The involvement of calpain-dependent proteolysis of the tumor suppressor NF2 (merlin) in schwannomas and meningiomas

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Neurofibromatosis type 2 (NF2) protein, also known as merlin or schwannomin, is a tumor suppressor, and *NF2* is mutated in most schwannomas and meningiomas. Although these tumors are dependent on NF2, some lack detectable *NF2* mutations, which indicates that alternative mechanisms exist for inactivating merlin. Here, we demonstrate cleavage of merlin by the ubiquitous protease calpain and considerable activation of the calpain system resulting in the loss of merlin expression in these tumors. Increased proteolysis of merlin by calpain in some schwannomas and meningiomas exemplifies tumorigenesis linked to the calpain-mediated proteolytic pathway.

Neurofibromatosis type 2 (NF2) is an autosomal dominantly-inherited disease with an incidence of approximately 1:40,000 (ref. 1). The disease is characterized by a predisposition for the development of specific intracranial tumors including vestibular schwannomas (formerly called acoustic neurinomas) and meningiomas[2]. These tumors, including both sporadically occurring and NF2 cases, account for about 30% of adult neoplasms in the central nervous system[3]. The *NF2* gene is on chromosome 22q12 and encodes a protein of 595 amino acids, designated merlin or schwannomin[4,5]. Its sequence is similar to that of the band 4.1 superfamily of proteins, especially ezrin, radixin and moesin (ERM proteins). These family members are thought to have important roles in linking the cell membrane with the cytoskeleton[6]. Such proteins share a conserved N-terminal 30-kDa domain (the ERM-homologous domain) that binds to membrane proteins, whereas a highly charged α-helix rich domain at the C terminus is thought to associate with cytoskeletal proteins[7].

Mutations in *NF2* have been found in approximately 50–80% of NF2 tumors[1,8], 15–35% of sporadic meningiomas[9–12] and 20–60% of sporadic schwannomas[10,11,13,14], as well as other tumors, including breast, colorectal cancers and mesotheliomas[15–17]. There are some salient features in *NF2* mutations. There is a high frequency of nonsense mutations, frameshift mutations and splice-site mutations resulting in merlin presumably truncated mostly in its N-terminal half[18,19]. In contrast, missense mutations occur with an extremely low frequency. Another feature is high penetrance, predicted from the development of the tumors in most NF2 patients. These features indicate that the development of NF2-related tumors (schwannomas and meningiomas here) depends on the loss of merlin function. The fact that such truncated merlins have never been detected[20–22] indicate that they might be unstable and degraded if synthesized in the cell. In addition, we have reported that merlin binds to five putative cellular proteins (p165, p145, p125, p85 and p70), named merlin-binding proteins, in the N-terminal region of a 400-amino-acid domain containing almost the entire ERM-homologous domain[23]. Most truncated forms of merlin are therefore unlikely to interact with merlin-binding proteins. Recent protein analyses showed that expression of merlin was reduced or absent in most sporadically occurring meningiomas, schwannomas and ependymomas[20–22]. Stemmer-Rachamimov *et al.* reported that 12 of 22 schwannomas had no merlin expression despite a lack of biallelic *NF2* inactivation[20]. Therefore, we suggest that post-translational regulation of merlin as well as *NF2* mutations may be involved in the loss of merlin expression, and that increased degradation of merlin results in its inactivation.

Here, we first demonstrate the calpain-mediated proteolysis of merlin. Calpains are a large family of calcium-dependent neutral cysteine proteases present in animal cells[24,25]. Two isozymes of the ubiquitous calpain, μ- and m-calpains, have similar biochemical features except for the calcium concentration they require for activation *in vitro*; μ- and m-calpains require micromolar and millimolar levels of calcium, respectively. Until now, calpain was thought to be activated as a cellular receptor in response to calcium mobilization, not only in physiological states but also in various pathological conditions. Therefore, calpain is regarded as a biomodulator acting through limited proteolysis of relevant substrate proteins, including cytoskeletal and membrane proteins, enzymes such as kinases and phosphatases, and transcription factors[24,25,26]. We also demonstrate here that the loss of merlin expression in schwannomas and meningiomas without *NF2* mutations could result from activation of the calpain system. These

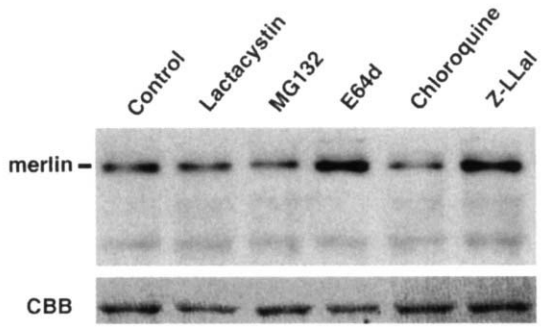

Fig. 1 Metabolic stabilization of merlin by calpain inhibitors. Primary cultured human fibroblasts were treated with protease inhibitors for 24 hours: lactacystin (5 μM), MG132 (10 μM), E64d (15 μM), chroloquine (100 μM), and Z-LLal (5 μM). Both E64d and Z-LLal, which specifically inhibit cysteine proteases or calpain, increased the amount of merlin. Western blot analysis was done using the anti-merlin antibody C-18. Coomassie brilliant blue staining of the gel showed equal amounts of proteins were loaded in all lanes.

findings shed light on new roles of the calpain-dependent proteolytic pathway in the fields of tumor biology and medicine.

Metabolic stabilization of merlin by calpain inhibitors

Many intracellular regulatory proteins such as p53, p27 and β-catenin are probably controlled by their selective degradation rather than by protein synthesis. To evaluate the proteolytic system of merlin, we used membrane-permeable specific inhibitors of various proteases on primary cultured human fibroblasts for 24 hours. Western blot analysis was done using an antipeptide antibody against a C-terminal, 18-residue sequence of human merlin (C-18) (Fig. 1*a*). The use of a cysteine protease inhibitor (E64d) and a specific calpain inhibitor, carbobenzoxyl-leucinyl-leucinal[27] (Z-LLal), increased the amount of an approximately 70-kDa, full-length merlin through metabolic stabilization, indicating the constitutive degradation of merlin by the calpain system in intact cells. Neither the proteasome inhibitors[28] lactacystin and MG132 nor the lysosome inhibitor chloroquine affected the amount of merlin. Coomassie brilliant blue staining of the gel showed that equal amounts of proteins were loaded in all lanes.

