of carboxyl terminus (ΔC) or of ATPase domain (ΔATP), was produced as previously reported (Imai *et al.* 2002).

Purification of recombinant proteins

Recombinant glutathione S-transferase-fused hSOD1 (GST-hSOD1) with or without FLAG-tag at the 3′ terminus was generated as previously reported (Kang and Eum 2000; Urushitani *et al.* 2002) with minor modifications. After digestion of GST-hSOD1 ± FLAG to be released from GST, the hSOD1 proteins were demetallated by overnight incubation in 100 mM EDTA, followed by additional overnight incubation in acetate pH 3.8. Finally, they were dialyzed against a buffer containing 50 mM Tris-HCl pH 7.5 and 100 mM

NaCl (Buffer A). Metallation was performed by incubation in two equivalent parts of zinc chloride for 24 h, followed by further incubation with two-equimolar copper chloride for 24 h. The activity of demetallated or remetallated recombinant SOD1 was confirmed using a SOD1 activity assay kit (Dojindo, Kumamoto, Japan), in which dismutase activity against the superoxide anion, generated from the reaction of xanthine with xanthine oxidase, was quantified (data not shown). For several experiments, metallated SOD1 proteins were incubated with hydrogen peroxide for 1 h at room temperature, followed by overnight dialysis against buffer A. Demetallated, metallated or oxidized SOD1 was stored at −80°C until use. Recombinant 6 × His-tagged CHIP (His-CHIP) and HA-tagged ubiquitin were produced as previously reported (Hatakeyama *et al.* 2001; Imai *et al.* 2002).

Cultures and transfection

Murine neuroblastoma cell-line, Neuro2a cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS). Transfection was performed using Lipofectamin PLUS (Invitrogen) according to the manufacturer's protocol. The medium was replaced with one containing 5 mM dibutylyl cyclic-AMP (db-cAMP) 3 h after transfection to differentiate cells. Twenty-four hours after transfection, cells were harvested and processed for immunoprecipitation or immunoblotting.

Immunoblotting

Cultured cells were harvested and lysed by the following buffers depending on the experiment: TNG-T buffer consisting of 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100 and a protease inhibitor cocktail (Roche); RIPA buffer consisting of 20 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet-P40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail; and denaturing buffer consisting of 20 mM Tris-HCl pH 7.5, 1% SDS, 2 mM EDTA and 1 mM dithiothreitol (DTT). Cells were briefly sonicated on ice in TNG-T buffer or RIPA buffer, or were sonicated and boiled for 5 min at 95°C in the denaturing buffer before centrifugation (10 400 *g* for 30 min). The supernatant fluids were analyzed for immunoprecipitation or western blotting after protein concentration was determined by the Coomassie protein assay kit or bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). In most experiments, the lysates were denatured using equal amounts of 4% SDS sample buffer containing 2-mercaptoethanol (2ME) for 5 min at 95°C, separated by SDS- polyacrylamide gel electrophoresis (denaturing-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). In several experiments investigating the proportion of dimer and monomer in SOD1 protein, lysates were incubated with equal amounts of 1% SDS sample buffer without 2ME for 15 min at 37°C before SDS-PAGE (partially denaturing PAGE). A western blot image was obtained using an enhanced chemiluminescence detection kit (ECL; Amersham Bioscience, Piscataway, NJ, USA) and quantified using Scion Image software (Scion Corp., Frederick, MD, USA) in several experiments.

Immunoprecipitation and *in vivo* ubiquitination assay

For immunoprecipitation, different cell lysis buffers were used depending on *in vivo* experiments. The TNG-T buffer was used to study the interaction between SOD1 and Hsp/Hsc70. For *in vivo* ubiquitination assay, RIPA buffer was used. Twenty-four hours after transfection of the cells in 6-well culture plates, cell lysates prepared as described previously were incubated with anti-V5 antibody and protein G sepharose to immunoprecipitate Hsp70-V5 (×500 dilution), or anti-FLAG affinity gel (Sigma, St Louis, MO, USA) to immunoprecipitate SOD1-FLAG, for overnight or 1 h at 4°C, respectively. Immunoprecipitates were washed five times in RIPA buffer and eluted in 2% SDS sample buffer by boiling for 5 min at 95°C. In partially denaturing PAGE, immunoprecipitates were eluted in 0.5% SDS sample buffer without 2ME for 15 min at 37°C. The eluate was analyzed by western blotting.

In *in vivo* ubiquitination assay, HA-ubiquitin was expressed in Neuro2a cells in addition to other constructs. After immunoprecipitation as described above, western analysis was carried out using anti-HA antibody as previously reported (Urushitani *et al.* 2002). However, since non-covalent interaction was not completely disrupted by RIPA buffer, it is unclear whether polyubiquitinated species are SOD1. Therefore, when covalently ubiquitinated molecules were examined, cells were solubilized in denaturing buffer containing 1% SDS (see above) with 5 min boiling. Subsequently, the lysate was diluted in a 10-fold volume of dilution buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA) before immunoprecipitation. Covalent ubiquitination of the target proteins was evaluated using antibodies against HA or the substrate proteins (Jiang *et al.* 2001).

Monomer-dimer transition study of SOD1

SOD1 possesses an unusual intrasubunit disulfide bond to stabilize its dimer formation (Abernethy *et al.* 1974). The holo-state SOD1 dimer contact is tight enough to be resistant to mild SDS denaturation. On the other hand, a reducing agent such as DTT or 2ME is required for complete monomerization in addition to boiling with SDS (Abernethy *et al.* 1974). Remetallated recombinant SOD1-FLAG proteins were treated by H_2O_2 (0.1 or 1 mM) for 1 h at room temperature or by DTT (0.1, 1.0 or 10 mM) for 1 h at 37°C. Proteins were incubated with an equal amount of 1% SDS sample buffer without 2ME for 15 min at 55°C, before SDS-PAGE (partially denaturing PAGE) and western blotting using anti-SOD1 antibody as described above.

In vitro binding assay

We investigated the molecular features of SOD1 protein that interact with Hsc70 by *in vitro* binding study. First, mouse monoclonal anti-Hsp/Hsc70 antibody was incubated overnight with Protein G sepharose (1 μg per 10 μL sepharose) at 4°C. Recombinant SOD1-FLAG proteins (0.4 μM), which were demetallated, remetallated or treated with hydrogen peroxide (0.1 or 1.0 mM) or 1 mM DTT as described above, and bovine Hsc70 (0.2 μM), were mixed in a binding buffer consisting of 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and 0.5 mg/mL bovine serum albumin (BSA) for 1 h at 4°C. The mixture was incubated overnight with the antibody-bound protein G sepharose at 4°C. The immunoprecipitates were washed with wash buffer (20 mM HEPES, pH 7.4, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitor cocktail) and eluted by 2% SDS sample suffer with boiling for 5 min. The eluate was probed by rabbit polyclonal anti-hSOD1 antibody.

In vitro ubiquitination assay

In vitro ubiquitination assay was performed as previously described with minor modifications (Hatakeyama *et al.* 2001). A 1 μL aliquot of 10 μM bovine Hsc70 was reacted with E1 (100 nM), E2 (ubcH5a; 1 μM), 0.4 μg/μL HA-ubiquitin and 1 μL 10 μM His-CHIP in the ubiquitination assay buffer (20 mM Tris-HCl pH 7.5, 10 mM KCl, 2 mM ATP, 2 mM phosphocreatine, 0.05 U/μL phosphocreatine kinase, 2 mM DTT and 5 mM $MgCl_2$) at a volume of 20 μL for 30 min at 37°C. The reaction was terminated by adding 4% SDS sample buffer for 5 min at 95°C. Polyubiquitination was evaluated by western blotting using antibodies against the substrates and HA (3F10; Roche).

Degradation assay

Neuro2a cells were transiently transfected with SOD1-FLAG and HA-ubiquitin with or without CHIP. At 12 h after transfection, cells were treated with 10 μg/mL cycloheximide (CHX) to prevent protein synthesis, and were harvested 0, 6 and 24 h after CHX treatment by denaturing buffer with boiling for 5 min. The same volume of lysates was analyzed by western blotting using anti-FLAG antibody. The remaining SOD1-FLAG was quantified by densitometry using Scion Image software. Using this method, we confirmed that wild-type *SOD1-FLAG was stable until 24 h after* CHX treatment, whereas mutant SOD1-FLAG proteins were more rapidly degraded. This degradation was reversed by proteasome inhibitor (data not shown). These data were the same as those from a pulse-chase experiment reported previously (Johnston *et al.* 2000).

Assay for interaction of ubiquitinated Hsp/Hsc70 with mutant SOD1

We investigated the interaction of mutant SOD1 with ubiquitinated Hsp/Hsc70 by the following two methods. In one experiment, Neuro2a cells were transiently transfected with SOD1-FLAG and HA-ubiquitin, and cell lysates were immunoprecipitated by anti-FLAG affinity gel. The immunoprecipitates were washed in ATP-containing buffer (10 mM ATP, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ and protease inhibitor cocktail) for 15 min at 37°C with gentle shaking (ATP wash). The affinity gel was then washed three times in RIPA buffer and the eluate was assayed by western blotting using antibodies against Hsp/Hsc70 and HA.

In a separate experiment, we employed sequential immunoprecipitation of mutant SOD1-interacting protein. Cell lysates of Neuro2a transfected with SOD1-FLAG, Hsp70-V5 and HA-ubiquitin were immunoprecipitated by anti-FLAG affinity gel; the beads were subsequently incubated in the denaturing buffer described above for 5 min at 95°C. The eluate was diluted 10-fold with dilution buffer and sequentially immunoprecipitated by anti-V5 antibody with protein G sepharose at 4°C overnight. The beads were eluted by 2% SDS sample buffer, and the eluate was analyzed by western blotting using anti-HA antibody.

Multiubiquitin-proteasome binding assay

The S5a component of the 19S complex in the 26S proteasome is a non-specific multiubiquitin binding site (Walters *et al.* 2002). Human S5a cDNA was cloned from a polyA-linked cDNA pool from a human cell line (SH-SY-5Y) by conventional PCR protocols. The primer pair used GCCGAATTCAAGATGGTGTTGGAAAGCACTA as the forward primer and GCCCTCGAGTCACTTCTTGTCTTCCTCCTT as the reverse. The PCR product was digested by EcoRI/XhoI and subcloned into pcDNA3 which was then modified to contain the FLAG tag sequence at the 5′ side of the multiple cloning sites. After verification by sequencing, the FLAG-S5a construct was subcloned into pET28a(+) at a HindIII site. Recombinant FLAG-S5a protein was induced by Isopropyl-b-D-thiogalactopyranoside (IPTG) by the protocol described above, and immunopurified using anti-M2 FLAG affinity gel (Sigma). After termination of *in vitro* ubiquitination of Hsc70 and CHIP in a 20 μL reaction mixture as described above, 80 μL 40 mM Tris-HCl (pH 7.4) were added to the reaction mixture and then inserted into a column containing FLAG-S5a. The reaction mixture was incubated for 1 h at 4°C; the column was then washed four times in RIPA buffer and eluted with 2% SDS sample buffer. Samples were immunoblotted using anti-HA antibody.

Primary spinal cord culture

Primary dissociated cultures from embryonic mouse spinal cord were prepared as described elsewhere (Urushitani *et al.* 2002). Cultures were assayed 7–8 days after plating. For immunocytochemical analysis, cultures were fixed with 4% paraformaldehyde (PFA) and were reacted with antibodies against CHIP (× 1000), NeuN (Chemicon, Temecula, CA, USA; ×500), glial fibrillary acidic protein (GFAP; Dako Glostrup, Denmark) and SMI32 (Steinberger monoclonals, Baltimore, MA, USA). Alexa 448 or 546 (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. Cultures were observed under a confocal laser microscope (Olympus; Tokyo, Japan).

Immunohistochemistry of mice

The transgenic mouse line expressing the mutant B6SJL-TgN[SOD1-G93A]1Gur^{df} was purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mouse genotypes were determined by PCR as previously reported (Gurney *et al.* 1994). Post-symptomatic transgenic mice at the age of 8 months were killed and perfusion fixed by 4% PFA. Fixed-frozen sections of the spinal cord were prepared for immunohistochemistry using affinity-purified rabbit antibody against CHIP (1 μg/mL) and polyclonal antibodies to ubiquitin (× 500; Dako, Glostrup, Denmark) and to hSOD1 (× 1000; StressGen). Primary antibodies were visualized by the avidin-biotin-immunoperoxidase complex (ABC) method using the appropriate Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and 3.3′-diaminobenzidine tetrahydrochloride (DAB; Nacalai Tesque, Kyoto, Japan). The experiments were performed in accordance with the RIKEN Guide for Animal Care for Research Use.

Results

Hsp/Hsc70 interacts with apo- or monomerized SOD1

Mutant SOD1 interacts with Hsp70 under heat-shocked conditions in Neuro2a cells stably expressing hSOD1 (Okado-Matsumoto and Fridovich 2002), and detergent-insoluble mutant SOD1 is co-immunoprecipitated with Hsp70 (Shinder *et al.* 2001). As shown in Fig. 1a (1), the antibody against both Hsp and Hsc70 recognized a molecule

co-immunoprecipitated with mutant, but not with wild-type, SOD1 in Neuro2a cells (Fig. 1a). Since the expression of inducible Hsp70 was very low (data not shown) under normal conditions, the main binding partner of mutant SOD1 appeared to be Hsc70. Since the binding manner of Hsp70 with mutant or wild-type SOD1 was not affected by the addition of FLAG-tag (Fig. 1a, 2), most of the experiments in this study were performed using carboxy-terminally FLAG-tagged SOD1 (SOD1-FLAG) proteins.

