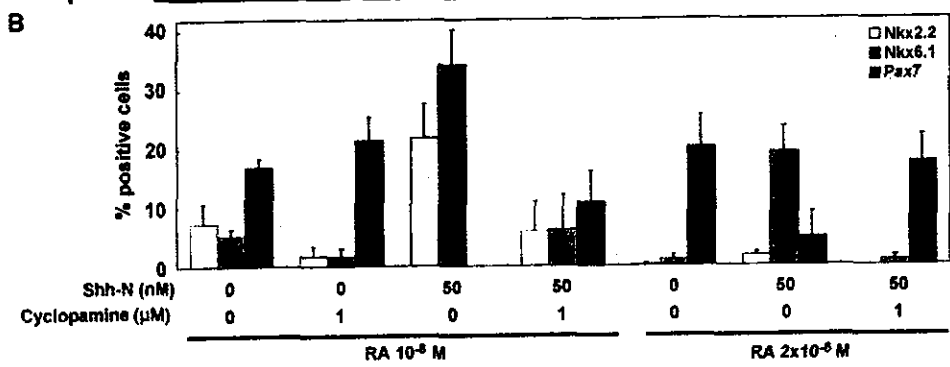
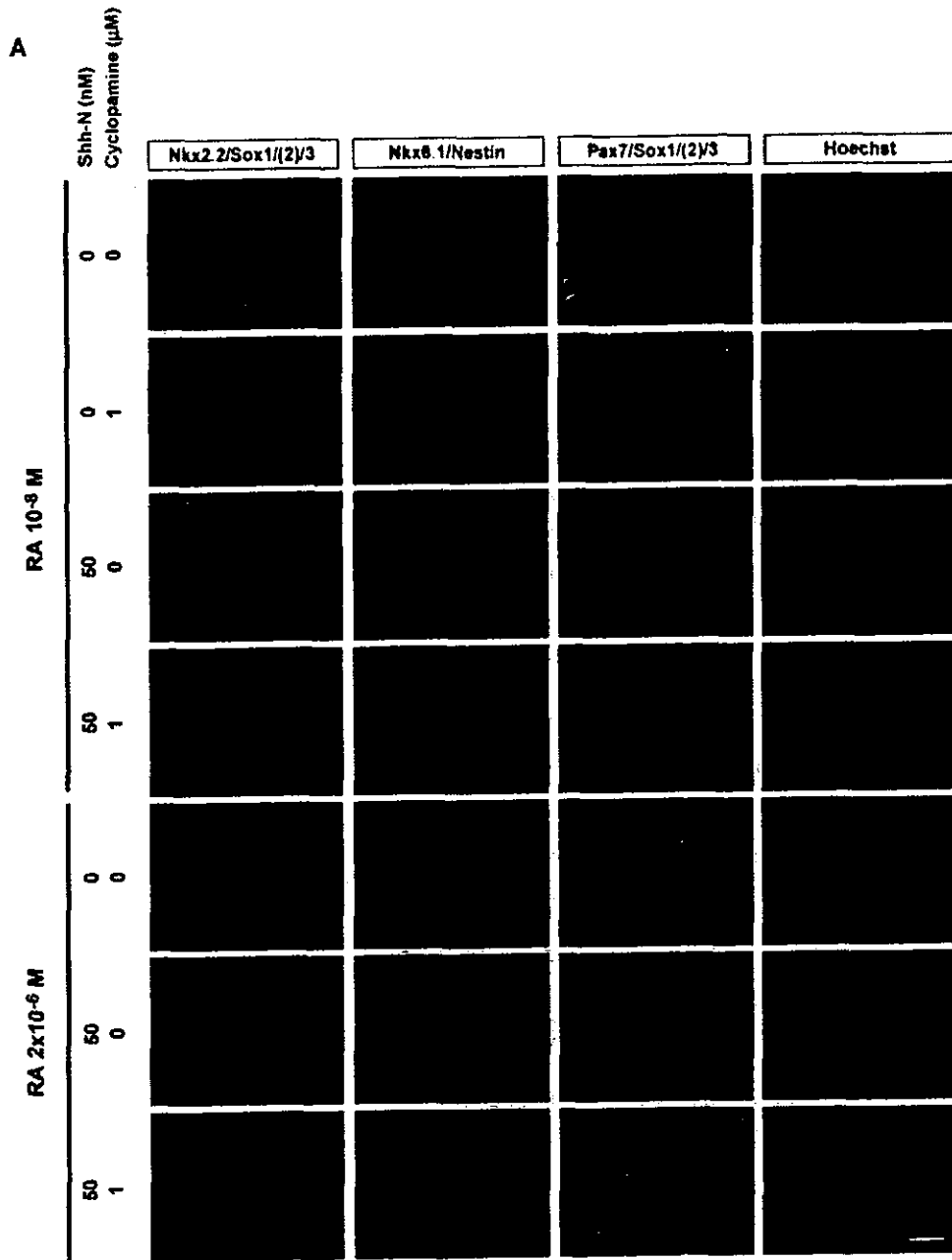