In vitro proteolysis of merlin by calpains

To investigate whether merlin is a substrate for calpain, we incubated *in vitro*-translated, [^{35}S] methionine-labeled merlin with purified calpain in various concentrations of $CaCl_2$. Full-length merlin was digested by both μ- and m-calpains (0.05 units) in a calcium-dependent manner (Fig. 2*a* and *b*, respectively). The cleavage was followed by the appearance of an approximately 35-kDa proteolytic fragment (Fig. 2*a* and *b*, arrow). The merlin cleavage by both calpains was inhibited by Z-LLal (5 μM). In the absence of added calpain, high concentrations of calcium were not associated with this digestion.

Next, we demonstrated the calpain-dependent proteolysis of native merlin protein from U-251MG malignant glioma cells. The merlin in cytosolic S-100 extracts from the cells was incubated with m-calpain in the presence of 10 mM $CaCl_2$, and western blot analysis was done using the C-18 antibody. Purified m-calpain digested merlin in a both dose-dependent and time-dependent manner (Fig. 2*c* and *d*, respectively), and this proteolysis was similarly abolished by the addition of Z-LLal. No proteolytic fragment from merlin was detected by C-18, indicating that such intermediates are unstable and degraded by cellular factors. These findings demonstrate merlin proteolysis by calpain *in vitro*.

Bacterially expressed merlin deletion mutants fused to GST were used for *in vitro* proteolysis, and the cleavage products were analyzed by silver staining to determine the proteolytic sites of

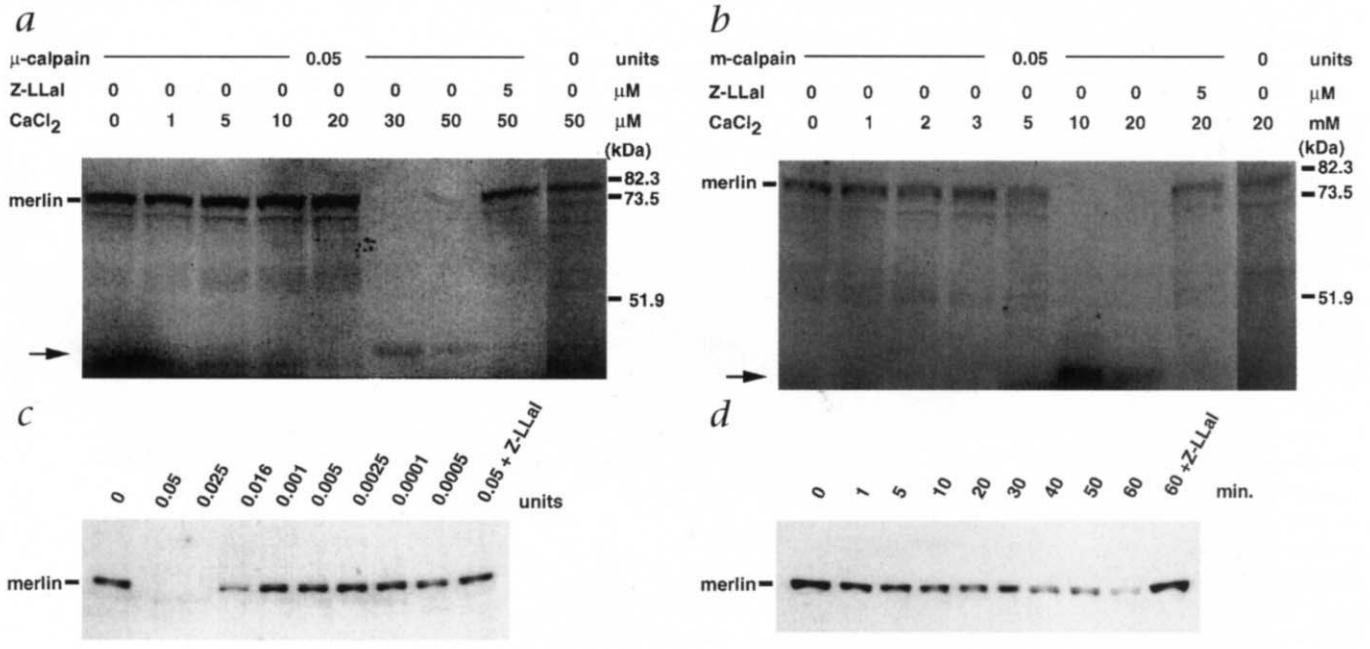

Fig. 2 *In vitro* proteolysis of merlin by purified calpains. [^{35}S] methionine-labeled merlin was incubated with purified μ-calpain (***a***) and m-calpain (***b***) at the indicated concentrations of $CaCl_2$ for 30 min, resolved by SDS–PAGE and visualized by autoradiography. Digestion of merlin in a calcium-dependent manner resulted in an approximately 35-kDa proteolytic fragment (arrow). Molecular weights are given in kilodaltons on the far right. ***c*** and ***d***, Native merlin in S-100 cytosolic extracts from U-251MG cells was similarly incubated with m-calpain in 10 mM $CaCl_2$. The merlin cleavage was found to be calpain-dependent (*c*) and time-dependent (*d*). Western blot analysis used the C-18 antibody. Z-LLal (5 μM) blocked all digestion.