We next investigated the molecular features of mutant SOD1 that are recognized by Hsp/Hsc70. Mutant SOD1 is reported to take several abnormal conformations including non-native oligomers (Johnston *et al.* 2000), detergent- insoluble species (Shinder *et al.* 2001) and oxidative stress-induced aggregation (Urushitani *et al.* 2002). To evaluate the molecular size of mutant SOD1 recognized by Hsp/Hsc70 *in vivo*, detergent-soluble fractions of neuro2a cells transfected with SOD1-FLAG and Hsp70-V5 were immunoprecipitated using anti-V5 antibody. The immunoprecipitates were incubated in a 0.5% SDS sample buffer without 2ME at 37°C, and were separated in partially denaturing PAGE. Figure 1(b) shows that a considerable amount of SOD1 dimer is preserved under these conditions (lanes 2–5 in the 5% input samples). However, most of the immunoprecipitated SOD1 with Hsp70 was monomeric mutant SOD1 [lanes 3 and 4 in the immunoprecipitation (IP) samples]. For further analysis, recombinant SOD1-FLAG proteins were demetallated, remetallated, or treated with DTT or H_2O_2, and were separated in partially denaturing SDS-PAGE. Figure 1c (1) indicates that increasing concentrations of DTT shifted recombinant SOD1 protein from dimer to monomer (Fig. 1c, 1: lanes 2–5, 9–12), which is consistent with a previous report (Tiwari and Hayward 2003). On the other hand, metallation and mild oxidative conditions stabilized dimerization of SOD1 (Fig. 1c, 1: lanes 2, 6, 9 and 13). A considerable amount of monomer is found in apo-state SOD1, as is shown in lanes 1 and 8 (arrowheads). Under extreme oxidative conditions, aggregate formation was facilitated (Fig. 1c, 1: lanes 7 and 14). Next, *in vitro* binding experiments between these recombinant SOD1-FLAG proteins and bovine Hsc70 revealed that Hsc70 interacted with DTT-treated or apo-SOD1-FLAG proteins in both wild-type and mutant (Fig. 1c, 2: lanes 1, 5, 6 and 10). We could not detect an obvious interaction between Hsc70 and holo-SOD1-FLAG or H_2O_2-treated SOD1-FLAG (Fig. 1c, 2: lanes 2–4 and 7–9). These results indicate that Hsp/Hsc70 favorably interacts with mutant SOD1 by targeting either a nascent monomer before metallation or a monomerized SOD1 in a reducing environment.

CHIP interacted with and promoted the degradation of mutant SOD1

The carboxyl terminus of Hsc70-interacting protein (CHIP) is a chaperone-dependent ubiquitin ligase (Connell *et al.* 2001; Murata *et al.* 2001; Alberti *et al.* 2002). We next tested our hypothesis that Hsp/Hsc70-CHIP is involved in the proteasomal degradation of mutant SOD1. When human SOD1 was overexpressed in Neuro2a cells, endogenous CHIP was co-immunoprecipitated with mutant SOD1, but not with wild-type (Fig. 2a). Overexpressed CHIP also interacted only with mutant SOD1 proteins (data not shown). Moreover, CHIP reduced the accumulation of detergent-insoluble mutant SOD1 in a dose-dependent manner (Fig. 2b). Degradation assay of total cell lysates revealed that CHIP reduced the remaining mutant SOD1 after protein synthesis was prevented by CHX (Fig. 2c). Both effects were reversed by the proteasomal inhibitor, lactacystin (data not shown). These results indicate that CHIP interacts with and promotes the proteasomal degradation of mutant SOD1.

CHIP does not target mutant SOD1 but its interacting proteins

To investigate whether mutant SOD1 is a substrate of CHIP, we performed an *in vivo* ubiquitination assay in which cells were solubilized in RIPA buffer, with immunoprecipitation carried out before lysate denaturing (Urushitani *et al.* 2002). In this condition, overexpression of Hsp70 or CHIP with mutant SOD1 strongly augmented the association of the polyubiquitinated species to mutant SOD1, most prominently in the presence of both Hsp70 and CHIP (Fig. 3a, upper panel). CHIP contains two functional domains: the tetratricopeptide repeat (TPR) and U box domains, which are necessary for Hsp/Hsc70 interaction and ubiquitin ligase activity, respectively. Mutant CHIP with these two domains deleted did not promote mutant SOD1-polyubiquitin complex formation (Fig. 3b). However, when the membrane was re-probed by anti-SOD1 antibody, we found mono or diubiquitinated SOD1 both in wild-type and mutants, but no polyubiquitinated SOD1 corresponding to Hsp70 or CHIP overexpression (Fig. 3a, lower panel). This was unexpected, since we had assumed mutant SOD1 was a substrate of CHIP and that immunoprecipitates consisted of polyubiquitinated mutant SOD1. These results indicate, however, that other proteins that are ubiquitinated by Hsp/Hsc70 and CHIP form complexes with mutant SOD1.

RIPA buffer still preserves appreciable non-covalent interaction as seen in mutant SOD1 and Hsp/Hsc70 (our consistent observation). Therefore, we employed an immunoprecipitation assay in which lysates were denatured by 1% SDS and boiled before immunoprecipitation to preserve only covalent binding. Under these conditions, we can detect the ubiquitination of Hsp70 by CHIP as shown in Fig. 3c (1). In contrast, no substantial effect of either Hsp70 or CHIP on mutant SOD1 ubiquitination was observed (Fig. 3c, 2; compare lane 4 with lane 6 in Hsp70 or lane 8 in CHIP), although Hsp70 and CHIP augmented the association of polyubiquitinated species with mutant SOD1 in RIPA buffer (Fig. 3c, 2; compare lanes 5, 7 and 9). On the other hand, we noted oligoubiquitination of wild-type SOD1-FLAG both in

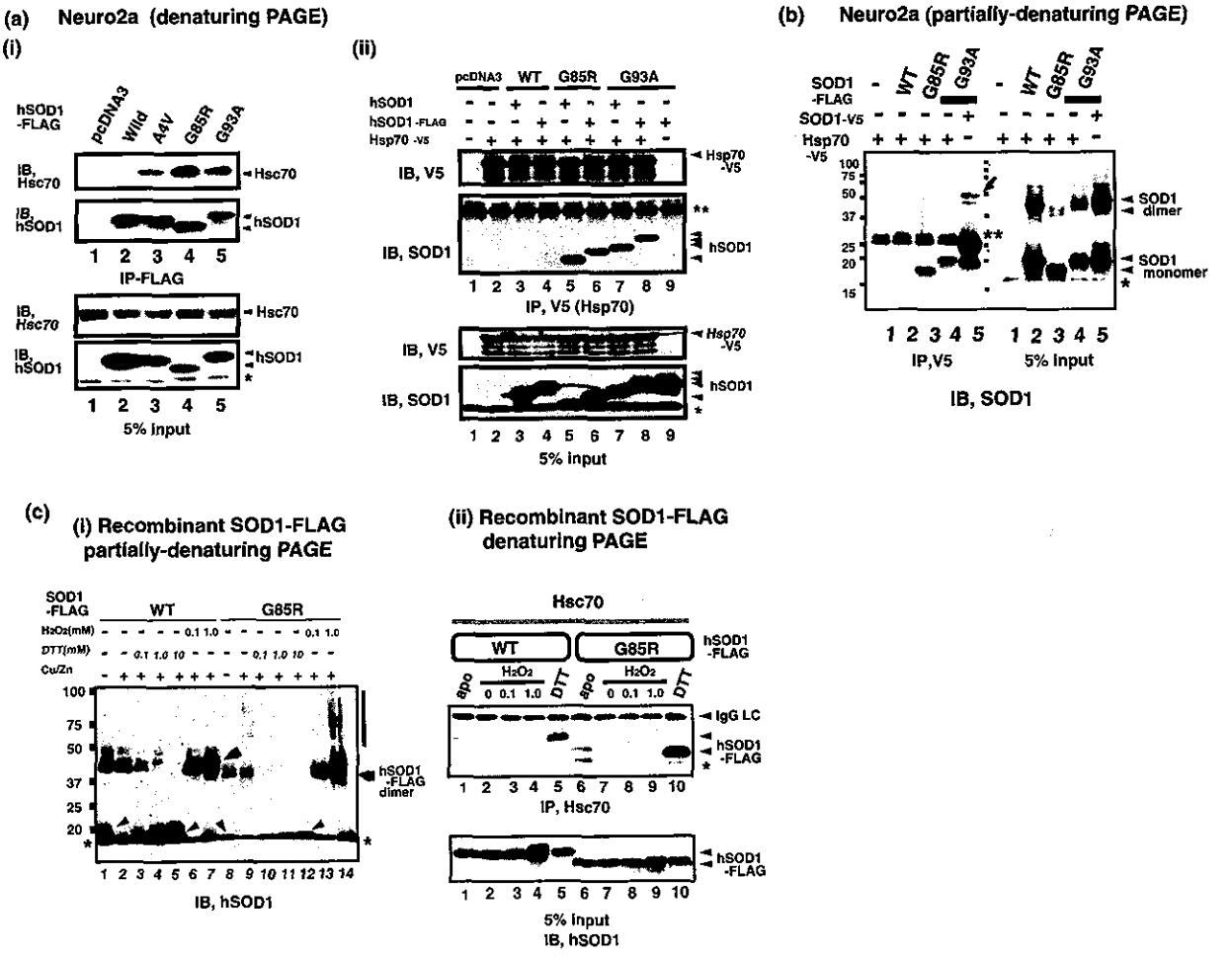


Fig. 1 Hsp/Hsc70 preferentially interacts with hSOD1 monomer or apo-state SOD1. (a) Mutant–specific interaction of SOD1 and Hsp/Hsc70 *in vivo*. (1) Neuro2a cells in 6-well culture plates were transiently transfected with hSOD1-FLAG (2 μg/well). Lysates were immunoprecipitated with anti-FLAG affinity gel, and blots were probed with anti-hSOD1 or anti-Hsp/Hsc70 antibodies. (2) Overexpressed Hsp70 interacted with mutant SOD1 with or without FLAG-tag. Neuro2a cells were transfected with Hsp70-V5, and hSOD1 with or without FLAG tag, at the C′ terminus. The cell lysates were immunoprecipitated by anti-V5 antibody, and the precipitates were probed by anti-hSOD1 or anti-V5 antibody. Asterisk indicates endogenous mice SOD1. Double asterisks indicate IgG light chain. In both (1) and (2), the immunoprecipitates were incubated in 2% SDS sample buffer containing 2ME for 5 min at 95°C before SDS-PAGE (denaturing PAGE). (b) The predominant species of mutant SOD1 that interacts with Hsp70 was a detergent-soluble monomer *in vivo*. Neuro2a cells were transiently transfected with SOD1-FLAG (1 μg/well) and Hsp70-V5 (1 μg/well) in a 6-well culture plate. The 1% TritonX 100-soluble lysates were immunoprecipitated by anti-V5 antibody. The lysates of 5% input lysates were incubated with the same volume of 1% SDS sample buffer without 2ME for 15 min at 37°C, and immunoprecipitates were eluted by 0.5% SDS sample buffer without 2ME for 15 min at 37°C. Samples were resolved in 10–20% gradient SDS-polyacrylamide gel (partially denaturing PAGE), and blots were analyzed by western blotting using anti-SOD1 antibody. Asterisk indicates endogenous mouse SOD1, and double asterisks show IgG light chain. For control in native PAGE, SOD1-V5 and SOD1-FLAG were transfected and immunoprecipitated by anti-V5 antibody. Arrow indicates dimeric SOD1 (G93A) formed by SOD1-V5 and SOD1-FLAG. (c) Apo-state or SOD1 monomer was recognized by Hsc70 *in vitro*. (1) Effect of redox conditions on dimer stabilization and monomerization. *E. coli*-purified SOD1-FLAG proteins were demetallated (lanes 1 and 8) and remetallated (lanes 2–7, 9–14). The recombinant proteins were treated with the indicated concentrations of DTT for 1 h at 37°C (lanes 3–5, 10–12) or treated with H_2O_2 for 1 h at room temperature (lanes 6 and 7, 13 and 14). The reaction mixtures were reacted with sample buffer without 2ME for 10 min at 55°C, and were resolved in 10–20% gradient SDS-polyacrylamide gel (partially denaturing PAGE). The blot was probed by anti-SOD1 antibody. Arrows indicate dimeric SOD1, whereas arrowheads indicate the reduced form of monomeric SOD1. These are present in lanes 3–5 in the wild-type, and 10–12 in G85R. In lanes 1, 7, 8 and 14, the amounts of monomeric SOD1-FLAG proteins increased (arrowheads). The asterisk indicates a possibly non-reduced monomeric SOD1. (2) *In vitro* binding experiment showing the interaction of demetallated or monomerized mutant SOD1-FLAG with Hsc70. Recombinant SOD1-FLAG proteins that were demetallated, remetallated, or treated with H_2O_2 (0.1 or 1.0 mM) or DTT (1 mM), were incubated with bovine Hsc70, followed by immunoprecipitation using anti-Hsp/Hsc70 antibody and western blotting using anti-SOD1 antibody. The asterisk indicates possible degradation product of SOD1 protein under apo-state or reducing environment.

RIPA buffer and denaturing buffer (arrowhead). To confirm this, Neuro2a cells were transfected with HA-ubiquitin and hSOD1, with or without FLAG tag, followed by immunoprecipitation using anti-HA antibody and western analysis using anti-SOD1 antibody. As shown in Fig. 3c (3), wild-type SOD1 appeared to be oligoubiquitinated to the same extent as mutant SOD1, irrespective of FLAG tag. These results strongly indicate that CHIP promotes proteasomal degradation of mutant SOD1 by ubiquitination of its binding partners.

Mutant SOD1-associated Hsp/Hsc70 was ubiquitinated and interacted with 26S proteasome subunit

Since CHIP did not ubiquitinate mutant SOD1 despite promoting mutant SOD1 degradation, it is possible that the ubiquitination of Hsp/Hsc70 was involved in proteasomal degradation of mutant SOD1. To test this, we first employed an ATP wash of co-immunoprecipitates containing mutant SOD1 from cell lysates to remove attached Hsp/Hsc70. The wash of the precipitates in ATP containing buffer at 37°C released Hsp/Hsc70 together with substantial ubiquitinated species (Fig. 4a, 1). Next, we proved directly that mutant SOD1-associated Hsp70 was ubiquitinated by sequential immunoprecipitation. The lysates from Neuro2a cells transfected with SOD1-FLAG, Hsp70-V5 and HA-ubiquitin were immunoprecipitated with anti-FLAG antibody, and then by anti-V5 antibody, sequentially. As shown in Fig. 4a (2), mutant SOD1-associated Hsp70 bound covalently with HA-tagged ubiquitin. Since a polyubiquitin chain is the recognition signal for ubiquitinated molecules to be delivered to 26S proteasome, we investigated whether polyubiquitinated Hsp/Hsc70 might translocate mutant SOD1 by S5a co-immunoprecipitation assay using recombinant proteins. S5a is a subunit of 26S proteasome that interacts with the polyubiquitin chain (van Nocker *et al.* 1996). First, we performed an *in vitro* ubiquitination experiment to make CHIP ubiquitinate Hsc70. CHIP also autoubiquitinates (Fig. 4b, 1). Next, the reaction mixture was incubated with recombinant FLAG-S5a subunit and was immunoprecipitated with anti-FLAG. As shown in Fig. 4b (2), a polyubiquitinated species, assumed to be Hsc70, interacted with the S5a subunit. This interaction was polyubiquitin-specific because no monoubiquitin (arrowhead), monoubiquitinated Hsc70 (arrow) or CHIP (double arrow heads) were observed as clearly as polyubiquitin. However, mutant SOD1 was not detected in this assay because the reaction buffer of the *in vitro* ubiquitination experiments contained ATP and $MgCl_2$, which may have had similar effects as the ATP wash (data not shown).