Fig. 7. Shh-N mediates RA-dependent dorso-ventral specification of ES-cell-derived neural progenitors. (A) Expression of Shh and its active N-terminal truncated form, Shh-N, in EBs cultured for 6 days, was analyzed by Western blotting. EBs were exposed to various concentrations of RA. Quantitative analysis was performed with Scion Image. The amounts of proteins were normalized to those of α-tubulin (B) ($n = 3$, mean \pm SEM, *, $P < 0.05$ vs. control. †, $P < 0.05$ vs. RA 2×10^{-6} M). (C) RT-PCR analysis of *shh* and *bmp4*. Shh-N was more highly expressed in EBs treated with low-RA (10^{-9} M– 10^{-8} M). (D) RT-PCR analysis of RA-exposed EBs treated with Shh-N and its inhibitor cyclopamine. Shh-N and cyclopamine were added together with RA on day 2. (E) Summary of expression patterns in vitro corresponding to in vivo. Cells from low-RA-treated EBs were a mixed population of dorsal-to-ventral neural progenitors and were capable of being dorsalized by inhibiting Shh signaling with cyclopamine. By contrast, exogenous Shh-N induced ventral neural progenitors were capable of being dorsalized by cyclopamine. Cells from high-RA-treated EBs showed dorsal positional identities. However, addition of Shh-N increased the number of ventral neural progenitors, and they were also capable of being dorsalized by cyclopamine treatment.

Fig. 8. Dorso-ventral specification of RA treated EBs is altered by Shh-N and cyclopamine. The alteration of dorso-ventral identity by RA, Shh-N, and cyclopamine was confirmed by the immunostaining of dissociated EBs with antibodies against Pax7 as a marker for dorsal neural progenitors, against Nkx6.1 as a marker for ventral neural progenitors, and against Nkx2.2 as a marker for ventralmost neural progenitors in combination with Group B1 Sox or Nestin as a marker for neural progenitors. Immunoreactive cells as a percentage of the total number of cells counted on the basis of the nuclear staining with hoechst33342 are shown in B ($n = 3$, mean \pm SEM). Scale bar: 50 μm.



number of Nkx6.1- or Nkx2.2-positive ventral neural progenitors in EBs treated with low-RA. The observation in EBs treated with high-RA is consistent with the previous report (Wichterle et al., 2002).

One of the major dorsolateralizing molecules in the CNS, *bmp4* (Casparly and Anderson, 2003; Jessell, 2000; Knecht and Bronner-Fraser, 2002), was not very strongly affected by RA, Shh-N, or cyclopamine, suggesting a lesser contribution of the BMP signal to this RA-mediated dorso-ventral specification (Fig. 7C, data not shown). These results indicate that differentiating EBs were ventralized by low-RA through induction of endogenous Shh-N protein, and that the effect was abrogated by cyclopamine and enhanced by the addition of exogenous Shh signal (Figs. 7D,E and 8).

Positional identity regulated by the RA concentration is mainly determined during the first 2 days of exposure to RA

According to the RT-PCR analysis of EBs cultured according to the 2-/4+ protocol, the expression patterns of most of the regionally specific markers were determined by day 4 and maintained unchanged thereafter. This observation raised two possibilities. One possibility is that the first 2 days of exposure to RA are critical to the determination of positional identity, and the second is that the effect of the RA concentration was altered during the later culture period by degradation of RA. To determine which of these possibilities was true, we performed a RT-PCR analysis of EBs cultured according to other protocols in which the times when RA is added or the duration of exposure to RA (2-/2+/2-, 2-/2+/2+, and 4-/4+ protocols) is different from the 2-/4+ protocol (Suppl. Fig. 1). Expression of *oct3/4* had been maintained before the addition of RA, but no expression of other markers except *otx2*, whose mRNA was expressed even in undifferentiated ES cells, had been detected in any of the culture protocols, including the 4-/4+ protocol. The expression patterns of most of regionally specific markers were determined by day 4 of the 2-/2+/2- and 2-/2+/2+ or by day 6 of the 4-/4+ protocol, and were virtually the same in all protocols as observed in the 2-/4+ protocol, and they were maintained thereafter as well (Suppl. Fig. 2). Overall, positional identity is determined during the first 2 days of exposure to RA and is maintained thereafter regardless of the presence or absence of RA in later culture periods.

Discussion

The pluripotent embryonic stem cell is a valuable in vitro model for studying the effects of various factors on cell lineage decisions in very early embryonic stages of mammalian development, and the effect of RA signaling on the differentiation of ES cells and neural induction, in particular, has been extensively studied. In addition to previous reports showing that RA promotes neural differ-

entiation of ES cells and caudalization of the positional identity of their progeny (Bain et al., 1995, 1996; Fraichard et al., 1995; Gajovic et al., 1997; Renoncourt et al., 1998; Strubing et al., 1995; Wichterle et al., 2002), the results of the present study demonstrate the novel and precise actions of RA on neural differentiation and acquisition of positional identity by ES-cell-derived neural cells.