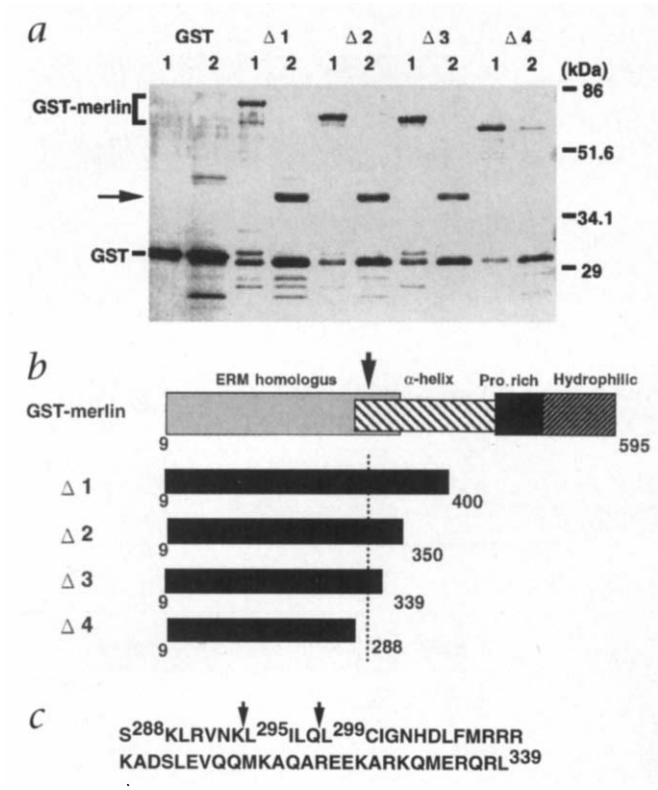

Fig. 3 Identification of calpain cleavage sites in bacterially expressed GST–merlin fusion proteins. ***a***, Recombinant GST–merlin deletion mutants were incubated in the absence (lane 1) and presence (lane 2) of m-calpain (0.05 units). Samples were analyzed to SDS–PAGE and silver staining. The arrow indicates an approximately 35-kDa proteolytic fragment in samples from Δ1, Δ2 and Δ3, but not Δ4. GST–merlin and GST alone are indicated. ***b***, Full-length merlin compared with merlin deletion mutants Δ1–Δ4. The downward arrow and dotted line indicate the predicted calpain proteolytic site. ***c***, The amino acid sequence of merlin near the calpain cleavage site. Arrows indicate preferential cleavage sites determined by C-terminal amino acid sequencing of the proteolytic fragment.

merlin by m-calpain (Fig. 3*a* and *b*). The digestion of C-terminal deletion mutants Δ1, Δ2, and Δ3 resulted in an N-terminal, 35-kDa proteolytic fragment and GST alone, and the predicted small cleavage fragments containing the C-terminal portion were barely visible on the gel. In contrast, the Δ4 mutant yielded no such 35-kDa fragment, and the amount of Δ4 was decreased, probably because of the instability of the mutant protein. Bovine serum albumin (a control) was not digested with calpain and existed stably for as long as several hours in a similar reaction (data not shown). In addition, western blot analysis using an anti-GST monoclonal antibody showed that calpain cleavage also occurred in the boundary between GST and the ERM-homologous domain of merlin in mutants Δ1–Δ4 (data not shown). These results indicate that the sequence of amino acids 288–339 of merlin, which are in the boundary between the ERM-homologous and α-helix domains, may be more susceptible to calpain.

The Δ2 protein was incubated with purified m-calpain, separated by SDS–PAGE, and transferred to a Teflon membrane. A protein band corresponding to the 35-kDa fragment was subjected to the automated C-terminal amino acid sequence analysis[29,30]. The first three cycles of the C-terminal analysis resulted in the identification of cycle-1 Gln(Q), cycle-2 Leu(L), Asn(N) and cycle-3 Val(V). In comparing this with the cleaved site sequence (Ser(288)–Leu(339)) of merlin, two prominent sequences, V(292)-N-K(294) and I(296)-L-Q(298), were found to be well matched to the results of the C-terminal analysis. Thus, the calpain preferential cleavage site of merlin could be on the N-terminal sides of both Leu(295) and Leu(299) (Fig. 3*c*).

Proteolysis of HA-tagged merlin transfected into COS-7 cells

To investigate the cleavage of merlin by calpain in cultured cells, we transfected two plasmids into COS-7 cells that enable us to detect the N-terminal portion of the protein using anti-HA antibody: the HA-tagged, full-length merlin expression plasmid pCGN-NF2 and the HA-tagged, Δcalpain merlin expression plasmid pCGN-NF2(Δcalpain), which produces HA-tagged merlin lacking the calpain cleavage sequence (amino acids 295–299; LILQL) (Fig. 4*a*). The cells expressing full-length merlin were treated with the calcium ionophore ionomycin (5 μM), for 0–30 min to activate intracellular calpain, in a medium containing 10 mM $CaCl_2$. Western blot analysis demonstrated that the amount of HA-tagged full-length merlin decreased at 15 and 30 minutes after the ionomycin treatment, in a time-dependent manner (Fig. 4*b*). The reduction was completely blocked by Z-LLal (5 μM). In addition to the full-length merlin, a slower-migrating band appeared, which seemed to be an unrelated 'background' band as it was also produced in mock-transfected cells. The presumed cleavage product, approximately 35 kDa in size, was found at 5, 15 and 30 minutes after the ionomycin treatment, corresponding with the calpain digestion of the full-length merlin. The appearance of the proteolytic fragment was blocked by Z-LLal (5 μM). In contrast, the amount of HA-tagged Δcalpain merlin did not diminish even with ionomycin treatment (Fig. 4*c*), strongly supporting the data resulting from *in vitro* proteolysis of merlin by calpain (Fig. 3). There was no proteolytic product from the HA-tagged Δcalpain merlin in ionomycin-treated cells. To detect the cleavage fragment (which was expected to be unstable in the cell), western blot analysis was done using increased amounts of cell lysate (about 75 μg protein per lane) on a 10–20% gradient gel. The proteolytic band is indicated separately because its detection required longer exposure of the film (Fig. 4*b*). Thus, calpain-dependent proteolysis of merlin in cell culture was demonstrated to be consistent with the data from the *in vitro* analyses.