Neuron-specific distribution of CHIP and its co-localization with mutant SOD1-containing aggregates in the spinal motor neurons from end-stage hSOD1^{G93A} mice

CHIP mRNA was originally reported to be most abundant in skeletal muscle and heart, with somewhat lower expression levels in pancreas and brain tissue (Ballinger *et al.* 1999). In the present study, CHIP was exclusively expressed in neurons in primary mouse embryonic spinal cord culture in which approximately 90% of CHIP-positive cells were merged with the neuronal marker anti-NeuN (Fig. 5a, 1–3), but not with anti-GFAP antibody (Fig. 5a, 7–9). Motor neurons, identified by SMI32-positivity and their characteristic shape, also expressed CHIP (Fig. 5a, 4–6). The surviving motor neurons in the mutant SOD1-related ALS patients or mouse models display ubiquitinated SOD1-immunopositive inclusions, designated Lewy body-like hyaline inclusions (LBHI) (Kato *et al.* 1997). Therefore, we examined whether LBHIs in the spinal cord sections from symptomatic hSOD1^{G93A} transgenic mice immunostained with CHIP antibody (Fig. 5b, 1–3). Immunohistochemistry revealed that LBHIs are frequently immunostained by anti-CHIP antibody (Fig. 5b, 4 and 5), as well as by anti-ubiquitin (Fig. 5b, 2) and anti-SOD1 (Fig. 5b, 3) antibodies. These results suggest that CHIP is involved in the formation of LBHIs in the motor neurons of mutant SOD1 transgenic mice.

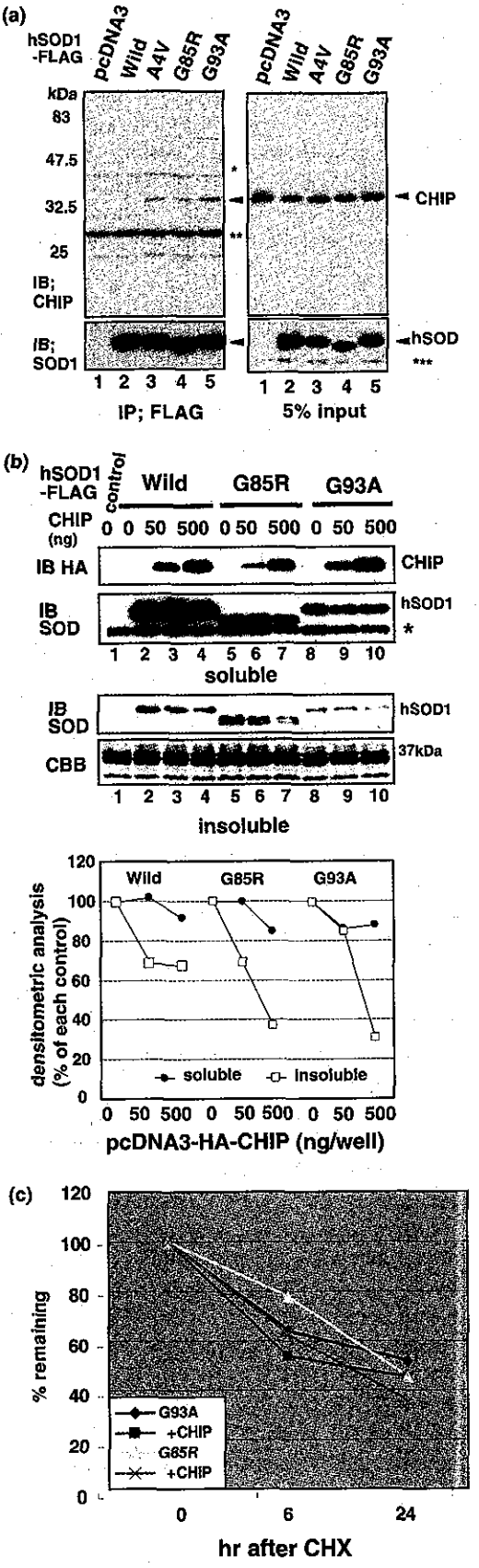
Discussion

In the present report, we demonstrated the roles of Hsp/Hsc70 and CHIP in degradation of mutant SOD1 at the proteasome. Hsp/Hsc70 preferentially interacted with the apo-state, or monomeric form, of mutant SOD1. CHIP promoted the degradation of mutant SOD1 at the proteasome in a chaperone-dependent manner. Surprisingly, this effect was mediated by polyubiquitination of Hsp/Hsc70, not mutant SOD1. Hence, we proposed a novel cascade for mutant SOD1 metabolism as shown in Fig. 6. Although Hsp/Hsc70 attempts to fold nascent monomeric SOD1, this function may be less effective in mutant SOD1 than in wild-type. Accordingly, the Hsp/Hsc70 may be ubiquitinated by its ubiquitin ligase including CHIP, resulting in recruitment of mutant SOD1 to the proteasome. Along with proteasomal impairment (Urushitani *et al.* 2002), the polyubiquitin complex may result in the formation of pathological aggregate, a hallmark of ALS.

Hsp/Hsc70 and mutant SOD1 monomers

Hsc70 was shown to recognize the apo-state or DTT-treated form of SOD1 protein (Fig. 1c, 2). The fact that mutant SOD1 is monomerized and destabilized by reducing agents, whereas wild-type is not, indicates that the SOD1 monomer is a key molecule underlying the diverse pathological conditions seen in mutant SOD1-linked pathology (Tiwari and Hayward 2003). Apo-state SOD1 is also implicated in mutant SOD1-linked pathogenesis. For instance, apo-SOD1, in both wild-type and mutant forms, appears to enter into mitochondria (Okado-Matsumoto and Fridovich 2002). Physiologically, a proportion of wild-type SOD1 exists as a homodimeric apoenzyme *in vivo*, although apo-state SOD1 is more readily

Fig. 2 CHIP interacts with and promotes ubiquitin complex formation of mutant SOD1. (a) Interaction of overexpressed mutant SOD1 with endogenous CHIP *in vivo*. Neuro2a cells in 6-well culture plates were transiently transfected with hSOD1-FLAG (2 μg/well). Lysates were immunoprecipitated with anti-FLAG affinity gel and blots were probed with antibodies against CHIP and hSOD1. Single and double asterisks indicate IgG heavy and light chains, respectively. (b) CHIP degraded misfolded mutant SOD1 in a dose-dependent manner at proteasome. Neuro2a cells in 12-well culture plates were transiently transfected with hSOD1-FLAG (1 μg), together with HA-CHIP at the indicated concentrations (total DNA, 1.5 μg/well). Supernatant (soluble) and pellet (insoluble) fractions in TritonX 100-containing buffer were immunoblotted using anti-HA or anti-SOD1 antibody. Immunoblots were analyzed by densitometry, and each value was expressed as the percentage of vector control in each genotype. Coomasie brilliant blue (CBB) staining of the insoluble fraction showed that an equal amount of protein was analyzed in each lane. The asterisk indicates endogenous mouse SOD1. (c) CHIP promotes degradation of mutant SOD1. Neuro2a cells in 12-well culture plates were transiently transfected with SOD1-FLAG (G85R and G93A, 1 μg/well) with or without HA-CHIP (0.5 μg/well). At 16 h after transfection, cells were treated with CHX (10 μg/mL) to prevent protein synthesis. Cells were harvested immediately (0), 6 and 24 h after CHX treatment and solubilized in denaturing buffer with boiling for 5 min. Remaining SOD1-FLAG proteins were probed by anti-FLAG antibody, and scanned images were analyzed by densitometry. Each data value represents percentage of remaining SOD1-FLAG compared with the one immediately after CHX treatment.



monomerized than holoenzyme by non-denaturing SDS-PAGE (Bartnikas and Gitlin 2003). Moreover, recent data show that apo-state wild-type SOD1 and metal-deficient mutant SOD1 form a β-sheet structure that mimics amyloid β fibrils (Elam *et al.* 2003; Strange *et al.* 2003). Our data showing that Hsc70 interacts with this reportedly pathogenic SOD1 strongly suggests that this reaction plays a fundamental role in mutant SOD1 metabolism and pathology *in vivo*. In particular, the evidence that overexpressed Hsc70 interacts with soluble SOD1 allows us to hypothesize about another potentially beneficial role for Hsp/Hsc70 in mutant SOD1 metabolism in addition to the refolding of aggregated SOD1. Unexpectedly, Hsc70 did not interact with oxidized SOD1 as tightly as with apoenzyme or monomerized SOD1 (Fig. 1c, 2). One explanation for this phenomenon may be that oxidative conditions stabilized the intramolecular disulfide bond of SOD1 and supported its dimerization. Alternatively, aggregates or fragments formed by H_2O_2 may interact with one another and trap hydrophobic residues inside the aggregates.

The role of Hsp/Hsc70 and CHIP in mutant SOD1 degradation

Hsp70 is known to accelerate the proteasomal degradation of androgen receptors with abnormally expanded polyglutamine (Bailey *et al.* 2002; Adachi *et al.* 2003). CHIP may be involved in this metabolism since CHIP reportedly promotes the ubiquitination of wild-type androgen receptors (Cardozo

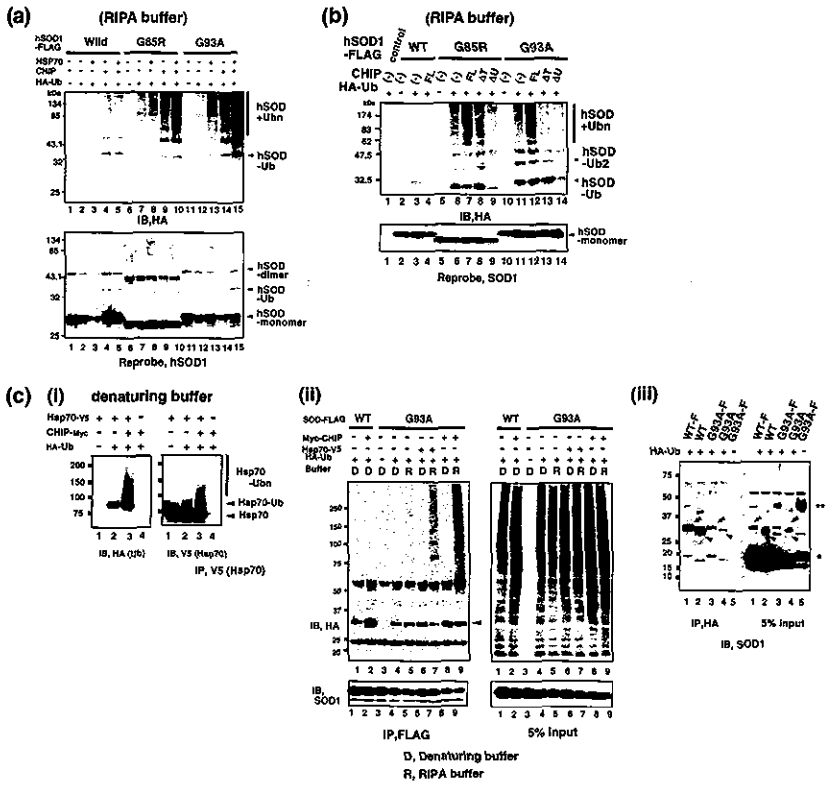


Fig. 3 CHIP does not target mutant SOD1, but ubiquitinates its interacting proteins. (a) Hsp70 and CHIP promoted formation of a complex consisting of hSOD1 and polyubiquitinated species, but did not covalently ubiquitinate SOD1 *in vivo*. Neuro2a cells in 6-well culture plates were transiently transfected with hSOD1-FLAG (1 μg/well), together with Hsp70-V5 (0.5 μg), Myc-CHIP (0.5 μg) or HA-Ub (total DNA, 2.5 μg/well). Cells were solubilized by RIPA buffer with sonication on ice, and lysates were immunoprecipitated in anti-FLAG affinity gel. Immunoprecipitates were incubated in 2% SDS sample buffer for 5 min at 95°C before resolving in 13% SDS-polyacrylamide gel (denaturing after immunoprecipitation). Blots were probed by anti-HA antibody and were reprobed by anti-SOD1 antibody. SOD1-Ub indicates monoubiquitinated SOD1. SOD-Ubn indicates complex of mutant SOD1 and polyubiquitinated species (does not necessarily mean covalently polyubiquitinated SOD1). (b) The effect of CHIP on the formation of the mutant SOD1-polyubiquitin complex required both TPR and U-box domains. Neuro2a cells in 6-well culture plates were transiently transfected with hSOD1-FLAG (1.5 μg), Myc-CHIP full-length (FL), deletion mutant of tetratricopeptide repeat domain (ΔTPR) or of U-box domain (ΔU; 0.5 μg), and HA-ubiquitin (HA-Ub; 0.5 μg) (total DNA, 2.5 μg/well). Immunoprecipitation and western analysis were performed in the same way as in (a). hSOD1-Ub_2 and -Ub indicate di and monoubiquitination of hSOD1, respectively. hSOD1 + Ubn indicates polyubiquitinated species associated with mutant hSOD1. (c) *In vivo* ubiquitination experiment showing polyubiquitin is not covalently bound to mutant SOD1. (1) *In vivo* ubiquitination using denaturing buffer showing Hsp70 is a substrate of CHIP. Neuro2a cells were transiently transfected with Hsp70-V5 and HA-ubiquitin (HA-Ub) with or without CHIP. Cells were solubilized by denaturing buffer containing 1% SDS buffer with boiling for 5 min. After diluting with a 10-fold volume of the dilution buffer, lysates were immunoprecipitated by anti-V5 antibody (denaturing before immunoprecipitation). Eluate was resolved in 5–20% gradient SDS polyacrylamide gel, and blots were probed by antibody against V5 or HA. Both panels indicate that Hsp70 is covalently bound to polyubiquitin in the presence of CHIP. (2) Mutant SOD1 is not a substrate of CHIP *in vivo*. Neuro2a cells were transiently transfected with hSOD1-FLAG, HA-Ub, Hsp70-V5 and Myc-CHIP as indicated. D indicates that cells were solubilized in denaturing buffer condition [samples were denatured before immunoprecipitation as in (1)], whereas R indicates that cells were solubilized in RIPA buffer condition [samples were denatured after immunoprecipitation as in (a) and (b)]. Immunoprecipitates and 5% input were resolved in 5–20% gradient SDS-polyacrylamide gel, and blots were probed by anti-HA or anti-SOD1 antibody. No significant elevation of ubiquitination was observed in lane 8 compared with 4, as in Fig. 3c (1). In denaturing buffer condition, only mono-ubiquitinated SOD1 in both wild-type and mutant was observed (arrowhead). (3) Both wild-type and mutant SOD1 are oligoubiquitinated. Neuro2a cells in 6-well plates were transfected with hSOD1 with or without FLAG tag (–F) and HA-Ub. Cell lysates in RIPA buffer were immunoprecipitated with anti-HA cross-linked beads and analyzed using antihSOD1 antibody.

et al. 2003). However, our data indicate that CHIP promotes the degradation of mutant SOD1 through a mechanism different from that for other CHIP substrates, such as glucocorticoid receptor (Connell *et al.* 2001), heat-denatured luciferase (Murata *et al.* 2001) and phosphorylated tau (Shimura *et al.* 2003).