Effect of the concentration of RA on ES cell differentiation

It is well known that exposure of growing EBs to high-RA markedly increases the rate of neural differentiation, whereas low-RA induces more mesodermal cells (Rohwedel et al., 1999). Higher concentrations of RA also promote faster differentiation of ES cells, as indicated by the pattern of *oct3/4* expression, which was down-regulated more rapidly in EBs exposed to higher concentrations of RA, and down-regulated more at day 6 than at day 4 (Fig. 2). The result of this study showed that RA also concentration-dependently facilitates terminal differentiation of neural cells derived from ES cells. The expression levels of markers of differentiated neurons and glia, i.e., of β III-tubulin and GFAP, respectively, was higher in EBs treated with higher concentrations of RA, whereas the expression levels of markers of undifferentiated neural cells, i.e., of Nestin, Group B1 Sox, Olig2, and *sox1* mRNA, was inversely correlated with the concentration of RA (Figs. 2 and 3). The findings are consistent with a high-RA enhancing differentiation of neural progenitor cells, as described previously (Bain et al., 1995, 1996; Fraichard et al., 1995; Gajovic et al., 1997; Renoncourt et al., 1998; Strubing et al., 1995; Wichterle et al., 2002).

Down-regulation of Wnt signaling has been shown to be one of the mechanisms involved in RA-induced neural differentiation of mouse ES cells (Aubert et al., 2002). Interestingly, β -catenin, which is a key molecule in Wnt signaling, has been shown to interact directly with retinoid receptor RAR, but not with RXR, in a retinoid-dependent manner, and as a result retinoids decrease β -catenin-Lef/Tcf-mediated transactivation in cultured cells in a dose-dependent manner (Easwaran et al., 1999). Wnt3a signaling through Lef/Tcf1 has also been implicated in suppression of neural differentiation and induction of mesodermal differentiation in the mouse embryo (Galceran et al., 1999; Yamaguchi et al., 1999; Yoshikawa et al., 1997). These findings raise the possibility that the one of the effects of RA in EBs is to inhibit the Wnt- β -catenin anti-neural pathway by up-regulation of Secreted frizzled-related protein 2 (*Sfrp2*) (Aubert et al., 2002) and/or sequestration of β -catenin in a concentration-dependent manner, thereby resulting in the promotion of neural differentiation, and inversely in the suppression of mesodermal differentiation.

FGFs are another molecules that may be involved in the neurogenesis related to RA signaling. RA has been shown to promote neuronal differentiation by repressing FGF signalings from the posterior neural plate. Caudal FGF signalings

have the opposite effects and repress *Raldh2* (RA synthesis) in the presomatic mesoderm and generic neuronal differentiation in chick early neural tube (Diez del Corral et al., 2003; Novitch et al., 2003). These observations raise the possibility that RA inhibits the action of endogenously generated FGFs in a concentration-dependent manner during the culture of EBs. Further study of the associations between these signals is required to clarify the mechanism underlying the RA-promoted neural differentiation of ES cells.

Acquisition of rostro-caudal identity depends on the concentration of RA

A previous study on chick embryos showed that the default identity of early neural tissue is a rostral location and that neural cells can be caudalized by exogenous factors, such as the caudalizing activity of paraxial mesoderm, FGFs, and retinoid from the mesoderm, which induce midbrain, hindbrain, and spinal cord characters, when applied during the appropriate period of development (Muhr et al., 1999).

RA is one of the factors, that has been shown to be involved in hindbrain patterning and the caudalization of neural tissues in the early embryonic CNS *in vivo* (Maden, 2002). The distribution of endogenous RA has been examined in mouse and chick embryos, by various methods, including HPLC (high-performance liquid chromatography) (Horton and Maden, 1995; Maden et al., 1998), the use of *LacZ* reporter cells (Maden et al., 1998; Wagner et al., 1992), and the use of *RAR β -LacZ* transgenic mice (Reynolds et al., 1991; Zimmer, 1992). This distribution of endogenous RA is correlated with the opposing action of the two main enzymes involved in RA-metabolism, RA-synthesizing enzyme, *Raldh2*, which is most strongly expressed in the paraxial mesoderm adjacent to the rostral spinal cord with the rostral boundary of the presumptive first somite (Berggren et al., 1999), and the catabolizing enzyme, *Cyp26a1*, which is expressed in anterior neuroepithelium. These spatially distributed enzymes create a rostro-caudal RA concentration gradient *in vivo* (Abu-Abed et al., 2001; Fujii et al., 1997; Maden et al., 1998; Sakai et al., 2001; Swindell et al., 1999), with the peak RA concentration occurring at the hindbrain/spinal cord boundary, with levels gradually decreasing anterior and posterior to it. Furthermore, it has been suggested that the patterning of the rhombomere is influenced over time by the constant supply of RA from the paraxial mesoderm, where the neuroepithelium grows and moves away from this source of RA. These findings imply that the more posterior rhombomeres that develop later than the more anterior rhombomeres may have been exposed to higher concentration of RA, leading to the expression of more posterior genes (such as posterior *hox* genes), which require a higher concentration of RA for activation *in vivo* (Maden, 2002). Our findings are consistent with the above-described putative regulatory mechanism of hindbrain/rostral spinal cord positional