Merlin and μ-calpain expression in tumors lacking *NF2* mutations

The fact that some meningiomas and schwannomas lack biallelic *NF2* inactivation prompted us to investigate whether merlin proteolysis is involved in such NF2-related tumors. Both mutation and protein analyses were performed in the same tumor tissues. This study included a total of twenty-four tumors consisting of three meningiomas from NF2 patients, six sporadic meningiomas, thirteen schwannomas from NF2 patients, and two sporadic schwannomas. Malignant glioma cells, which are not usually related to NF2, were used as a control in the study.

A protein truncation test[8] was done first, to detect translation-terminating mutations using RT-PCR for full-length *NF2* cDNA followed by *in vitro* transcription and translation. Seventeen of twenty-four (70.8%) tumors had premature termination of translation. In contrast, [^{35}S] methionine-labeled full-length merlin was found in the remaining seven cases which included three sporadic meningiomas and one meningioma and three schwannomas from the NF2 patients. Results from representatives of each of the three tumor types, sporadic meningiomas (M1, M2 and M3), schwannomas from the NF2 patients (S1, S2 and S3), and control glioma cells (U-251MG, RBR17T and MS638), are

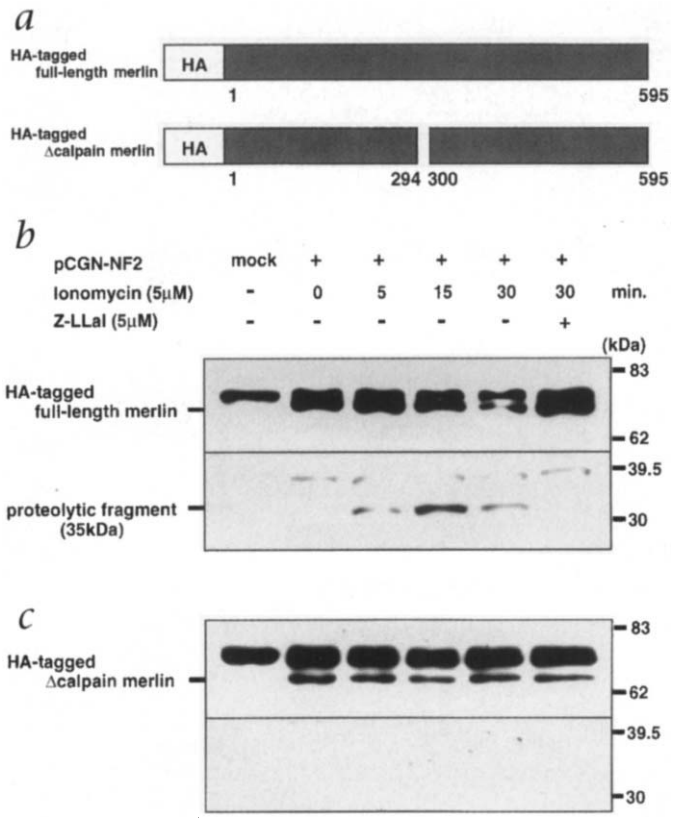

Fig.4 Calpain-dependent proteolysis of HA-tagged merlin in COS-7 cells. ***a***, HA-tagged full-length merlin compared with HA-tagged Δcalpain merlin lacking the calpain cleavage site (amino acids 295–299). ***b*** and ***c***, Cells were transfected by pCGN-NF2 and pCGN-NF2(Δcalpain) expression vectors and treated with 5 μM ionomycin to activate intracellular calpain; western blot analysis of cell lysates was done using anti-HA antibody to detect the N-terminal portion of HA-merlin. The amount of full-length HA-merlin decreased in the presence of ionomycin, and the 35-kDa cleavage product appeared correspondant with the proteolysis on a long (1 hour) exposure of the film; the cleavage was inhibited by Z-LLal (*b*). HA-tagged Δcalpain merlin, in contrast, was not digested in ionomycin-treated cells (*c*).

shown in Fig. 5*a*. There were no other small truncated products in these samples. We did not find any missense mutations in these tumors. RT-PCR analysis demonstrated the presence of *NF2* transcript in the NF2-related tumors as well as in glioma cells (Fig. 5*b*). Western blot analysis using the C-18 antibody showed the loss of merlin in both meningiomas and schwannomas, although the tumors expressed *NF2* mRNA translatable into full-length merlin (Fig. 5*c*). Merlin was expressed in control glioma cells. We also analyzed the status of μ-calpain activation in this study because its activation can occur with physiological levels of calcium. Three forms of μ-calpain are obtained on autolytic activation of the N-terminal portions: 80-kDa pre-activated (inactive), 78-kDa intermediate, and 76-kDa activated[31]. A marked activation of μ-calpain was found in the M2, M3, S2, and S3 tumors. The amounts of the 78-kDa and 76-kDa forms might be underestimated because of their instability, but this μ-calpain activation seemed to be absent in glioma cells. Results with β-tubulin showed that similar amounts of protein were loaded on the gel. Billger *et al.* have reported the proteolysis of tubulin by calpain[32], and thus merlin might be targeted by the calpain system in NF2-related tumors. These lines of evidence indicate the development of tumors through degradation of merlin by activated calpain.

Marked activation of μ-calpain in sporadic meningiomas

We also demonstrated the activation of μ-calpain in sporadic meningiomas and primary cultured cells from these tumors by immunochemical analyses using an antibody against activated μ-calpain. This antibody reacts specifically with the activated form and does not react with the pre-activated or intermediate forms of μ-calpain[33]. Activated μ-calpain was stained considerably in the cytoplasm of both meningioma cells in the tissues (Fig. 6*a* and *c*) and in their primary cultured cells (Fig. 6*b* and *d*, respectively). In contrast, the activated form was not detected in interstitial non-tumor cells in the meningioma tissues (Fig. 6*a* and *c*), two independent glioma tissues (Fig. 6*e* and *f*) or U-251MG glioma cells (Fig. 6*g*). The treatment of U-251MG cells with ionomycin (5 μM) resulted in the activation of pre-existing μ-calpain (Fig. 6*h*), indicating that μ-calpain is not normally activated in these cells. Other malignant glioma cell lines (RBR17T and MS638) produced similar results (data not shown). These data indicate that the calpain system is constitutively activated in meningioma cells, possibly leading to the degradation of merlin.