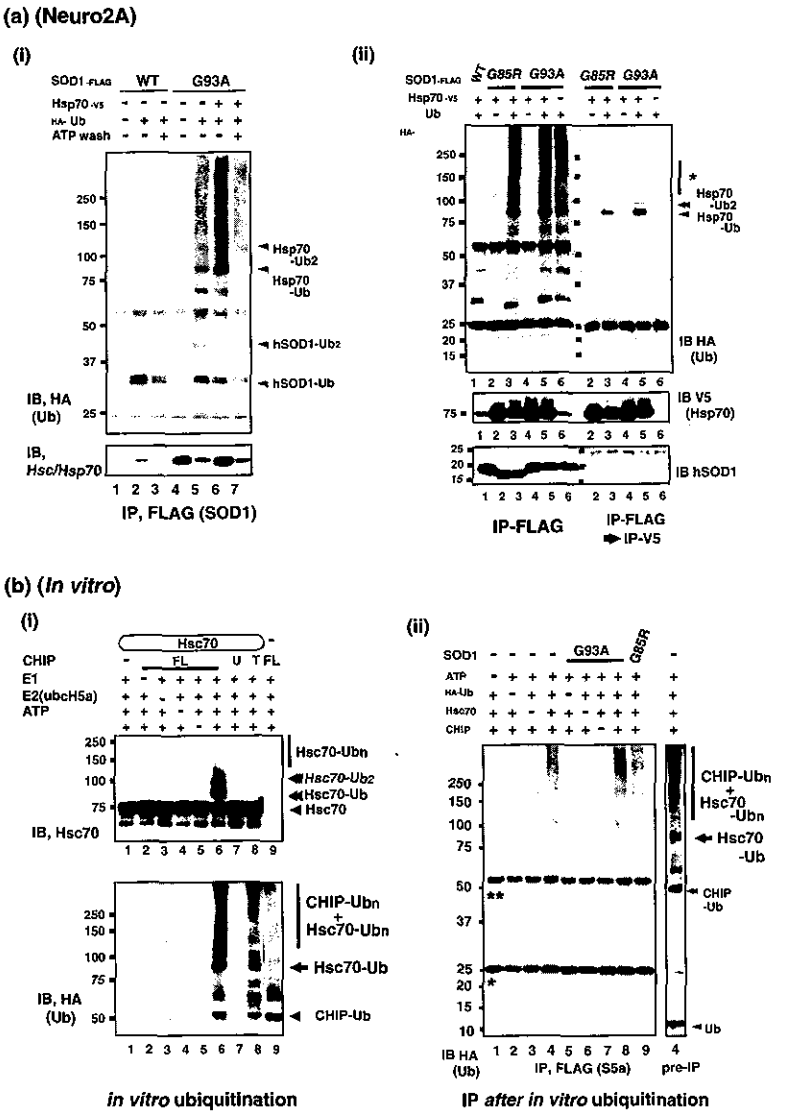


Fig. 4 Hsp/Hsc70 associated with mutant SOD1 is ubiquitinated. (a) Mutant *SOD1-associated Hsp/Hsc70 polyubiquitinated in vivo.* (1) Polyubiquitinated species were released from mutant SOD1 together with Hsp/Hsc70 by ATP wash *in vivo.* Neuro2a cells in 6-well plates were transfected with hSOD1-FLAG (1 μg/well) and HA-ubiquitin (HA-Ub, 1 μg/well) and solubilized in RIPA buffer with sonication. Lysates were immunoprecipitated by anti-FLAG affinity gel. Immunoprecipitates were washed in the buffer containing 10 mM ATP and 5 mM $MgCl_2$ for 15 min at 37°C. *Eluates were resolved in* 5–20% gradient SDS-polyacrylamide gel, and blots were probed by anti-HA and anti-Hsp/Hsc70 antibodies. (2) Mutant SOD1-associated Hsp70 was ubiquitinated. Neuro2a cells were transfected with hSOD1-FLAG, Hsp70-V5 and HA-ubiquitin. Cells were solubilized in RIPA buffer with sonication and the supernatant fluid was immunoprecipitated by anti-FLAG affinity gel. Half of the immunoprecipitates were eluted by 2% SDS-sample buffer with boiling, and the rest were sequentially immunoprecipitated by anti-V5 antibody (see Experimental procedures). The eluates from the first and second immunoprecipitates were resolved in 5–20% gradient SDS polyacrylamide gel, and the blot was probed by anti-HA antibody. (b) Polyubiquitinated Hsc70 associated with the S5a subunit of the 26S proteasome. (1) *In vitro* ubiquitination experiments demonstrating that CHIP ubiquitinated Hsc70 in the presence of E1, E2 and ATP. *CHIP* also ubiquitinated *itself* (*auto*-ubiquitination) as shown by the blot probed by anti-HA antibody (lower panel). The reaction mixture was resolved in 5–20% gradient SDS-polyacrylamide gel, and the blot was probed with anti-HA or anti-Hsp/Hsc70 antibody. (2) After *in vitro* ubiquitination reaction with or without SOD1 protein as in (1), reaction mixtures were incubated with FLAG-tagged recombinant S5a protein and immunolinked to anti-FLAG affinity gel. The reaction mixture was resolved in 5–20% gradient SDS-polyacrylamide gel, and the blot was probed with anti-HA or anti-Hsp/Hsc70 antibody. The sample from lane 4 was loaded as a control without immunoprecipitation. The asterisk indicates IgG light chain, whereas double asterisks indicate IgG heavy chain.

We initially expected CHIP to ubiquitinate mutant SOD1, and CHIP did promote the formation of mutant SOD1-polyubiquitin complex (Fig. 3a). However, mutant SOD1 is not covalently polyubiquitinated, while its binding partner, including Hsp/Hsc70, was linked to polyubiquitin by covalent bond [Figs 3c (2) and 4a]. Taken with the finding that polyubiquitinated species containing Hsc70 associate with the S5a subunit of the 26S proteasome, it is plausible that ubiquitinated Hsp/Hsc70 accompanies mutant SOD1 to the 26S proteasome. However, it is still unclear how mutant SOD1, without a polyubiquitin chain, degrades at the 26S proteasome. Several oxidized proteins are reportedly degraded at the 20S proteasome in an ATP- or ubiquitin-independent manner (Ferrington *et al.* 2001; Shringarpure *et al.* 2003), which is a possible explanation for proteasomal degradation of mutant SOD1 since SOD1 itself is modified by oxidation (Andrus *et al.* 1998; Urushitani *et al.* 2002). An alternative explanation may be that a co-chaperone, BAG-1, might play a role in recruiting mutant SOD1 at the proteasome, since BAG-1 accepts substrate proteins from Hsp/Hsc70 and promotes CHIP-induced degradation (Demand *et al.* 2001; Alberti *et al.* 2002). Our data indicate that interaction with Hsp/Hsc70 determines the degradation fate of mutant SOD1. Recent findings that cultured motor neurons have limited Hsp70 expression (Bruening *et al.* 1999) and promoter activity (Batulan *et al.* 2003) suggest that insufficient proteasomal degradation of mutant SOD1 may lead to aggregate formation in motor neurons.

Molecular features of mutant SOD1-ubiquitin complex

From the *in vivo* ubiquitination study under the denaturing condition, it is shown that both wild-type and mutant SOD1 are oligoubiquitinated (Fig. 3b, 2 and 3). Moreover, we observed no overt polyubiquitination product of mutant SOD1 in a series of *in vitro* and *in vivo* ubiquitination experiments using S100 lysates from HeLa cells, HEK293T cells and Neuro2a cells (data not shown). Because mutant SOD1 readily aggregates or fragments (Urushitani *et al.* 2002), it is sometimes difficult to clearly differentiate the oligoubiquitination of aggregated SOD1 from the polyubiquitinated form. Although the physiological function of oligoubiquitination of SOD1 is unknown, it is unlikely that this is the signal for proteasomal degradation. However, it is still possible that there might be other ubiquitin chain elongation factors, namely E4, that recognize oligoubiquitinated species (Koegl *et al.* 1999), and a chaperone-associated E4 may explain the difference between wild-type and mutants. Furthermore, other ubiquitin ligases, such as dorfin (Niwa *et al.* 2002), may co-operate with chaperone-CHIP machineries. Nevertheless, our data indicate that the mutant SOD1-polyubiquitin complex is composed of various polyubiquitinated species including Hsp70 and/or CHIP. Immunohistochemical analysis revealed that Lewy body-like hyaline inclusions (LBHIs) in the remaining motor neurons

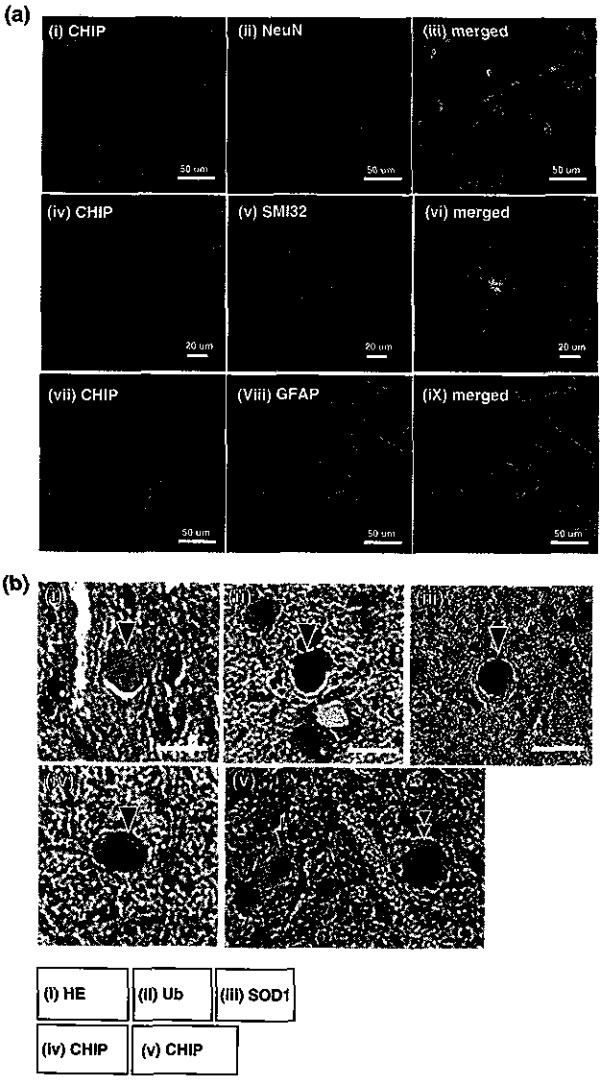


Fig. 5 CHIP is expressed abundantly in neurons, and Lewy body-like hyaline inclusions (LBHIs) are CHIP-immunoreactive. (a) CHIP is expressed predominantly in neurons including motor neurons of embryonic spinal cord cultures (E12). Dissociated cultures at 8 days after plating were fixed and doubly-stained by CHIP (1, 4, 7) and various cell markers (2, anti-NeuN; 5, anti-non-phosphorylated neurofilament H (SMI32); 8, anti-GFAP antibodies, respectively). 3, 8, and 9 are merged images from 1–2, 4–5 and 7–8, respectively. (b) CHIP-immunoreactive Lewy body-like hyaline inclusions (LBHI) in the spinal cord of a 180-day-old hSOD1^{G93A} transgenic mouse. Panel 1 shows a typical LBHI with eosinophilic structure in hematoxylin-eosin (H&E) preparation. Panels 2, 3 and 4 exhibit immunostaining of ubiquitin, mutant SOD1 and CHIP, respectively. In panel 5, various CHIP staining patterns are seen in LBHIs: core-staining (arrow) and diffuse-staining (arrowheads) patterns. Panels 1–5 are × 400 and 2–5 are weakly counterstained with hematoxylin. Scale bar, 20 μm.

of hSOD1^{G93A} transgenic mice were CHIP immunoreactive (Fig. 5b). These aggregates are highly Hsc70-immunoreactive (Watanabe *et al.* 2001), which, together with our