specification correlated with the RA concentration gradient *in vivo* in the following manner. The default positional identity of ES-cell-derived neural cells is specified as anteriormost forebrain, which was acquired in the control and Noggin-exposed EBs. EBs treated with low-RA were specified as midbrain to hindbrain, which is generated earlier and require lower concentrations of RA *in vivo*, whereas EBs treated with high-RA were specified as posterior hindbrain to rostral spinal cord, which is generated later and requires higher concentrations of RA *in vivo*. In addition, the fact that even the EBs treated with high-RA expressed genes specific to rostral (*hoxc4* to *hoxc6*), but not to caudal spinal cord (*hoxc8* to *hoxc10*) is consistent with the putative gradient of endogenous RA *in vivo* with a higher concentration in the rostral spinal cord, and the proposed role of RA in rostral spinal cord determination (Liu et al., 2001). Moreover, other factors may be involved in the activation of RA-responsive genes and the specification of positional identity, such as RA binding proteins, including cellular retinoic acid binding protein (CRABP) 1, which limits the access of RA to the nuclear retinoid receptors. The spatiotemporal pattern of expression of CRABP1 suggests that the fine regional control of availability of RA to the nuclear receptors may also play an important role in the organization of the central nervous system and the differentiation of its progenitors *in vivo* (Leonard et al., 1995; Maden, 2001; Maden et al., 1992; Ruberte et al., 1993). The role of these RA binding proteins in the regulation of *in vitro* differentiation of ES-cell-derived neural cells should be investigated further in the future.

RA also affects dorso-ventral positional identity

In contrast to the acquisition of rostro-caudal identity, dorso-ventral identity was analyzed in terms of expression of the transcriptional control of the homeodomain (HD) and basic helix-loop-helix (bHLH) proteins. Previous studies have emphasized the role of *Shh* signaling in establishing the pattern of expression of ventral spinal cord patterning genes (Jessell, 2000). RA has also been reported to contribute to the ventral patterning of the spinal cord; that is, to the induction of ventral interneurons (V0 and V1) by inducing class I genes, including *Dbx1*, *Dbx2*, *Evx1*, *Evx2*, and *En* (Pierani et al., 1999), and to the specification of limb level motor neuron subtypes by the expression of *Raldh2* in LMC (Sockanathan and Jessell, 1998). Furthermore, recent studies have revealed involvement of RA from the paraxial mesoderm in the timing of neurogenesis and the patterning of the ventral spinal cord regulating the expression of class I and class II genes via inhibition of FGF signals and in combination with *Shh* signals (Diez del Corral et al., 2003; Novitch et al., 2003). However, the results of our study showed that the concentration of RA to which EBs were exposed was critical for acquisition of dorso-ventral identity by differentiating ES cells, and the concentration dependency showed a bell-

shaped pattern. This was shown by the pattern of the expression of class I and class II genes (Figs. 6A,B), which determines the dorso-ventral progenitor domains of developing hindbrain and spinal cord. EBs exposed to high-RA exhibit mainly dorsal phenotypes, whereas EBs exposed to low-RA exhibit more ventral phenotypes (Figs. 6–8). The expression pattern of *olig2*, higher at day 4 in EBs treated with high-RA and at day 6 in those treated with low-RA, seems to conflict with this finding; however, there are several possible explanations. One is that this alteration of the expression pattern of *olig2* mimics that in vivo according to the stage of development, with expression in most of the undifferentiated neural/glia progenitor cells in the ventral half of the spinal cord occurring around the period of neural tube closure and later being restricted to the motor neuron domain (pMN domain) of the ventral ventricular region, where the progenitors of motor neurons and oligodendrocytes arise sequentially (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2001). Thus, both the cells collected at day 4 from EBs exposed to high-RA and those collected at day 6 from EBs exposed to low-RA may consist of multipotent neural progenitors expressing *Olig2*. Furthermore, the role of RA in motor neuron development, such as its effect on the expression of bHLH and HD transcription factors, including *Olig2*, varies with the stage of development, according to a previous study that analyzed chick spinal cord development (Novitsch et al., 2003). Similar alteration of the effects of RA may occur in our culture system and be another possible explanation for the sequential expression pattern of *olig2* in EBs exposed to high-RA.