Calpain inhibition restores Merlin in cultured meningioma cells

To test directly whether the loss of merlin results from dysregulated calpain-dependent proteolysis, primary cultured meningioma cells and control fibroblasts were incubated in the presence of Z-LLal (5 μM). Western blot analysis (using C-18) of parallel cultures showed a rapid increase in the amount of merlin at six hours after the treatment of meningioma cells (Fig. 7*a*). In contrast, a slow increase in the amount of the protein was observed for as long as 24 hours in fibroblasts (Fig. 7*b*), similar to that shown in Fig. 1. A faint band at 0 hours in meningioma cells was probably merlin derived from fibroblasts in the primary culture. Although the proteolytic fragment was almost undetectable in this experiment, our data do not exclude the possibility of the presence of such unstable intermediates of a proteolytic process. Collectively, these findings demonstrate that calpain-mediated degradation specifically contributes to the absence of merlin in meningioma cells without *NF2* mutations.

Discussion

Inactivation of tumor suppressors has been found in a variety of sporadic human tumors and inherited cancer syndromes. As with gene mutations, abnormal protein metabolism probably leads to similar tumor formation. We have presented here evidence that the proteolysis of merlin mediated by activated calpain is involved in the development of some schwannomas and meningiomas.

Proteolysis of merlin by calpain. The calpain-mediated cleavage of merlin *in vitro* and in cell culture corresponds with the findings that many cytoskeleton-associated proteins are substrates for calpain[34–39]. It also indicates that merlin plays a part in the calcium signaling pathway. Both merlin and calpain molecules were immunochemically stained in the cytoplasm along the cytoskeleton and cell membranes, supporting the idea of possible interactions between merlin and calpain (data not shown). The boundary between the ERM-homologous and α-helix domains of merlin was susceptible to calpain digestion. NXXY motifs in the vicinity of calpain cleavages were found in integrin β3 subunit and other calpain substrates[40]. Correspondingly, the NISY sequence (amino acids 263–266) N-terminal to the proteolytic sites of merlin might be involved in such cleavage. In *in vitro* proteolysis, calpain cleav-

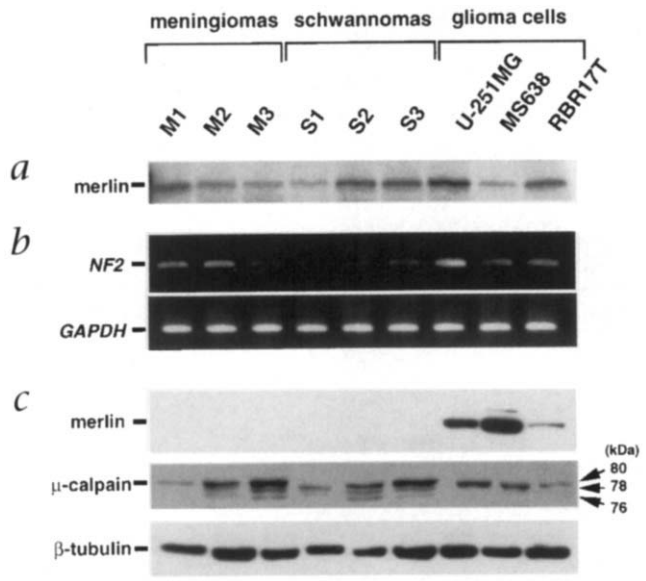

Fig. 5 Expression analysis of *NF2* mRNA/merlin and μ-calpain in meningiomas, schwannomas and malignant glioma cells without *NF2* mutations. ***a***, Protein truncation test of merlin. ***b***, RT-PCR analysis of *NF2* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). ***c***, Western blot analysis of merlin, μ-calpain and β-tubulin. Samples analyzed: meningiomas (M1, M2, M3), schwannomas (S1, S2, S3), and malignant glioma cells (U-251MG, MS638, RBR17T). Merlin was lost from both meningioma and schwannoma tissues, which expressed *NF2* mRNA that can be translated into full-length merlin *in vitro*. Activated (76 kDa) and intermediate (78 kDa) forms of μ-calpain were found in M2, M3, S2, and S3.

age of the protein yielded an approximately 35-kDa fragment containing the ERM-homologous domain. We also demonstrated the presence of the cleavage product related to the calpain digestion of full-length merlin in cell culture. The instability of the cleavage products in the cells is indicated by the fact that truncated merlins synthesized from mutated *NF2* genes have never been detected[20–22]. Both truncated and digested merlin are thought to result in similar inactivation, due to gene mutations and increased proteolysis, respectively, and they are not able to interact with the putative merlin-binding proteins. Thus, calpain cleavage in the cell could lead to termination of the function of merlin.

The activity of the calpain-mediated proteolytic system is determined by intracellular calcium mobilization, whereas phosphorylation of the substrate proteins either inhibits or promotes their degradation by calpain. The substrate proteins include endothelial actin-binding protein[38], platelet cortactin[39], connexin-32 (ref. 41), tau[42], and neurofilament proteins[43]. We previously showed that merlin is constitutively phospholylated mostly on serine residues[23]. The phosphorylation of merlin might affect the sensitivity to calpain cleavage of the protein. In the NF2-related tumors studied, β-tubulin, which is one of the calpain substrates[32], was normally expressed despite calpain activation. Thus, merlin seems to be selectively targeted by the calpain system through cellular factors in the NF2-related tumors.