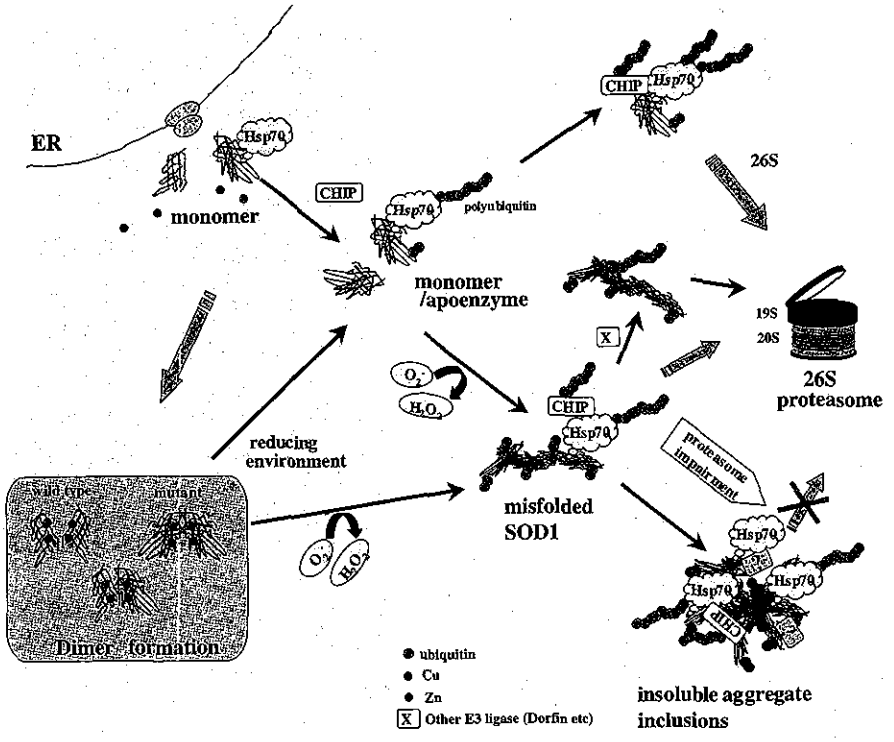


Fig. 6 Hypothetical mechanism for mutant SOD1 degradation. The nascent monomeric SOD1 before metallation in both wild-type and mutant may be bound to Hsp/Hsc70 for folding into the proper conformation. In the mutants, a considerable amount of SOD1 may not fold properly and may remain as a monomer. In the course of this process, Hsp/Hsc70 might be polyubiquitinated by CHIP and recruited to the 26S proteasome. Even in the dimeric forms, mutant SOD1 is susceptible to reducing conditions, resulting in a monomer, which may be recognized by Hsp/Hsc70. The oxidative stress augmented the abnormal conformation of mutant SOD1, and the proteasomal impairment under these conditions may result in the observed pathological ubiquitin-positive aggregation.

findings, suggests the involvement of Hsp/Hsc70-CHIP machinery in the metabolism of mutant SOD1 in the ALS mouse model containing mutant SOD1. Although we showed that CHIP was expressed predominantly in the neurons, including motor neurons of the spinal cord, western analysis of the primary astrocyte culture revealed that CHIP was also expressed in astrocytes (data not shown). In this regard, it would be interesting to examine whether CHIP is localized to the glial LBHIs in hSOD1^{G85R} transgenic mice and some human ALS cases (Bruijn *et al.* 1997; Kato *et al.* 1997). We have also observed that CHIP and Hsp/Hsc70 accumulate in insoluble fractions of the spinal cord lysates from mutant SOD1 transgenic mice as ALS progresses (unpublished observation). However, this is not transcriptionally regulated because the mRNA profile analyzed by TaqMan chemistry provided no significant alteration in CHIP expression patterns in motor neurons with respect to the disease course (data not shown).

In conclusion, we have provided evidence that proteasomal translocation of mutant SOD1 is regulated by the ubiquitination of Hsp/Hsc 70. Currently, it is widely accepted that neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and polyglutamine disease, are caused by the accumulation of misfolded proteins in neurons. Such aberrant proteins may be degraded in a similar manner, considering the interaction of Hsp/Hsc70 with disease-causing proteins.

Acknowledgements

We thank Dr Hidemi Misawa (Tokyo Metropolitan Institute for Neuroscience) for helpful discussions, and Dr Yuzuru Imai and Ms Mariko Soda for providing anti-CHIP antibody and constructs for Hsp70. We also thank Dr Yasuyuki Suzuki for his cooperation with our experiment, and for his generous gift of recombinant XIAP and Smac proteins. We also thank Dr Kazuhiro Yamanaka (Ludwig Institute for Cancer Research, San Diego, USA) for his critical advice on the *in vitro* ubiquitination assay. This work was supported by research grants from RIKEN BSI, a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid from the Japan Foundation for Neuroscience and Mental Health, and by grants from the Ministry of Health and Welfare, Japan.

References

Abernethy J. L., Steinman H. M. and Hill R. L. (1974) Bovine erythrocyte superoxide dismutase. Subunit structure and sequence

location of the intrasubunit disulfide bond. *J. Biol. Chem.* **249**, 7339–7347.

Adachi H., Katsuno M., Minamiyama M. *et al.* (2003) Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. *J. Neurosci.* **23**, 2203–2211.

Alberti S., Demand J., Esser C., Emmerich N., Schild H. and Hohfeld J. (2002) Ubiquitylation of BAG-1 suggests a novel regulatory mechanism during the sorting of chaperone substrates to the proteasome. *J. Biol. Chem.* **277**, 45920–45927.

Andrus P. K., Fleck T. J., Gurney M. E. and Hall E. D. (1998) Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *J. Neurochem.* **71**, 2041–2048.

Aravind L. and Koonin E. V. (2000) The U box is a modified RING finger – a common domain in ubiquitination. *Curr. Biol.* **10**, R132–R134.

Bailey C. K., Andriola I. F., Kampinga H. H. and Merry D. E. (2002) Molecular chaperones enhance the degradation of expanded polyglutamine repeat androgen receptor in a cellular model of spinal and bulbar muscular atrophy. *Hum. Mol. Genet.* **11**, 515–523.

Ballinger C. A., Connel P., Wu Y., Thompson L. J., Yin L. Y. and Patterson C. (1999) Identification of CHIP, a novel tetratricopeptide repeat-containing protein that interacts with heat shock proteins and negatively regulates chaperone functions. *Mol. Cell. Biol.* **19**, 4535–4545.

Bartnikas T. B. and Gitlin J. D. (2003) Mechanisms of biosynthesis of mammalian copper/zinc superoxide dismutase. *J. Biol. Chem.* **278**, 33602–33608.

Batulan Z., Shinder G. A., Minotti S., He B. P., Doroudchi M. M., Nalbantoglu J., Strong M. J. and Durham H. D. (2003) High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. *J. Neurosci.* **23**, 5789–5798.

Bercovich B., Stancovski I., Mayer A., Blumenfeld N., Laszlo A., Schwartz A. L. and Ciechanover A. (1997) Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70. *J. Biol. Chem.* **272**, 9002–9010.

Bruening W., Roy J., Giasson B., Figlewicz D. A., Mushynski W. E. and Durham H. D. (1999) Up-regulation of protein chaperones preserves viability of cells expressing toxic Cu/Zn-superoxide dismutase mutants associated with amyotrophic lateral sclerosis. *J. Neurochem.* **72**, 693–699.

Bruijn L. I., Becher M. W., Lee M. K. *et al.* (1997) ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* **18**, 327–338.

Cardozo C. P., Michaud C., Ost M. C., Fliss A. E., Yang E., Patterson C., Hall S. J. and Caplan A. J. (2003) C-terminal Hsp-interacting protein slows androgen receptor synthesis and reduces its rate of degradation. *Arch. Biochem. Biophys.* **410**, 134–140.

Cleveland D. W. and Rothstein J. D. (2001) From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. *Nat. Rev. Neurosci.* **2**, 806–819.

Connell P., Ballinger C. A., Jiang J., Wu Y., Thompson L. J., Hohfeld J. and Patterson C. (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat. Cell Biol.* **3**, 93–96.

Demand J., Alberti S., Patterson C. and Hohfeld J. (2001) Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling. *Curr. Biol.* **11**, 1569–1577.

Elam J. S., Taylor A. B., Strange R. *et al.* (2003) Amyloid-like filaments and water-filled nanotubes formed by SOD1 mutant proteins linked to familial ALS. *Nat. Struct. Biol.* **10**, 461–467.

Ferrington D. A., Sun H., Murray K. K., Costa J., Williams T. D., Bigelow D. J. and Squier T. C. (2001) Selective degradation of oxidized calmodulin by the 20 S proteasome. *J. Biol. Chem.* **276**, 937–943.

Gurney M. E., Pu H., Chiu A. Y., Dal Canto M. C., Polchow C. Y., Alexander D. D., Caliendo J., Hentati A., Kwon Y. W. and Deng H. X. (1994) Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science* **264**, 1772–1775.

Hatakeyama S., Yada M., Matsumoto M., Ishida N. and Nakayama K. (2001) U box proteins as a new family of ubiquitin-protein ligases. *J. Biol. Chem.* **276**, 33111–33120.

Imai Y., Soda M., Hatakeyama S., Akagi T., Hashikawa T., Nakayama K. I. and Takahashi R. (2002) CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol. Cell* **10**, 55–67.

Jiang J., Ballinger C. A., Wu Y., Dai Q., Cyr D. M., Hohfeld J. and Patterson C. (2001) CHIP is a U-box-dependent E3 ubiquitin ligase: identification of Hsc70 as a target for ubiquitylation. *J. Biol. Chem.* **276**, 42938–42944.

Johnston J. A., Dalton M. J., Gurney M. E. and Kopito R. R. (2000) Formation of high molecular weight complexes of mutant Cu, Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc. Natl Acad. Sci. USA* **97**, 12571–12576.

Julien J. P. (2001) Amyotrophic lateral sclerosis. Unfolding the toxicity of the misfolded. *Cell* **104**, 581–591.

Kang J. H. and Eum W. S. (2000) Enhanced oxidative damage by the familial amyotrophic lateral sclerosis-associated Cu, Zn-superoxide dismutase mutants. *Biochim. Biophys. Acta* **1524**, 162–170.

Kato S., Hayashi H., Nakashima K., Nanba E., Kato M., Hirano A., Nakano I., Asayama K. and Ohama E. (1997) Pathological characterization of astrocytic hyaline inclusions in familial amyotrophic lateral sclerosis. *Am. J. Pathol.* **151**, 611–620.

Koegl M., Hoppe T., Schlenker S., Ulrich H. D., Mayer T. U. and Jentsch S. (1999) A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**, 635–644.

Lindberg M. J., Tibell L. and Oliveberg M. (2002) Common denominator of Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis: decreased stability of the apo state. *Proc. Natl Acad. Sci. USA* **99**, 16607–16612.

Meacham G. C., Patterson C., Zhang W., Younger J. M. and Cyr D. M. (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol.* **3**, 100–105.

Murata S., Minami Y., Minami M., Chiba T. and Tanaka K. (2001) CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep.* **2**, 1133–1138.

Nakano R., Inuzuka T., Kikugawa K., Takahashi H., Sakimura K., Fujii J., Taniguchi N. and Tsuji S. (1996) Instability of mutant Cu/Zn superoxide dismutase (Ala4Thr) associated with familial amyotrophic lateral sclerosis. *Neurosci. Lett.* **211**, 129–131.

Niwa J., Ishigaki S., Hishikawa N., Yamamoto M., Doyu M., Murata S., Tanaka K., Taniguchi N. and Sobue G. (2002) Dorfin ubiquitylates mutant SOD1 and prevents mutant SOD1-mediated neurotoxicity. *J. Biol. Chem.* **277**, 36793–36798.

van Nocker S., Deveraux Q., Rechsteiner M. and Vierstra R. D. (1996) Arabidopsis MBP1 gene encodes a conserved ubiquitin recognition component of the 26S proteasome. *Proc. Natl Acad. Sci. USA* **93**, 856–860.

Okado-Matsumoto A. and Fridovich I. (2002) Amyotrophic lateral sclerosis: a proposed mechanism. *Proc. Natl Acad. Sci. USA* **99**, 9010–9014.

Rosen D. R., Siddique T., Patterson D. *et al.* (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **362**, 59–62.

Shimura H., Schwartz D., Gygi S. P. and Kosik K. S. (2003) CHIP-Hsc70 complex ubiquitinates phosphorylated Tau and enhances cell survival. *J. Biol. Chem.* **279**, 4869–4876.

Shinder G. A., Lacourse M. C., Minotti S. and Durham H. D. (2001) Mutant Cu/Zn-superoxide dismutase proteins have altered solubility and interact with heat shock/stress proteins in models of amyotrophic lateral sclerosis. *J. Biol. Chem.* **276**, 12791–12796.

Shringarpure R., Grune T., Mehlhase J. and Davies K. J. (2003) Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. *J. Biol. Chem.* **278**, 311–318.

Strange R. W., Antonyuk S., Hough M. A., Doucette P. A., Rodriguez J. A., Hart P. J., Hayward L. J., Valentine J. S. and Hasnain S. S. (2003) The structure of holo and metal-deficient wild-type human Cu, Zn superoxide dismutase and its relevance to familial amyotrophic lateral sclerosis. *J. Mol. Biol.* **328**, 877–891.

Tiwari A. and Hayward L. J. (2003) Familial amyotrophic lateral sclerosis mutants of copper/zinc superoxide dismutase are susceptible to disulfide reduction. *J. Biol. Chem.* **278**, 5984–5992.

Urushitani M., Kurisu J., Tsukita K. and Takahashi R. (2002) Proteasomal inhibition by misfolded mutant superoxide dismutase 1 induces selective motor neuron death in familial amyotrophic lateral sclerosis. *J. Neurochem.* **83**, 1030–1042.

Valentine J. S. and Hart P. J. (2003) Misfolded CuZnSOD and amyotrophic lateral sclerosis. *Proc. Natl Acad. Sci. USA* **100**, 3617–3622.

Walters K. J., Kleijnen M. F., Goh A. M., Wagner G. and Howley P. M. (2002) Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a. *Biochemistry* **41**, 1767–1777.

Watanabe M., Dykes-Hoberg M., Culotta V. C., Price D. L., Wong P. C. and Rothstein J. D. (2001) Histological evidence of protein aggregation in mutant SOD1 transgenic mice and in amyotrophic lateral sclerosis neural tissues. *Neurobiol. Dis.* **8**, 933–941.

Xu W., Marcu M., Yuan X., Mimnaugh E., Patterson C. and Neckers L. (2002) Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proc. Natl Acad. Sci. USA* **99**, 12847–12852.

[CANCER RESEARCH 64, 3452–3457, May 15, 2004]

Antiapoptotic Function of Apoptosis Inhibitor 2-MALT1 Fusion Protein Involved in t(11;18)(q21;q21) Mucosa-Associated Lymphoid Tissue Lymphoma

Yoshitaka Hosokawa,[1] Hiroko Suzuki,[1] Yasuyuki Suzuki,[2] Ryosuke Takahashi,[2] and Masao Seto[1]

[1]Division of Molecular Medicine, Aichi Cancer Center Research Institute, Nagoya, Japan, and [2]Laboratory for Motor System Neurodegeneration, RIKEN Brain Science Institute, Wako City, Saitama, Japan

ABSTRACT

t(11;18)(q21;q21) is a characteristic chromosomal translocation in mucosa-associated lymphoid tissue (MALT) type lymphoma, and this translocation results in fusion transcript of *apoptosis inhibitor 2* (*API2*), also known as *c-IAP2*, and *MALT translocation* gene 1 (*MALT1*). Although the API2-MALT1 fusion protein has been shown to enforce activation of nuclear factor κB signaling, its precise role in the apoptotic signaling pathway remains to be established. To identify proteins that bind the API2-MALT1 protein, we used coimmunoprecipitation and SDS-PAGE, followed by liquid chromatography-electrospray ionization tandem mass spectrometry. As a result, three important regulators of apoptosis, Smac, HtrA2, and TRAF2, and three other proteins were identified as potential API2-MALT1-binding proteins. Immunoprecipitation analyses verified that API2-MALT1 indeed binds to both exogeneous and endogeneous Smac proteins. It is especially noteworthy that stably transfected API2-MALT1 significantly suppressed both UV- and etoposide-induced apoptosis in HeLa cells, thus demonstrating for the first time that API2-MALT1 indeed possesses antiapoptotic function. Furthermore, API2-MALT1 significantly suppressed Smac-promoted apoptosis in UV-irradiated HeLa cells. Thus, our results provide direct experimental evidence that API2-MALT1 can confer resistance to apoptosis, at least in part, by neutralizing apoptosis promoted by Smac.