The ventralization of EBs treated with low-RA can be explained by the finding that the active form of Shh-N, which is secreted by the notochord and floor plate of the developing CNS and ventralizes gene expression of neural progenitors in a concentration-dependent manner in vivo (Jessell, 2000), is more highly expressed on day 6 in EBs treated with low-RA (Figs. 7A–C). The hypothesis that the concentration-dependent activity of RA that defines dorso-ventral identity is mediated by Shh-N was confirmed by the result of treatment with the inhibitor of Shh signaling, cyclopamine (Figs. 7D,E and 8) (Chen et al., 2002a,b; Incardona et al., 1998). Cyclopamine abrogated the ventralization activity of low-RA treatment, and addition of exogenous Shh-N more efficiently ventralized differentiating EB-derived cells that had been exposed to both low-RA and high-RA, in a cyclopamine-sensitive manner. The expression level of *bmp4*, which dorsalizes neural progenitor cells (Caspari and Anderson, 2003; Jessell, 2000; Knecht and Bronner-Fraser, 2002), was not very strongly affected by Shh-N or cyclopamine in EBs exposed to either low-RA or high-RA (Fig 7C, data not shown), indicating that BMP signaling is not the major contributor to RA-mediated dorso-ventral specification. Taken together, these findings suggest that Shh-N expressed in EBs exposed to low-RA may be one of the major signals that ventralize neural progenitors and induce

expression of class II genes in addition to class I genes, and that the lack of the shh signal in EBs exposed to high-RA may result in expression of only class I genes and a more dorsalized phenotype, which can be ventralized by exogenous Shh-N protein. The results of this study are consistent with a previous report that RA enhances expression of class I genes, but not of class II genes, in developing chick spinal cord (Diez del Corral et al., 2003; Novitsch et al., 2003), and that exogenous Shh-N is required in addition to high-RA for efficient generation of motor neurons during EB formation in vitro (Renoncourt et al., 1998; Wichterle et al., 2002). Because EBs exposed to low-RA are mixed populations and contain many mesodermal cells (Fig. 2) (Rohwedel et al., 1999), they may secrete larger amounts of Shh-N than EBs treated with high-RA, which contain smaller proportions of mesodermal cells.

The discrepancy in response to RA between full-length Shh expression and Shh-N expressions detected by Western blotting (Figs. 7A,B) may be another important finding in this study. In contrast to the expression of full-length Shh being observed in EBs treated with RA at concentration 10^{-8} M and above, generation of Shh-N was detected only in EBs exposed to lower concentrations of RA (Figs. 7A,B), indicating the possible existence of RA-dependent machinery controlling Shh-N production by modulating an auto-processing mechanism by the C-terminus of Shh, which processes full-length Shh into the N-terminus active form, or by altering degradation activity of Shh-N.

Use of mutant ES cells for *indian hedgehog (ihh)* and *smoothed (smo)* has shown that hedgehog signaling is also required for neural differentiation of mouse ES cells by RA (Maye et al., 2004). In our study, however, expression of Group B1 Sox and *sox1* mRNA in EBs treated with low- or high-RA and their dissociates were not down-regulated by cyclopamine (Fig. 8, data not shown), indicating that neural differentiation was not inhibited under our culture conditions even in the presence of cyclopamine. There are two possible explanations for this discrepancy. In our experiments, cyclopamine was added on day 2 after the start of ES cells differentiation, whereas in the mutant ES cells in which hedgehog signaling was disrupted it was disrupted at the start of differentiation, raising the possibility that hedgehog signaling may be one of the factors that is required for the initial commitment of neuroectodermal differentiation. The other possibility is that the concentration of cyclopamine used in our study may not have been adequate to completely block hedgehog signaling, and the residual signaling activity may have been sufficient for the transition of ES-cell-derived ectoderm into neuroectoderm, but not for the ventralization of neural cells.

The mechanism underlying these roles of hedgehog signals in differentiation and specification of ES-cell-derived neural cells needs to be elucidated in the future.

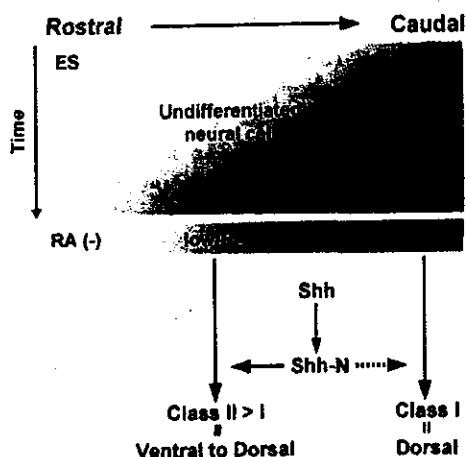


Fig 9. Schematic presentation of the concentration-dependent effects of RA on neural differentiation by mouse ES cells. RA simultaneously promotes both neural differentiation and caudalization in concentration-dependent manner. Low-RA induces a higher level of Shh-N, which endows ES-cell-derived neural progenitors with ventral identity, whereas high-RA poorly induces Shh-N, and they acquire dorsal neural identity instead.