Increased proteolysis of merlin in some NF2-related tumors. The significance of the calpain-mediated degradation of merlin was additionally emphasized by its association with tumors. Indeed, reports have shown reduced or absent expression of merlin in most sporadic meningiomas, schwannomas and ependymomas, although gene mutations were not fully analyzed[20–22]. Therefore, we did mutation and protein analyses in the same tissues and cells from meningiomas and schwannomas. Despite a relatively small sample size, protein truncation test analysis successfully identified NF2-related tumors which lacked any detectable *NF2* mutations. The M2, M3, S2 and S3 tumors were considered to have abnormal proteolysis because of their expression of *NF2* mRNA that can be translated into full-length merlin *in vitro*, their lack of native merlin and their constitutive activation of μ-calpain (Fig. 5). However, we did not exclude the possibility of decreased *NF2* transcription in the tumors, and preferentially predict the coexistence of both gene repression and abnormal proteolysis in same tumors (for example, M3 and S2).

An altered intracellular proteolytic system, particularly the ubiquitin–proteasome pathway, is associated with oncogenic processes in cells. Tumor suppressor protein p53, which is modified by the addition of ubiquitin by human E6AP ubiquitin ligase in the presence of E6 oncoprotein from papillomavirus, is rapidly degraded by the proteasome[44]. Cyclin-dependent kinase inhibitor p27 is a potential tumor suppressor, but its gene has not been found to be mutated in human tumors. Increased proteasomal degradation of p27 protein was found in primary breast cancer[45] and aggressive colorectal carcinomas[46]. These lines of evidence as well as our results indicate that the proteolytic system plays an important part in carcinogenesis and tumor cell proliferation. The increased proteolysis of merlin in some NF2-related tumors exemplifies a tumorigenesis linked to calpain-dependent pathway. Thus, the term 'proteolysis mutation' might be proposed for the inactivation of tumor suppressor protein caused by increased degradation.

Significance of merlin proteolysis in clinical medicine. NF2-related intracranial tumors are clinically severe despite their pathologically benign and slow-growing features. At present, the only therapy for these tumors is surgical removal, which is a burden for most patients. Some NF2 patients suffer from recurrent or nonresectable tumors. Our results indicate that the therapeutic application of calcium channel-blocking agents and certain calpain inhibitors could prevent growth and relapse of the tumors associated with abnormal proteolysis, although additional studies are needed. Indeed, the merlin production was restored in primary cultured meningioma cells in the presence of a calpain inhibitor. Moreover, calcium channel antagonists, including verapamil, nifedipine and diltiazem, inhibited the proliferation of meningioma cells in culture after growth factor stimulation[47,48].

The results presented here provide evidence of the physiologically regulated cleavage of merlin by calpain. Analysis of NF2-related tumors without *NF2* mutations demonstrated the absence of merlin resulting from excessive activation of the calpain system, indicating a new mechanism of tumorigenesis related to calpain-mediated proteolysis.

Methods

Cell culture. Malignant glioma cell lines (U-251MG, RBR17T and MS638) were maintained in RPMI 1640 (Life Technologies) supplemented 10% (v/v) with fetal calf serum (FCS). COS-7 cells and primary cultured human fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented 10% with FCS, and primary cultured meningioma cells were grown in DMEM supplemented 20% with FCS. Each medium contained 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. Primary cultured cells were established as described[47].

Antibodies, protease inhibitors and purified calpain. Anti-merlin polyclonal antibody (C-18) was obtained from Santa Cruz Biotechnology (Santa

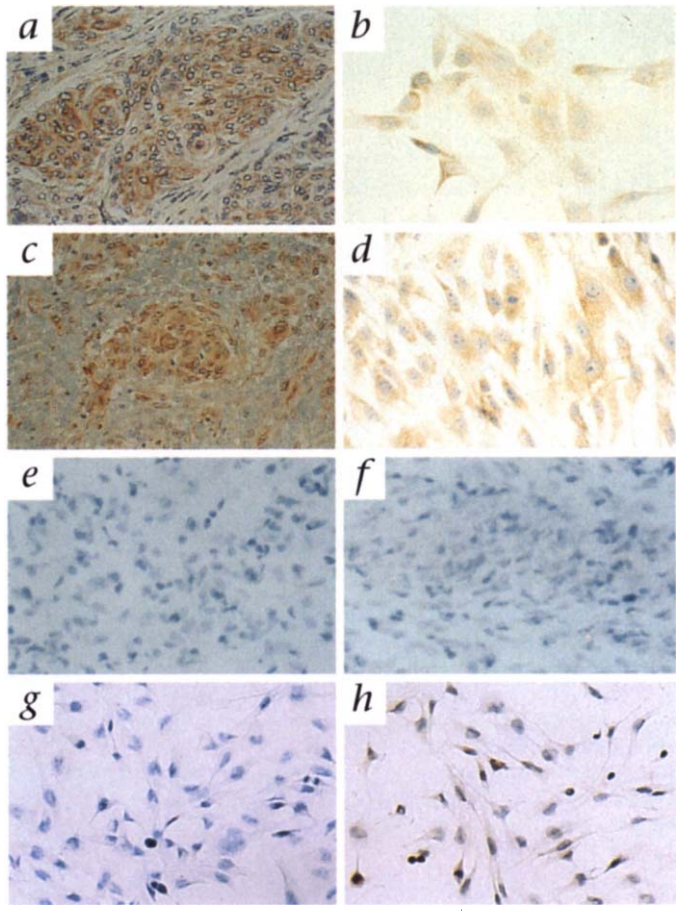

Fig. 6 Constitutive activation of μ-calpain in meningiomas and their primary cultured cells. Immunochemical analysis was done using an antibody against activated μ-calpain[33]. Activated μ-calpain was found in two distinct meningiomas (***a*** and ***c***) and their primary cultured cells (***b*** and ***d***, respectively). In contrast, the activated form of μ-calpain was not normally detected in interstitial non-tumor cells in the meningioma tissues (*a* and *c*), two independent glioma tissues (***e*** and ***f***) or U-251MG malignant glioma cells (***g***). Treatment with 5 μM ionomycin resulted in the activation of pre-existing μ-calpain in U-251MG cells (***h***).