INTRODUCTION

Extranodal lymphomas arising from the mucosa-associated lymphoid tissue (MALT) represent a subtype of B-cell non-Hodgkin's lymphoma with a distinct clinicopathological entity (1). Because of their supposed cell of origin, they are now recognized and defined as extranodal marginal zone lymphomas of MALT type in the revised European-American classification of lymphoid neoplasms (REAL) and the recently published WHO classification of malignant lymphomas (2, 3). According to the International Non-Hodgkin's Lymphoma Classification Project, MALT lymphoma comprises 7.6% of all non-Hodgkin's lymphomas and represents one of the most common non-Hodgkin's lymphomas (4). The majority of MALT lymphomas occur in the stomach, but this lymphoma may affect most organs, including the ocular adnexa, lung, salivary glands, thyroid, skin, and intestine.

Data on the molecular genetic mechanisms underlying the pathogenesis of MALT lymphomas are only now beginning to emerge. Their recurrent abnormalities include trisomies of chromosomes 3, 7, 12, and 18, t(1;14)(p22;q32), and t(11;18)(q21;q21) (5–9). The *BCL10* gene was isolated from the breakpoint region of the t(1;14) in MALT lymphomas and subsequently shown to be proapoptotic (10, 11). On the other hand, the t(11;18) translocation is reported to be one of the most frequent and specific chromosomal translocations in MALT lymphomas (12), and a novel gene, named *MALT1* or *MLT*, was recently cloned by ourselves and others from the breakpoint of t(11;18). This aberration has been found to result in the fusion of two genes, *apoptosis inhibitor 2* [*API2* (also known as *c-IAP2*)] at 11q21 and the novel gene *MALT1* at 18q21 (13–15), generating the API2-MALT1 fusion protein. More recently, a novel t(14;18) translocation involving the immunoglobulin gene at 14q32 and the *MALT1* gene at 18q21 has been reported (16, 17), and the actual frequency of this translocation in MALT lymphoma is roughly 10% (18, 19).

API2 is a member of the inhibitor of apoptosis (*IAP*) protein gene family, which includes *X-IAP, API1 (c-IAP1), API2 (c-IAP2)*, and *ML-IAP*, and has three baculovirus IAP repeat domains, one caspase recruitment domain, and one RING finger domain (20, 21). MALT1 is a novel protein that contains a death domain, two immunoglobulin-like domains, and a caspase-like domain (13–15, 22). It was demonstrated that MALT1 and BCL10 form a strong complex and that these proteins synergize in nuclear factor (NF)-κB activity (22, 23). Together with the findings for *BCL10* and *MALT1* knockout mice, these results suggest that both MALT1 and BCL10 link antigen receptor signaling to NF-κB activation (24–26). The caspase-like domain of MALT1 was demonstrated to be essential for NF-κB activity (22, 23), but functions of the death domain and immunoglobulin-like domains remain to be identified. It was also demonstrated that API2-MALT1 can induce NF-κB activation through its homodimerization mediated via the API2 portion of the fusion protein, whereas full-length API2, MALT, or their truncated forms cannot (22, 23), raising the possibility that the oncogenic role of API2-MALT1 may be mediated by deregulated NF-κB activity. Given that API2 is a member of the IAP family, it can be hypothesized that API2-MALT1 may exert an antiapoptotic effect as well. However, experimental evidence for such an antiapoptotic effect has yet to be presented.

In this study, we first tried to identify the binding proteins of API2-MALT1 to further delineate its apoptotic and oncogenic functions. To this end, we used electrospray ionization tandem mass spectrometry analysis of the coimmunoprecipitates of transiently transfected API2-MALT1 in cultured cells and were able to identify Smac (27, 28) as consisting of several API2-MALT1-binding proteins. We also demonstrated for the first time that API2-MALT1 can significantly suppress both UV- and etoposide-induced apoptosis in HeLa cells and that this suppression may be mediated, at least in part, by neutralizing apoptosis promoted by Smac.

MATERIALS AND METHODS

Plasmids and Antibodies. The plasmids encoding FLAG-API2-MALT1 (pcDNA3-FLAG-API2-MALT1 and PCXN2-FLAG-API2-MALT1) have been described elsewhere (29), as has the plasmid for COOH-terminal myc-tagged Smac (pcDNA3-Smac-myc) (30). To generate the plasmids for pRK5-FLAG-API2ΔC (amino acids 1–442) and pRK5-FLAG-MALT1ΔN (amino acids 217–813), the corresponding fragments were PCR amplified using KOD Taq polymerase from their full-length cDNAs, digested with the appropriate enzymes, and then subcloned into pRK5-FLAG-N plasmids. Proper construction of these plasmids was confirmed by DNA sequencing with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA). The pEGFP-C plas-

Received 11/25/03; revised 2/16/04; accepted 3/5/04.

Grant support: A Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare and a Grant-in-Aid for Science in Primary Areas (Cancer Research) from the Ministry of Education, Science, Sports and Culture, Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Yoshitaka Hosokawa, Division of Molecular Medicine, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya 464-8681, Japan. Phone: 81-52-762-6111, ext.7083; Fax: 81-52-763-5233; E-mail: yhosokaw@aichi-cc.jp.

mid was purchased from Clontech. Anti-FLAG M2 monoclonal antibody and polyclonal antibody against myc (A-14) were obtained from Santa Cruz Biotechnology. Anti-FLAG monoclonal antibody-coupled agarose beads were obtained from Sigma (St. Louis, MO).

Purification of API2-MALT1-Binding Proteins. 293T cells were maintained in Iscove's medium supplemented with 10% FCS in a 5% CO_2 incubator at 37°C. The pcDNA3 FLAG-tagged API2-MALT1 plasmid or the empty plasmid was transiently transfected into 293T cells using Effectene Reagent (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. At 24 h after transfection, the cells were homogenized in lysis buffer [10 mM Tris (pH 8.0), 120 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100] with Complete Protease Inhibitors (Roche Diagnostics, Tokyo, Japan). The homogenate was centrifuged twice at 10,000 × g for 15 min. The soluble fraction of the suspension was then incubated for 3 h, immunoprecipitated with anti-FLAG M2-agarose beads (Sigma), and washed five times in lysis buffer without protease inhibitors. The fractions eluted with 0.2 M glycine-HCl (pH 2.8) were neutralized, concentrated by freeze drying, and separated by 10% SDS-PAGE (15 × 15 cm). The bands detected by Coomassie Brilliant Blue staining were excised for in-gel digestion.

High-Performance Liquid Chromatography Tandem Mass Spectrometry Analysis. In-gel digestion was performed by trypsin digestion at 35°C overnight as described elsewhere (31). The digest was analyzed directly by means of nanoscale high-performance liquid chromatography coupled to a tandem mass spectrometer (Q-Tof2; Micromass) equipped with a nanoelectrospray ionization source. The eluate was analyzed by tandem mass spectrometry (Q-Tof2) performed by Applied Bioscience (Tokushima, Japan). A database search of tandem mass spectra was performed using a Mascot Search Program (Matrix Science Ltd., London, United Kingdom).

Immunoprecipitation and Western Blot Analysis. 293T cells were transiently transfected using Effectene Reagent (Qiagen K.K.) or LipofectAMINE 2000 reagent (GIBCO-BRL, Tokyo, Japan) according to the manufacturer's instructions. For immunoprecipitation, a total of 8 × 10^5 293T cells were placed on a 3.5-cm dish or a 6-well dish, washed the following day, and then transfected with a total of 0.5–1.0 μg of plasmid DNA. After 6 h of incubation, the medium was replaced with fresh complete medium. At 24 h after transfection, the cells were homogenized in lysis buffer [20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, and 10% glycerol] with Complete Protease Inhibitors (Roche Diagnostics). Cellular debris was removed by centrifugation at 10,000 × g for 20 min, and the supernatant was incubated first with anti-FLAG monoclonal antibody at 4°C for 2 h and then with protein G-Sepharose at 4°C for 2 h. Immunoprecipitates were washed five times in lysis buffer without protease inhibitors. After this, immunoprecipitates were lysed with 1× sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.05% bromphenol blue] and boiled for 3 min. It should be noted that the lysis buffer solubilizes mitochondrial membranes and thus releases Smac without the need for any apoptotic stimuli.

The samples were separated electrophoretically on SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and the membrane was visualized with an enhanced chemiluminescence detection kit (Amersham-Japan, Tokyo, Japan).

Establishment of Stable Transfectants Expressing API2-MALT1. The linearized pCXN2-FLAG-tagged API2-MALT or the control pCXN2 plasmid was transfected into HeLa cells using LipofectAMINE 2000 reagent (GIBCO-BRL) according to the manufacturer's instructions. At 24 h after transfection, the cells were seeded in several dilutions. At 48 h after transfection, Geneticin (IBL, Fujioka, Japan) was added to the medium at a final concentration of 1 mg/ml. This was then diluted over subsequent days by adding fresh medium. On the seventh day, Geneticin was diluted to a final concentration of 100 μg/ml. The surviving cells were grown for 1 more week and analyzed by Western blot analysis using an anti-FLAG monoclonal antibody for API2-MALT protein expression. One part of the cell population was seeded into 96-well plates at a density of 0.3 cells/well and propagated at 100 μg/ml Geneticin. The surviving colonies were again analyzed by Western blot analysis for API2-MALT1 protein expression. Two independent clones expressing API2-MALT1 were selected for further study. The two clones were maintained in Iscove's medium supplemented with 10% FCS and 500 μg/ml Geneticin.

Cell Death Assay. Stable transfectants with or without API2-MALT1 expression were seeded at 3 × 10^5 cells/3.5-cm dish in Iscove's medium supplemented with 10% FCS but without Geneticin. After 24 h, the medium was removed, and the cells were exposed to UV irradiation (200 J/m^2) with Stratalinker UV cross-linker 1800 (Stratagene, La Jolla, CA). The UV-treated cells were then cultured in fresh medium for 6 h. The transfectants were also treated with etoposide (100 μM) or DMSO for 12 h. Apoptosis was assessed by determining the percentages of cells that had condensed chromatin detected by staining with Hoechst 33342 (1 μg/ml) in serum-free medium for 10 min after fixation with 4% paraformaldehyde. At least 100 chromatins were counted in every sample.

For the apoptosis assay of HeLa cells transiently transfected with Smac-myc vector, the stable transfectant cells were transiently transfected with 0.5 μg of pEGFP-C plasmid (Clontech) plus either 2 μg of pcDNA3-Smac-myc or 2 μg of pcDNA3 plasmid control per dish. At 6 h after transfection, the cells were seeded at 4 × 10^5 cells/3.5-cm dish in Iscove's medium supplemented with 10% FCS but without Geneticin. At 24 h after transfection, the medium was removed, and the cells were UV irradiated (200 J/m^2) with Stratalinker UV cross-linker 1800. The treated cells were then cultured in fresh medium for 6 h. The cells were harvested, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (1 μg/ml) in serum-free medium for 10 min. After washing, the cell suspensions were dropped onto a slide. Green cells that also exhibited condensed chromatin were counted by means of the excitation from blue light (excited green fluorescent protein) and UV (excited Hoechst 33342). Apoptosis was expressed as the percentage of green cells with condensed chromatin.

Assay of DEVDase Activity. DEVDase activity was measured by using the Apo-ONE homogeneous caspase-3/7 assay kit (Promega, Tokyo, Japan) according to the manufacturer's instructions. The homogeneous caspase buffer and the caspase substrate Z-DEVD-R110 (rhodamine 110; bis-*N*-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide) were mixed and added to each well of a 96-well plate that contained or did not contain samples (5 × 10^5 cells/well). The contents of the wells were gently mixed using a plate shaker at 300–500 rpm for 1 h at room temperature. Fluorescence of liberated R110 was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. DEVDase activity was expressed as an arbitrary unit.

RESULTS

Identification of API2-MALT1-Binding Proteins. The t(11;18) translocation, most commonly encountered in MALT lymphoma, was found to result in the fusion of an IAP, API2, and a novel protein, MALT1 (13–15). Although API2-MALT1 was shown to activate NF-κB activity through its homodimerization, it remains to be established whether this fusion protein can indeed exert an antiapoptotic function, which may be relevant to the pathogenesis of MALT lymphoma. As a first step in addressing this fundamental issue, we used a proteomic approach to identify API2-MALT1-binding proteins.

293T cells were transiently transfected with either an expression plasmid for NH_2-terminal FLAG-tagged API2-MALT1 or an empty plasmid. Cell lysates were prepared and incubated with anti-FLAG monoclonal antibody-coupled agarose beads. After extensive washing, associated proteins were eluted under acidic conditions from the beads and separated by SDS-PAGE. Five candidate protein bands were observed in a Coomassie Blue-stained gel (Fig. 1), but not in the control immunoprecipitate. These bands were excised from the gel and digested in gel with trypsin. The digested peptides were then analyzed directly by nanoscale high-performance liquid chromatography coupled to tandem mass spectrometry equipped with a nanoelectrospray ionization source. A database search of tandem mass spectra was performed with a Mascot Search Program.