RA is one of the most important inductive signals in vertebrate ontogeny and can be used to induce neural differentiation of mouse ES cells *in vitro*. However, its actions are complicated and difficult to deal with at will, because RA has the ability to induce various types of cells depending on its concentration, and it simultaneously affects both the timing of differentiation and the acquisition of positional identity, including rostro-caudal and dorso-ventral identity during neural differentiation (Fig. 9). Separation of these two phenomena is desirable to investigate the underlying mechanisms, and separation may have been accomplished, in part, by using SDIA, which is a culture protocol that induces neural cells without RA treatment. Thus, previous studies have shown involvement of RA at a single concentration in the caudalization of ES-cell-derived neural cells (Mizuseki et al., 2003; Wichterle et al., 2002). However, it is still not easy to separate these two phenomena completely during the neural induction of ES cells, because they are simultaneously affected by RA *in vivo* in combination with other signals, such as FGF and Shh signals, as shown by previous studies (Appel and Eisen, 2003; Diez del Corral et al., 2003; Novitch et al., 2003).

The present study identified detailed gene expression profiles and clarified the effects of the concentration of RA on ES cell differentiation, neuralization, and positional specification, though it may be impossible to map the patterns of expressions of the regional specific markers observed in ES-cell-derived neural cells directly to parallel expression of the markers *in vivo*. In combination with the RA-independent neural induction method using Noggin, this information will enable us to establish a strategy that will allow control of both the differentiation and the positional identity of neural cells

derived from mouse ES cells through EB formation *in vitro*, and it may be applicable to human ES cells, raising the possibility of application to the treatment of neurological diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.07.038.

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Physical and Functional Interaction between Dorfin and Valosin-containing Protein That Are Colocalized in Ubiquitylated Inclusions in Neurodegenerative Disorders*

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Dorfin, a RING-IBR type ubiquitin ligase (E3), can ubiquitylate mutant superoxide dismutase 1, the causative gene of familial amyotrophic lateral sclerosis (ALS). Dorfin is located in ubiquitylated inclusions (UBIs) in various neurodegenerative disorders, such as ALS and Parkinson's disease (PD). Here we report that Valosin-containing protein (VCP) directly binds to Dorfin and that VCP ATPase activity profoundly contributes to the E3 activity of Dorfin. High through-put analysis using mass spectrometry identified VCP as a candidate of Dorfin-associated protein. Glycerol gradient centrifugation analysis showed that endogenous Dorfin consisted of a 400–600-kDa complex and was co-immunoprecipitated with endogenous VCP. *In vitro* experiments showed that Dorfin interacted directly with VCP through its C-terminal region. These two proteins were colocalized in aggresomes in HEK293 cells and UBIs in the affected neurons of ALS and PD. VCP^{R524A}, a dominant negative form of VCP, reduced the E3 activity of Dorfin against mutant superoxide dismutase 1, whereas it had no effect on the autoubiquitylation of Parkin. Our results indicate that VCPs functionally regulate Dorfin through direct interaction and that their functional interplay may be related to the process of UBI formation in neurodegenerative disorders, such as ALS or PD.

motor neuron degeneration in the spinal cord, brain stem, and cortex. Two genes, CuZn-superoxide dismutase (SOD1) and amyotrophic lateral sclerosis 2 have been identified as responsible genes for familial forms of ALS. Using mutant SOD1 transgenic mice, the pathogenesis of ALS has been partially uncovered. The proposed mechanisms of the motor neuron degeneration in ALS include oxidative toxicity, glutamate receptor abnormality, ubiquitin proteasome dysfunction, inflammatory and cytokine activation, dysfunction of neurotrophic factors, damage to mitochondria, cytoskeletal abnormalities, and activation of the apoptosis pathway (1, 2).

In a previous study (3), we identified several ALS-associated genes using molecular indexing. Dorfin was identified as one of the up-regulated genes in ALS, which contains a RING-IBR (in between ring finger) domain at its N terminus and mediated ubiquitin ligase (E3) activity (3, 4). Dorfin colocalized with Vimentin at the centrosome after treatment with a proteasome inhibitor in cultured cells (4). Dorfin physically bound and ubiquitylated various SOD1 mutants derived from familial ALS patients and enhanced their degradation, but it had no effect on the stability of wild-type SOD1 (5). Overexpression of Dorfin protected neural cells against the toxic effects of mutant SOD1 and reduced SOD1 inclusions (5).

Recent findings indicate that the ubiquitin-proteasome system is widely involved in the pathogenesis of Parkinson's disease (PD), Alzheimer's disease, polyglutamine disease, and Prion diseases as well as ALS (6). From this point of view, we previously analyzed the pathological features of Dorfin in various neurodegenerative diseases and found that Dorfin was predominantly localized not only in Lewy body (LB)-like inclusions in ALS but also in LBs in PD, dementia with Lewy bodies, and glial cell inclusions in multiple system atrophy (7). These characteristic intracellular inclusions composed of aggregated, ubiquitylated proteins surrounded by disorganized filaments are the histopathological hallmark of aging-related neurodegenerative diseases (8).