Cruz, California); anti-GST monoclonal antibody, from MBL (Nagoya, Japan); anti β-tubulin (MAB065) antibodies, from Chemicon (Temecula, California); and anti-HA monoclonal antibody (12CA5), from Boehringer. A polyclonal antibody against μ-calpain and a polyclonal antibody against human post-autolytic (activated) μ-calpain were prepared as described[31,33]. Calpain inhibitor carbobenzoxyl-leucinyl-leucinal (Z-LLal) and proteasome inhibitor carbobenzoxyl-leucinyl-leucinyl-leucinal (CBZ-LLLal, MG132) were supplied by Peptide Institute (Osaka, Japan); lactacystin was obtained from Kyowa (Tokyo, Japan); cysteine protease inhibitor E64d, from Sigma; lysosome inhibitor chloroquine, from Wako (Osaka, Japan); and calcium ionophore (ionomycin), from Calbiochem (La Jolla, California). Purified μ-calpain and m-calpain were obtained from Chemicon (Temecula, California) and Sigma, respectively.

Plasmid construction and transfection. The 1.7-kb human *NF2* cDNA containing the entire coding sequence was isolated by RT-PCR, double-digested with *Bam*HI and *Eco*RI, and subcloned into a pGEX-2TH bacterial expression vector to generate the plasmid pGEX-2TH-NF2. The truncated constructs were made as described[23]. The full-length *NF2* cDNA was PCR-amplified from the pGEX-2TH-NF2 plasmid using the primers NF2-HA sense (5′–GCCCTCTAGAATGGCCGGGGCCATCGCTTCCCGCAT-GAGCTTCAGCTCTCTCAAG–3′) containing an *Xba*I site (underlined) and NF2-HA-AS (5′–GGGTGGTACCTAGAGCTCTTCAAAGAAGG–3′) containing a *Kpn*I site (underlined). The PCR fragment was double-digested with *Xba*I and *Kpn*I, and ligated into a pCGN mammalian expression vector to generate the plasmid pCGN-NF2. Merlin expression vector pCGN-NF2(Δcalpain), tagged with HA epitope and lacking a calpain proteolytic site, was generated by the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California) using the pCGN-NF2 plasmid as a template and primers NF2/E9F1 (5′–TCAAAGCTCCGTGTTAATAAGTG-TATCGGGAACCATGATCTATTT–3′) and NF2/E9R1 (5′–ATCATGGTTCCC-GATACACTTATTAACACGGAGCTTTAGGGA–3′). To express merlin tagged with the HA epitope, pCGN-NF2 and pCGN-NF2(Δcalpain) were transfected into COS-7 cells by a liposome-mediated gene transfer method[49].

Western blot analysis. Cell lysates containing equal amounts of protein (15 μg) were separated by 8% SDS–PAGE and were transferred to a nitrocellulose membrane by a constant current of 150 mA for 1 hour. The membrane was blocked for 1 hour at room temperature with phosphate-buffered saline (PBS) containing 10% nonfat dry milk, and then incubated with either C-18 or anti-HA antibodies in PBS containing 0.03% Tween 20 for 1 hour. After being washed with PBS containing 0.3% Tween 20, the membrane was incubated with species-appropriate HRP-conjugated secondary antibodies for 40 min. After the membrane was washed with PBS containing 0.3% Tween 20, bands were visualized using an ECL detection system (Amersham).

RNA extraction and cDNA synthesis. Total RNA was extracted from the tumor specimens by a modified guanidium thiocyanate-phenol-chloroform extraction method[50]. First-strand cDNA was synthesized from the RNA (5 μg) with SuperScript II reverse transcriptase (Life Technologies) using an oligo-dT primer. Amplification for full-length *NF2* cDNA was done by two-step PCR using rTth DNA polymerase (Perkin Elmer, Norwalk, Connecticut), which has proofreading activity. The first PCR step used NF2-NST (5′-TGGCCCTGAG-GCCTGTGCAGCAA-3′) as a sense primer, and NF2-AS2 (5′-TCTACGCGGC-CGCAGTTCAAGGCAATTGCACATAA-3′) as an antisense primer, and comprised 30 cycles; each cycle consisted of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 2 min. The first PCR product was used as a template in the second step, in which T7PS1-NF2 (5′-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGGGCTCAGAGT-

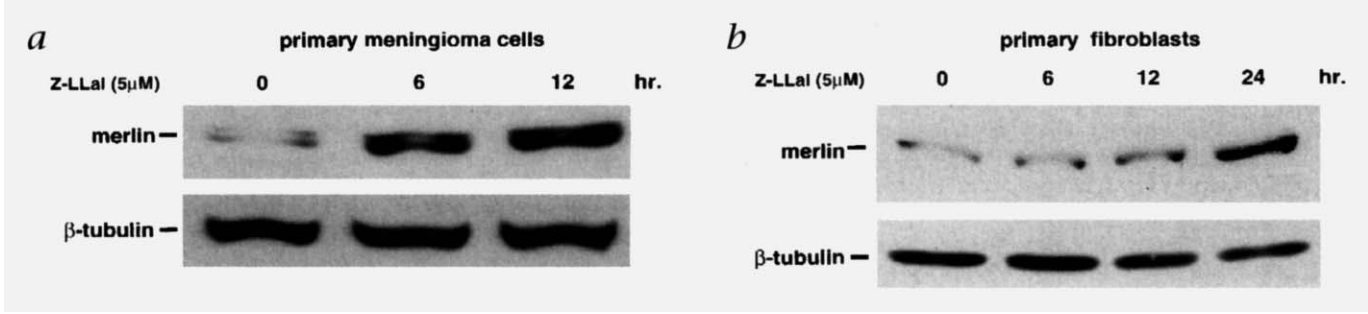

Fig. 7 Restoration of merlin in primary meningioma cells with calpain inhibition. Primary cultured meningioma cells and control fibroblasts were incubated in the presence of Z-LLal (5 μM). Western blot analysis using C-18 showed a rapid increase in the amount of merlin at 6 hours after Z-LLal treatment of meningioma cells (***a***). In contrast, a slow increase in the amount of the protein was observed for as long as 24 hours in fibroblasts (***b***). The findings indicate that calpain-mediated degradation contributes to the absence of merlin in meningioma cells.