Table 1 summarizes the peptides detected by mass spectrometry and their assigned proteins. Several peptides of HtrA2 and Smac were detected in bands D and E, respectively. The detection of HtrA2 and Smac as API2-MALT1-binding proteins is not surprising, in view of previous reports that these two proteins were identified as X-IAP-binding proteins (27, 28, 30, 32). HtrA2 is a mitochondrial serine protease and is released from mitochondria to the cytoplasm during apoptosis (30, 33–35). It also interacts with and cleaves IAPs, relieving caspase inhibition and promoting apoptosis (36, 37). Smac is also

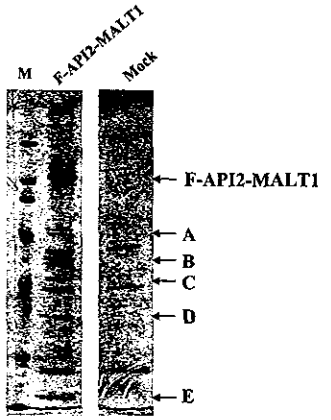


Fig. 1. Detection of apoptosis inhibitor 2 (API2)-MALT1-binding proteins. Overexpressed FLAG-tagged API2-MALT1 in 293T cells was affinity-purified using anti-FLAG M2 monoclonal antibody-coupled agarose beads. Eluted fractions were subjected to SDS-PAGE and stained with Coomassie Blue. Coeluted proteins with affinity-purified API2-MALT1 are represented by *A–E*.

normally a mitochondrial protein and, like HtrA2, is released to the cytoplasm concurrent with cytochrome *c* release during apoptosis (27, 28). It was found to interact with the baculovirus IAP repeat domain of IAPs. The binding of Smac to IAPs relieves the binding of IAPs to caspases, thus promoting caspase-mediated apoptosis. Several peptides of TRAF2 were detected in band 2. Because TRAF2 plays a crucial role in the tumor necrosis factor-stimulated apoptotic pathway, further study is warranted to examine the role of API2-MALT1 in this pathway. In addition to these regulators of apoptosis, three other proteins, Hsc70 (heat shock 70-kDa protein 8), GRP75 (stress-70 protein) and KIAA1892, were also detected in bands A and C. Identification of two members of the heat shock protein family of 70 kDa (HSP70) is intriguing because they not only function as molecular chaperones to facilitate protein folding and oligomerization, but also have been shown to exert antiapoptotic activity (38). KIAA1892 is a novel hypothetical protein whose function is currently unknown but is presumed to have WD-40 repeats. Given the fact that this novel protein has the potential to bind API2-MALT1, it will be interesting to further examine its function in the context of apoptosis as well as NF-κB signaling.

Unexpectedly, no peptides corresponding to BCL10 were detected that have been shown to form a complex with MALT1 through immunoglobulin-like domains (22, 23). To clarify this point, we performed Western blot analysis of API2-MALT1 immunoprecipitate samples (one-fourth of the proteins shown in Fig. 1) using BCL10 antibody. This analysis could not detect any apparent band corresponding to BCL10 (data not shown). One explanation for this nondetection would be that the amount of BCL10, if any, was undetectable in Coomassie Blue staining or below the detection limit of Western blot analysis. It is also conceivable that API2-MALT1 may have bypassed the normal BCL10 signaling pathway.

Interaction between API2-MALT1 and Smac. To verify the interaction between API2-MALT1 and Smac, expression plasmids for NH_2-terminal FLAG-tagged API2-MALT1 (F-API2-MAL1), COOH-terminal-deleted API2 (F-API2ΔC), NH_2-terminal-deleted MALT1 (F-MALT1ΔN), or an empty plasmid were transiently transfected together with an expression plasmid for Smac with a COOH-terminal myc tag (Smac-myc) into 293T cells. Transfected cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody and then immunoblotted with anti-Myc monoclonal antibody. As shown in Fig. 2, the mature form of Smac-myc was coimmunoprecipitated with API2-MALT1 and API2ΔC. This indicates that API2-MALT1 indeed associates with exogeneously expressed Smac in the cells.

We next sought to examine whether API2-MALT1 interacts with endogenous Smac in cells. Expression plasmids for F-API2-MALT1, F-API2ΔC, F-MALT1ΔN, or an empty plasmid were transiently transfected into 293T cells. Transfected cell lysates were immunoprecipitated with anti-FLAG monoclonal antibody and then immunoblotted with anti-Smac monoclonal antibody. As shown in Fig. 3, endogeneous Smac was also immunoprecipitated with API2-MALT1, which strongly suggests that the interaction between API2-MALT1 and Smac could be physiologically significant. Interaction of API2-MALT1 with endogenous HtrA2 and TRAF2 was also confirmed by immunoprecipitation analysis (data not shown).

Table 1 *List of proteins identified as API2-MALT1-binding proteins*

Proteins	Bands	Peptides detected
HSC70 (HSP73)	Band A	VEIIANDQGNR
		NQVAMNPTNTVFDAK
		RFDDAVVQSDMK
		HWPFMVVNPAGRPK
		SFYPEEVSSMVLTK
		IINEPTAAAIAYGLDKK
GRP75 (75-kDa glucose-regulated protein, Stress-75 protein)		VQQTVQQDLFGR
TRAF2	Band B	VAMTAEACSR
		LDQDKIEALSSK
		DLAMADLEQK
		IYLNGDGTGR
Precursor HtrA2		YIGVMMLTLSPSILAELQLR
		AGLRPGDVILAIGEQMVQNAEDVYEAVR
KIAA1892	Band C	TLLATGGDNPNSLAIYR
HtrA2	Band D	AVPSPPPASPR
		SQYNFIADVVEK
		LLSGDTYEAVVTAVDPVADIATLR
		EPLPTLPLGR
		QGEFVVAMGSPFALQNTITSGIVSSAQRPAR
		VTAGISFAIPSDR
Smac	Band E	SEPHSLSSEALMR
		AVYTLTSLYR
		MNSEEEDEVMQVIIGAR
		LETTWMTAVGLSEMAAEAAYQTGADQASITAR
		LQVEEVHQLSR
		LAEAQIEELR
		AESEQEAYLRED

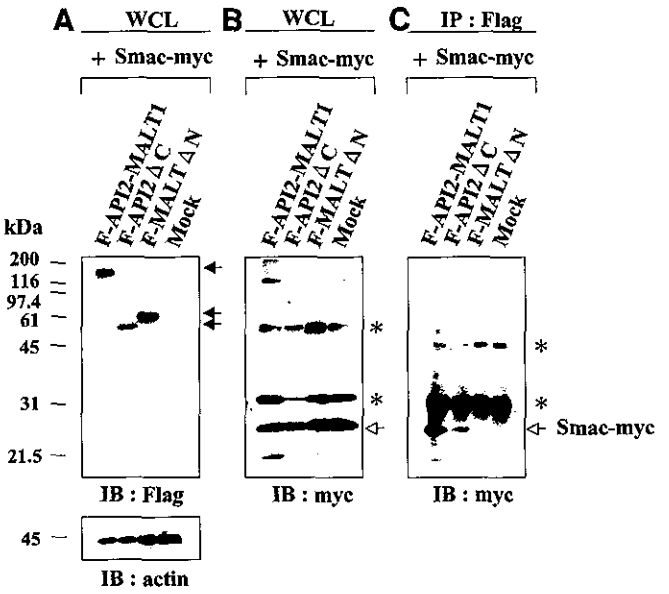


Fig. 2. Apoptosis inhibitor 2 (API2)-MALT1 binds to exogeneous Smac. FLAG-tagged API2-MALT1 plasmid, FLAG-tagged API2ΔC (amino acids 1–442), or FLAG-tagged MALT1ΔN [amino acids 217–813 (0.5 μg each)] was transfected with a COOH-terminal myc-tagged Smac plasmid (0.5 μg) into 293T cells, and whole cell lysates (*WCL*) were immunoprecipitated with anti-FLAG M2 monoclonal antibody. Whole cell lysates were analyzed with immunoblotting (*IB*) using anti-FLAG monoclonal antibody (*A*) or anti-Myc polyclonal antibody (*B*). Immunoprecipitates were analyzed with immunoblotting using anti-Myc polyclonal antibody (*C*). Nonspecific bands are shown by an *asterisk*.

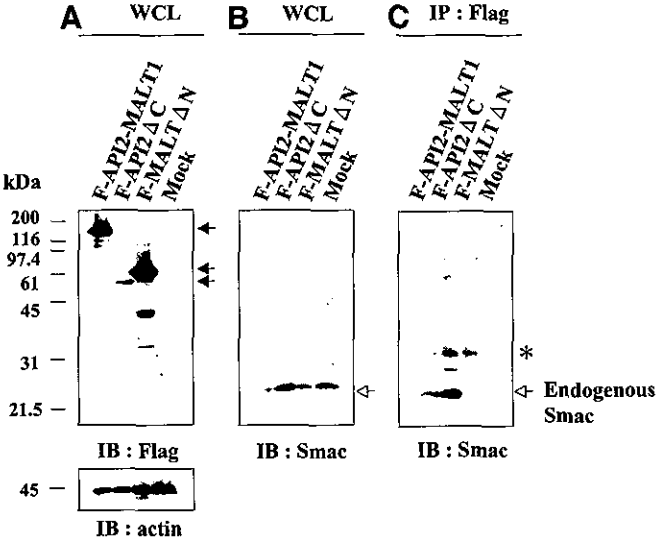


Fig. 3. Apoptosis inhibitor 2 (API2)-MALT1 binds to endogeneous Smac. Whole cell lysates (*WCL*) from the 293T cells transfected with a FLAG-tagged API2-MALT1 plasmid, FLAG-tagged API2ΔC (amino acids 1–442), or FLAG-tagged MALT1ΔN [amino acids 217–813 (0.5 μg each)] were immunoprecipitated with anti-FLAG M2 monoclonal antibody. Whole cell lysates were analyzed with immunoblotting (*IB*) using anti-FLAG monoclonal antibody (*A*) or anti-Smac polyclonal antibody (*B*). Immunoprecipitates were analyzed with immunoblotting using anti-Smac polyclonal antibody (*C*). Nonspecific bands are shown by an *asterisk*.

API2-MALT1 Confers Resistance to UV- and Etoposide-Induced Apoptosis. Because API2 is known to be an IAP (also known as c-IAP2), one can speculate that API2-MALT1 has a similar antiapoptotic function as well. Because no experimental evidence has been presented for such a function, however, we decided to test API2-MALT1 for this purpose. We chose a HeLa cell line in which several apoptotic stimuli including UV irradiation, chemotherapeutic agents, and tumor necrosis factor rapidly induce typical apoptotic changes characterized by chromatin condensation and DNA fragmentation. First, an API2-MALT1 expression plasmid was transiently transfected into HeLa cells, but the fusion protein was barely detectable by Western blot analysis of the whole cell lysates (data not shown). Therefore, we decided to establish stable transfectants expressing API2-MALT1. To this end, we transfected its linearized expression plasmid (PCXN2-FLAG-API2-MALT1) into HeLa cells, selected the cells with Geneticin, and finally cloned them by limiting dilution procedure. As a result, two independent clones expressing API2-MALT1, designated API2-MALT1-1 and API2-MALT1-2, were obtained and used for additional studies. As controls, two independent clones transfected with an empty plasmid only, designated Mock-1 and Mock-2, were also established.

It was noteworthy that when exposed to UV irradiation and treated with etoposide, both clones expressing API2-MALT1 (API2-MALT1-1 and API2-MALT1-2) showed significant attenuation, compared with that of the two control clones, in both UV- and etoposide-induced apoptosis as determined by the chromatin condensation (Figs. 4*A* and 5*A*). Thus, this provides for the first time direct evidence that API2-MALT1 can indeed confer resistance to apoptosis. We also measured DEVDase activity in these transfectants. Similarly, when exposed to UV irradiation or treated with etoposide, the two clones expressing API2-MALT1 showed significant attenuation of DEVDase activity (Figs. 4*B* and 5*B*), almost compatible with and further supporting the results of the apoptosis assay.

Because Smac has been shown to promote apoptosis in response to several stimuli such as UV irradiation that trigger the mitochondria-mediated apoptotic pathway, we examined the role of Smac in UV irradiation-induced apoptosis of the stable transfectants expressing API2-MALT1. To do so, we transiently transfected an expression plasmid for Smac-myc with a green fluorescent protein expression plasmid into the stable transfectants and then induced apoptosis by UV irradiation. Approximately 47–49% of Smac-transfected cells without expression of API2-MALT1 showed signs of apoptosis, as determined by the condensed chromatin. In contrast, only about 21–22% of Smac-transfected cells with stable expression of API2-MALT1 showed signs of apoptosis (Fig. 6). Thus, API2-MALT1 can confer significant resistance to apoptosis promoted by Smac in UV-irradiated HeLa cells.

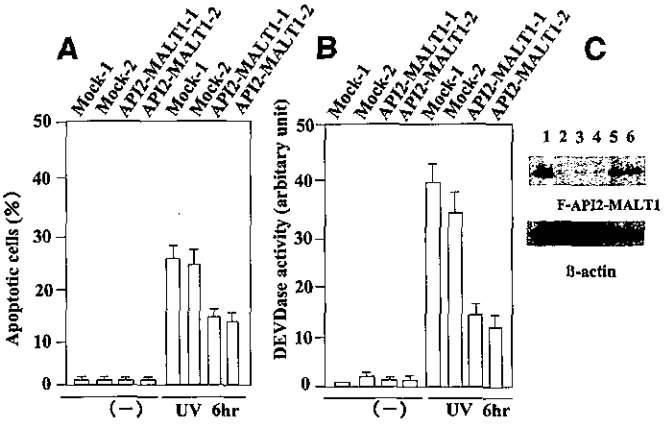


Fig. 4. Apoptosis inhibitor 2 (API2)-MALT1 confers resistance to apoptosis and suppresses DEVDase activity induced by UV irradiation. HeLa cells stably transfected with a FLAG-tagged API2-MALT1 plasmid (API2-MALT1-1 and API2-MALT1-2) or an empty plasmid (Mock-1 and Mock-2) were stimulated with UV irradiation (200 J/m^2). *A*, after 6 h, apoptosis was assessed by the presence of condensed chromatin. Data represent mean ± SD of triplicate samples. *B*, DEVDase activity (5×10^4 cells/sample) was also measured. *C*, whole cell lysates were analyzed with immunoblotting using anti-FLAG monoclonal antibody or anti-β-actin monoclonal antibody. *Lane 1*, 293T cells with API2-MALT1 expression (transient transfection); *Lane 2*, wild-type HeLa cells; *Lanes 3* and *4*, HeLa cells with empty vector (Mock-1 and Mock-2); *Lanes 5* and *6*, HeLa cells with API2-MALT1 stable expression (API2-MALT1-1 and API2-MALT1-2).