A structure called aggresome by Johnston *et al.* (9) is formed when the cell capacity to degrade misfolded proteins is exceeded. The aggresome has been defined as a pericentriolar, membrane-free, cytoplasmic inclusion containing misfolded ubiquitylated protein ensheathed in a cage of intermediate filaments, such as Vimentin (9). The formation of the aggresome mimics that of ubiquitylated inclusions (UBIs) in the affected neurons of various neurodegenerative diseases (10). Combined with the fact that Dorfin was localized in aggresomes in cultured cells and UBIs in ALS and other neurode-

Amyotrophic lateral sclerosis (ALS)¹ is one of the most common neurodegenerative disorders, characterized by selective

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¹ The abbreviations used are: ALS, amyotrophic lateral sclerosis; E3, ubiquitin ligase; ERAD, endoplasmic reticulum-associated degradation; LB, Lewy body; MS, mass spectrometry; LC-MS/MS, liquid chromatography coupled to electrospray tandem mass spectrometry; PD, Parkinson's disease; SOD1, CuZn-superoxide dismutase; UBI, ubiquitylated inclusions; VCP, valosin-containing protein; FLAG-Parkin, pcDNA3.1/FLAG-Parkin; Ub, ubiquitin; MBP, maltose-binding protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; HA, hemagglutinin; WT, wild type.

generative diseases, these observations suggest that Dorfin may have a significant role in the quality control system in the cell. The present study was designed to obtain further clues for the pathophysiological roles of Dorfin. For this purpose, we screened Dorfin-associated proteins using high performance liquid chromatography coupled to electrospray tandem mass spectrometry (LC-MS/MS). The results showed that Valosin-containing protein (VCP), also called p97 or Cdc48 homologue, obtained from the screening, physically and functionally interacted with Dorfin. Furthermore, both Dorfin and VCP proteins colocalized in aggresomes of the cultured cells and in UBIs in various neurodegenerative diseases.

MATERIALS AND METHODS

Plasmids and Antibodies—pCMV2/FLAG-Dorfin vector (FLAG-Dorfin^{WT}) was prepared by PCR using the appropriate design of PCR primers with restriction sites (ClaI and KpnI). The PCR product was digested and inserted into the ClaI-KpnI site in pCMV2 vector (Sigma). pEGFP-Dorfin (GFP-Dorfin), pCMX-VCP^{WT} (VCP^{WT}), and pCMX-VCP^{K524A} (VCP^{K524A}) vectors were described previously (5, 11). pcDNA/HA-VCP^{WT} (HA-VCP^{WT}) and pcDNA/HA-VCP^{K524A} (HA-VCP^{K524A}) were subcloned from pCMX-VCP^{WT} and pCMX-VCP^{K524A}, respectively, into pcDNA3.1 vectors (Invitrogen). The HA tag was introduced at the N terminus of VCP. pcDNA3.1/FLAG-Parkin (FLAG-Parkin) was generated by PCR using the appropriate design of PCR primers with restriction sites (EcoRI and NotI) from pcDNA3.1/Myc-Parkin (12). The FLAG tag was introduced at the N terminus of Parkin. To establish the RING mutant plasmid of Dorfin (FLAG-Dorfin^{C132S/C135S}), point mutations for Cys at positions 132 and 135 to Ser were generated by PCR-based site-directed mutagenesis using a QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). pcDNA3.1/HA-Ub (HA-Ub), pcDNA3.1/Myc-SOD1^{WT} (SOD1^{WT}-Myc), pcDNA3.1/Myc-SOD1^{G93A} (SOD1^{G93A}-Myc), and pcDNA3.1/Myc-SOD1^{G85R} (SOD1^{G85R}-Myc) were described previously (13, 14). Polyclonal anti-Dorfin (Dorfin-30 and Dorfin-41) and monoclonal anti-VCP antibodies were used as in previous reports (5, 15). The following antibodies were used in this study: monoclonal anti-FLAG antibody (M2; Sigma), monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-HA antibody (12CA5; Roche Applied Science), polyclonal anti-maltose-binding protein (MBP) antibody (New England Biolabs, Beverly, MA), polyclonal anti-Parkin (Cell Signaling, Beverly, MA), and polyclonal anti-SOD1 (SOD-100; Stressgen, San Diego, CA).

Cell Culture and Transfection—All media and reagents for cell culture were purchased from Invitrogen. HEK293 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5 units/ml penicillin, and 50 µg/ml streptomycin. HEK293 cells at subconfluence were transfected with the indicated plasmids using FUGENE6 reagent (Roche Applied Science). To inhibit cellular proteasome activity, cells were treated with 1 µM MG132 (benzoyloxycarbonyl-Leu-Leu-Leu-al; Sigma) for 16 h after overnight post-transfection. Cells were analyzed at 24–48 h after transfection.