GCAGGCCGTGGGGCGCGA-3′) (the T7 promoter sequence followed by an 8-base translation initiation signal is underlined) and NF2-HA-AS (5′-GGGTGGTACCTAGAGCTCTTCAAAGAAGG-3′) (the *KpnI* site is underlined) were used as primers to amplify the full-length *NF2*. The second PCR comprised 35 cycles with the same conditions as the first PCR.

***In vitro* transcription and translation.** The fragment of *NF2* cDNA with the T7 promoter (amplified by PCR as described above) was added to an RNA transcription mixture containing 0.5 mM of each NTP, 10 mM DTT, 10 units T7 RNA polymerase, and 10 units RNase inhibitor (Ambion, Austin, Texas). The samples were incubated at 37 °C for 1 hour, and then with 1 unit RNase-free DNase for an additional 20 min. The transcription product was added to the translation mixture which contained 100 mM potassium acetate, 500 mM magnesium acetate, 40% rabbit reticulocyte lysate and 9 μCi of [^{35}S] methionine (Amersham). The samples were incubated at 30 °C for 90 min, and were used for *in vitro* proteolysis assay. For the protein truncation test, the translated samples were diluted in SDS sample buffer (2% SDS, 10% glycerol, 0.1 M DTT, 120 mM Tris-HCl, pH 6.8, 0.02% bromophenol blue), boiled for 5 min and resolved by 8% SDS–PAGE. The gel was dried and subjected to autoradiography.

Preparation of S-100 fractions from U-251MG cells. Collected U-251MG cells were washed once with ice-cold PBS and centrifuged at 1000*g* for 5 min at 4 °C . The cell pellet was suspended in 5 volumes of an ice-cold hypotonic lysis buffer (20 mM HEPES pH 7.5, 10 mM potassium acetate, 1.5 mM magnesium acetate) without detergent. After incubation of the samples on ice for 15 min, lysis was completed using a Dounce homogenizer. The nuclei were removed by centrifugation at 2,000*g* for 5 min at 4 °C, and the supernatants were centrifuged at 10,000*g* for 1 hour. The resulting cytosolic cell extracts (S-100 fractions) were stored at –80 °C and used for *in vitro* proteolysis assay.

***In vitro* proteolysis assay.** The [^{35}S] methionine-labeled merlin, recombinant GST–merlin deletion mutants, and cellular merlin in the S-100 fraction from U-251MG cells were suspended in a proteolysis buffer (20 mM Tris-HCl pH 7.4, 10 mM 2-mercaptoethanol) on ice, and then incubated with purified m- or μ-calpain at various concentrations of $CaCl_2$, at 30 °C for 30 min. After SDS–PAGE, the gel was subjected to either autoradiography, western blot with C-18, or silver staining.

RT-PCR. The cDNA was amplified with primers specific for either *NF2* or *GAPDH* transcripts: for *NF2*, NF2-RTS1 (5′-CAAGTTTAACTCCTCAAAGCTTC-3′) and NF2-RTAS4 (5′-CGGCTTCCAGCACCTTCTGC-3′); for *GAPDH*, GAPDH-S (5′-AAGGCTGGGGCTCATTT-3′) and GAPDH-AS (5′-CCGTATTCATTGTCATACCA-3′). Amplification conditions were 95 °C for 5 min, and then 30 cycles of 95 °C for 1 min, 60 °C–62 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 7 min. Each PCR was done using cDNA products synthesized from total RNA (100 ng). PCR products were resolved on a 1% agarose gel.

C-terminal amino acid sequence analysis. The sample was analyzed using the HP241 C terminal Protein Sequence System (Hewlett-Packard)[29,30]. Detection and calibration of each amino residue was based on the standard of thiohydantoin-amino acid standards. The recovery of first three cycle residues of the sample was more than 10% of the total amount of sample applied to the Teflon membrane (100 pmol).

Immunocytochemistry. Primary cultured meningioma cells and malignant glioma cell lines were grown on glass slides, rinsed with PBS, and fixed in 4% paraformaldehyde in PBS for 30 min. After being washed with PBS, the cells were pretreated with 0.3% hydrogen peroxide in methanol for 1 hour and blocked with normal goat serum diluted 1:50 in PBS for 30 min. Then they were incubated with antibody against activated μ-calpain (1:100 dilution) in PBS containing 2% normal goat serum at 4 °C overnight. After being washed with PBS, the cells were incubated for 30 min with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, California) at a dilution of 1:200 in PBS containing 2% normal goat serum and treated with avidin-conjugated horseradish peroxidase for 30 min, with rinses in PBS between each step. The peroxidase substrate, 3,3′-diaminobenzine tetrahydrochloride-hydrogen peroxide (DAB) solution was used for 5 min at room temperature. After excess DAB was washed away, the glass slide was counterstained in hematoxylin, and mounted with Crystal/Mount (Biomeda; Foster City, California).

Immunohistochemistry. Frozen sections (6 μm in thickness) were fixed in 10% buffered formalin for 15 min. Immunochemical analysis was done using the Vecstatin ABC kit (Vector Laboratories, Burlingame, California). After nonspecific staining was blocked using normal goat serum, the samples were treated with antibody against activated μ-calpain (1:100 dilution) in PBS containing 2% normal goat serum for 1 hour. They were then washed with PBS and incubated with biotinylated goat anti-rabbit IgG (1:200 dilution) in PBS containing 2% normal goat serum for 30 min. After being treated with avidin-conjugated horseradish peroxidase for 30 min, samples were incubated in the DAB solution for 5 min at room temperature. The sections were also counterstained with hematoxylin and mounted with Crystal/Mount (Biomeda; Foster City, California).

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