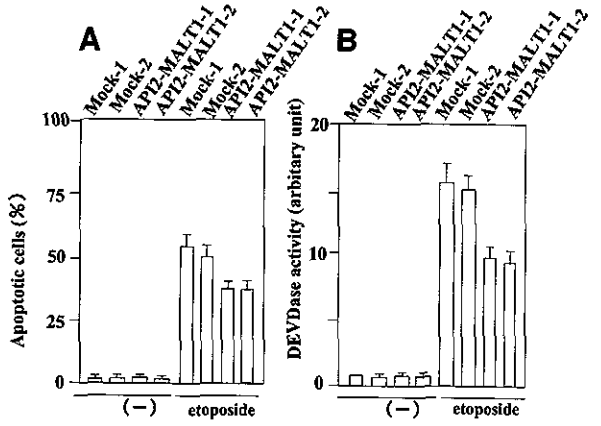


Fig. 5. Apoptosis inhibitor 2 (API2)-MALT1 confers resistance to apoptosis and suppresses DEVDase activity induced by etoposide treatment. HeLa cells stably transfected with a FLAG-tagged API2-MALT1 plasmid (API2-MALT1-1 and API2-MALT1-2) or an empty plasmid (Mock-1 and Mock-2) were treated with etoposide (100 μM) or DMSO for 12 h. *A*, apoptosis was assessed by the presence of condensed chromatin. *B*, DEVDase activity (5×10^4 cells/sample) was also measured. Data represent mean ± SD of triplicate samples.

DISCUSSION

Three distinct chromosomal translocations have been implicated in the pathogenesis of MALT lymphoma. The first, t(1;14), results in the juxtaposition of the *BCL10* gene to the immunoglobulin enhancer, in which BCL10 expression is thought to be deregulated (10, 11). The second, t(11;18), results in the synthesis of a novel fusion protein, known as API2-MALT1 (13–15). The more recently discovered third translocation, t(14;18), results in the juxtaposition of the *MALT1* gene to the immunoglobulin enhancer, in which deregulated MALT1 expression may occur (16, 17). We and others have recently shown (22, 23) that BCL10 and MALT1 form a specific complex within the cells and that these proteins synergize through homodimerization of MALT1 in activation of NF-κB. Furthermore, expression of API2-MALT1 was also shown to strongly activate NF-κB activity; however, ectopic expression of MALT1 alone, as can be expected to occur in MALT cases with t(14;18), was incapable of inducing NF-κB activity (22, 23). Thus, it remains to be established whether NF-κB activation by API2-MALT1 is indeed relevant to the pathogenesis of MALT lymphoma. Furthermore, no experimental evidence has been produced related to the fundamental question of whether API2-MALT1 can confer resistance to apoptosis.

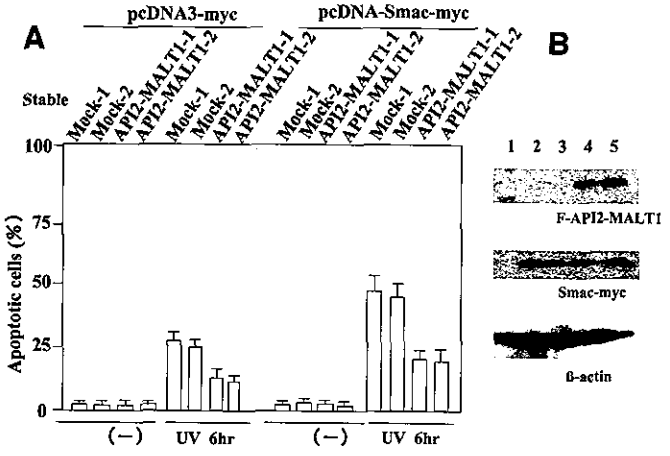


Fig. 6. Apoptosis inhibitor 2 (API2)-MALT1 confers resistance to apoptosis by action against the Smac-mediated apoptotic pathway. HeLa cells stably transfected with a FLAG-tagged API2-MALT1 plasmid (0.5 μg) or an empty plasmid (0.5 μg) were transiently transfected with a COOH-terminal myc-tagged Smac plasmid (2 μg) and a pEGFP plasmid (0.5 μg), incubated for 24 h, and then stimulated with UV irradiation (200 J/m^2). *A*, after 6 h, apoptosis was assessed by the presence of condensed chromatin and expressed as a percentage of apoptotic cells in total transfected green cells. Data represent mean ± SD from triplicate samples. *B*, whole cell lysates were analyzed with immunoblotting using anti-FLAG monoclonal antibody or anti-Myc polyclonal antibody or anti-β-actin monoclonal antibody. *Lane 1*, wild-type HeLa cells; *Lanes 2* and *3*, HeLa cells with empty vector; *Lanes 4* and *5*, HeLa cells with API2-MALT1 stable expression.

In the present study, we were able to identify API2-MALT1-binding proteins and to demonstrate for the first time that API2-MALT1 does exert an antiapoptotic effect against UV irradiation- and etoposide treatment-induced apoptosis in HeLa cells. This antiapoptotic effect may be mediated, at least in part, by action against the Smac-promoted apoptotic pathway. Because several apoptotic inhibitors have been shown to be up-regulated by NF-κB activation (39), our data do not entirely exclude the possibility that the antiapoptotic effect may be mediated, in part, by the secondary up-regulation of such apoptotic inhibitors. In a recent study, we examined whether API2-MALT1 has an antiapoptotic effect on murine interleukin 3-dependent hematopoietic Ba/F3 cells (27). For this purpose, stable transfectants expressing API2-MALT1 were established, and apoptosis was induced by interleukin 3 deprivation or UV irradiation. These results indicated that API2-MALT1 does not show obvious resistance to apoptosis induced by interleukin 3 deprivation or UV irradiation in Ba/F3 cells. Because NF-κB activation is generally thought to contribute to an antiapoptotic effect, we analyzed the nuclear NF-κB activity of these two cell lines by using the NF-κB ELISA kit (Oxford Biomedical Research, Inc.). However, this analysis revealed no significant difference in the amount of nuclear NF-κB between the two cell lines (data not shown), suggesting that the difference in antiapoptotic effect by API2-MALT1 may not be due to the difference in the cell lines' nuclear NF-κB activity. One reason for the difference between the results obtained for the HeLa and Ba/F3 cells would be that these two cell lines do not always use the same apoptotic signaling pathway.

We also examined the stability of ectopically expressed API2, MALT1, and API2-MALT1 by Western blot analysis of the cell lysates with or without treatment with MG132, a proteasome inhibitor (27). It was found that MALT1 is rapidly degraded via the ubiquitin-proteasome pathway, as is the case with API2. On the synthesis of fusion, API2-MALT1 was readily detectable even without MG132, suggesting that this fusion protein becomes stable against the ubiquitin-proteasome pathway (27). Thus, increased stability of API2-MALT1 would be expected to augment counteraction against or synergy with the functions of its binding partners including Smac, HtrA2, and TRAF2.

To the best of our knowledge, this is the first report demonstrating that API2-MALT1 acts as an antiapoptotic regulator, which may be mediated, at least in part, by neutralizing apoptosis promoted by Smac. It stands to reason that Smac would not be the only target for the antiapoptotic effect exerted by API2-MALT1, and indeed, we have identified TRAF2, HtrA2, KIAA1892, and HSP70s as other potential API2-MALT1-binding proteins. Given that Smac, TRAF2, and HtrA2 function as *bona fide* regulators of apoptosis, it is tempting to speculate that the KIAA1892 protein and two Hsp70s exert the same effect. Because recent studies of the *BCL10* and *MALT1* knockout mice provide evidence that both BCL10 and MALT1 participate in linking antigen receptor signaling and NF-κB activation (29–31) and exogeneously expressed API2-MALT1 was shown to enforce activation of NF-κB activity (27, 28), it will be of particular importance to pursue the possible roles of API2-MALT1-binding proteins in antigen receptor signaling. It is hoped that additional studies will provide further insight into the biological functions of API2-MALT1 and ultimately lead to new diagnostic and therapeutic advances for dealing with MALT lymphoma, a human lymphoma that has been attracting special attention.

REFERENCES

1. Isaacson P, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. A distinctive type of B-cell lymphoma. Cancer (Phila) 1983;52:1410–6.
2. Harris NL, Jaffe ES, Stein H, et al. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. Blood 1994;84:1361–92.
3. Isaacson PG, Muller-Hermelink HK, Piris MA, et al. Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma). In: Jaffe ES, Harris NL, Stein H, Vardiman JW, editors. WHO Classification of Tumours: Pathology and Genetics. Tumours of Haemopoietic and Lymphoid Tissues. Lyon, France: IARC Press; 2001. p. 121–6.
4. The Non-Hodgkin's Lymphoma Classification Project. A clinical evaluation of the International Lymphoma Study Group Classification of Non-Hodgkin's Lymphoma. Blood 1997;89:3909–18.
5. Horseman D, Gascoyne R, Klasa R, Coupland R. t(11;18)(q21;q21): a recurring translocation in *lymphomas of mucosa-associated* lymphoid tissue (MALT)? Genes Chromosomes Cancer 1992;4:183–7.
6. Wotherspoon AC, Pan L, Diss TC, Isaacson PG. Cytogenetic study of B-cell lymphoma of mucosa-associated lymphoid tissue. Cancer Genet Cytogenet 1992;58: 35–8.
7. *Wotherspoon* AC, Finn TM, Isaacson PG. Trisomy 3 in low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. Blood 1995;85:2000–4.
8. Dierlamm J, Pittaluga S, Wlodarska I, et al. Marginal zone B-cell lymphomas of different sites share similar cytogenetic and morphologic features. Blood 1996;87: 299–307.
9. Auer IA, Gascoyne RD, Connors JM, et al. t(11;18)(q21;q21) is the most common translocation in MALT lymphomas. Ann Oncol 1997;8:979–85.
10. Willis TG, Jadayel DM, Du M-Q, et al. Bcl10 is involved in t(1;14)(p22;q32) of MALT B-cell lymphoma and mutated in multiple tumor types. Cell 1999;96:35–45.
11. Zhang Q, Siebert R, Yan M, et al. Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). Nat Genet 1999;22:63–8.
12. Ott G, Katzenberger T, Greiner A, et al. The t(11;18)(q21;q21) chromosome translocation is a frequent and specific aberration in low-grade but not high-grade malignant non-Hodgkin's lymphomas of the mucosa-associated lymphoid tissue (MALT-) type. Cancer Res 1997;57:3944–8.
13. Akagi T, *Motegi M, Tamura A, et al. A novel gene,* MALT1 at 18q21, is involved in t(11;18)(q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Oncogene 1999;18:5785–94.
14. Dierlamm J, Baens M, Wlodarska I, et al. The apoptosis inhibitor gene API2 and a novel 18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood 1999;93:3601–9.
15. Morgan JA, Yin Y, Borowsky AD, et al. Breakpoints of the t(11;18)(q21;q21) in mucosa-associated lymphoid tissue (MALT) lymphoma lie within or near the previously undescribed gene MALT1 in chromosome 18. Cancer Res 1999;59:6205–13.
16. Sanchez-Izquierdo D, Buchonnet G, Siebert R, et al. MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-Hodgkin lymphoma. Blood 2003;101:4539–46.
17. Streubel B, Lamprecht A, Dierlamm J, et al. t(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. Blood 2003; 101:2335–9.
18. Murga Penas EM, Hinz K, Roser K, et al. Translocations t(11;18)(q21;q21) and t(14;18)(q32;q21) are the main chromosomal *abnormalities involving* MLT/MALT1 in MALT lymphomas. Leukemia (Baltimore) 2003;17:2225–59.
19. Remstein ED, Kurtin PJ, Einerson RR, Paternoster SF, Dewald GW. Primary pulmonary MALT lymphomas show frequent and heterogeneous cytogenetic *abnormalities*, including aneuploidy and translocations involving API2 and MALT1 and IGH and MALT1. Leukemia (Baltimore) 2004;18:156–60.
20. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis *proteins. Cell* 1995;83:1243–52.
21. Hofmann K, Bucher P, Tschopp J. The CARD domain: a new apoptotic signalling motif. Trends Biochem Sci 1997;22:155–6.
22. Uren AG, O'Rourke K, Aravind L, et al. Identification of paracaspases and metacaspases. two ancient families of caspase-like proteins, one of Which plays a key role in MALT lymphoma. Mol Cell 2000;6:961–7.
23. Lucas PC, Yonezumi M, Inohara N, et al. Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-κB signaling pathway. J Biol Chem 2001;276:19012–9.
24. Ruland J, Duncan GS, Elia A, et al. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-κB and neural tube closure. Cell 2001;104:33–42.
25. Ruland J, Duncan GS, Wakeham A, Mak TW. *Differential requirement for malt1 in* T and B cell antigen receptor signaling. Immunity 2003;19:749–58.
26. Ruefli-Brasse AA, French DM, Dixit VM. Regulation of NF-κB-dependent lymphocyte activation and development by paracaspase. Science (Wash DC) 2003;302: 1581–4.
27. *Du C, Fang M, Li Y, Li L, Wang X.* Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell 2000; 102:33–42.
28. Verhagen AM, Ekert PG, Pakusch M, et al. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell 2000;102:43–53.
29. Izumiyama K, Nakagawa M, Yonezumi M, et al. Stability and subcellular localization of API2-MALT1 chimeric protein involved in t(11;18)(q21;q21) MALT lymphoma. Oncogene 2003;22:8085–92.
30. Suzuki Y, Imai Y, Nakayama H, et al. Serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Mol Cell 2001;8:613–21.
31. Shevchenko A, Wilm M, Vorm O, *Mann M. Mass spectrometric* sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.
32. Vaux DL, Silke J. Mammalian mitochondrial IAP binding proteins Biochem Biophys Res Commun 2003;304:499–504.
33. Hegde R, Srinivasula SM, Zhang Z, et al. Identification of Omi/HtrA2 as a mitochondrial apoptotic serine protease that disrupts inhibitor of apoptosis protein-caspase interaction. J Biol Chem 2002;277:432–8.
34. Martins LM, Iaccarino I, Tenev T, et al. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem 2002;277:439–44.
35. Verhagen AM, Silke J, Ekert PG, et al. HtrA2 promotes cell death through its serine protease activity and its ability to antagonize inhibitor of apoptosis proteins. *J Biol* Chem 2002;277:445–54.
36. Srinivasula SM, Gupta S, Datta P, et al. Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/HtrA2. J Biol Chem 2003;278:31469–72.
37. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of *IAPs and their degradation in proteasomes in response* to apoptotic stimuli. Science (Wash DC) 2000;288:874–7.
38. Parcellier A, Gurbuxani S, Schmitt E, Solary E, Garrido C. Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem Biophys Res Commun 2003;304:505–12.
39. Mayo MW, Baldwin AS. The transcription factor NF-κB: control of oncogenesis and cancer therapy resistance. Biochim Biophys Acta 2000;1470:M55–62.