Protein Identification by LC-MS/MS Analysis—FLAG-Dorfin^{WT} was expressed in HEK293 cells (semiconfluent in a 10-cm dish) and then immunoprecipitated by anti-FLAG antibody. The immunoprecipitates were eluted with a FLAG peptide and then digested with Lys-C endopeptidase (*Achromobacter* protease I). The resulting peptides were analyzed using a nanoscale LC-MS/MS system as described previously (16). The peptide mixture was applied to a Mightysil-PR-18 (1-µm particle, Kanto Chemical Corp., Tokyo) column (45 × 0.150 mm ID) and separated using a 0–40% gradient of acetonitrile containing 0.1% formic acid over 30 min at a flow rate of 50 nL/min. Eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-ToF Ultima; Micromass, Manchester, UK). MS and MS/MS spectra were obtained in data-dependent mode. Up to four precursor ions above an intensity threshold of 10 cps were selected for MS/MS analysis from each survey scan. All MS/MS spectra were searched against protein sequences of Swiss Prot and RefSeq (NCBI) using batch processes of the Mascot software package (Matrix Science, London, UK). The criteria for match acceptance were the following: 1) when the match score was 10 over each threshold, identification was accepted without further consideration; 2) when the difference of score and threshold was lower than 10 or when proteins were identified based on a single matched MS/MS spectrum, we manually confirmed the raw data prior to acceptance; 3) peptides assigned by less than three y series ions and peptides with +4 charge state were all eliminated regardless of their scores.

Recombinant Proteins and Pull-down Assay—We used pMALp2 (New England Biolabs) and pMALp2T (Factor Xa cleavage site of pMALp2 was replaced with a thrombin recognition site) to express fusion proteins with MBP. To produce the full-length (residues 1–838) Dorfin (MBP-Dorfin^{full}), N-terminal (residues 1–367) Dorfin (MBP-Dorfin^N), and C-terminal (residues 368–838) Dorfin (MBP-Dorfin^C), the PCR fragments were amplified from pcDNA4/HisMax-Dorfin (4) by using the appropriate PCR primers with restriction sites (FbaI and HindIII) and then ligated into pMAL-p2 vectors. To produce the MBP-Parkin protein, full-length PARKIN cDNA was inserted into the EcoRI-NotI sites of pMALp2T. All of the MBP-tagged recombinant proteins were purified from *Escherichia coli* BL21-codon-plus. The detail of the purification method of MBP-tagged proteins was described previously (17). Recombinant GST fusion VCP^{WT} and VCP^{K524A} proteins were also generated from *E. coli* lysate and purified with glutathione-Sepharose. Recombinant His-VCP^{WT} and His-VCP^{K524A} proteins were purified from insect cells using baculovirus. The detail of purification of these recombinant VCP proteins was described previously (15). Binding experiments were performed with proteins carrying different tags. His- or GST-VCP were mixed with MBP fusion proteins: MBP-Dorfin^{full}, -Dorfin^N, -Dorfin^C, -Parkin, and -mock. His-VCP and GST-VCP proteins were precipitated by Ni²⁺-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA), and glutathione-Sepharose (Amersham Biosciences), respectively. Binding was performed with 1–3 µg of each protein in 300 µl of binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 10% glycerol, 0.5 mg/ml bovine serum albumin, 1 mM dithiothreitol) for 1 h at 4 °C. Then 15 µl of beads were added and incubated for 30 min. The beads were washed by binding buffer three times and eluted with sample buffer and analyzed by SDS-PAGE followed by Western blotting using specific antibodies.

Glycerol Gradient Centrifugation—Cultured cells or mouse tissues were homogenized in 1 ml of PBS with protease inhibitor (Complete Mini; Roche Applied Science). Supernatants (1 mg of protein for cultured cells, 5 mg of protein for mouse tissues, and 0.1 mg of recombinant His-VCP protein) were used as the samples after 10,000 × g centrifugation for 20 min. The samples (1.0 ml) were loaded on top of a 34-ml linear gradient of glycerol (10–40%) prepared in 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol in 40 PA centrifuge tubes (Hitachi, Tokyo), and centrifuged at 4 °C and 80,000 × g for 22 h using a Himac CP100a centrifuge system (Hitachi). Thirty fractions were collected from the top of the tubes. Two hundred µl of each fraction was precipitated with acetone, and the remaining pellet was lysed with 50 µl of sample buffer and then used for SDS-PAGE followed by Western blotting.

Immunological Analysis—Cells (4 × 10⁶ in a 6-cm dish) were lysed with 500 µl of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, and 1 mM EDTA) with protease inhibitor mixture (Complete Mini) 24–48 h after transfection. The lysate was then centrifuged at 10,000 × g for 10 min at 4 °C to remove debris. A 10% volume of the supernatants was used as the "lysate" for SDS-PAGE. When immunoprecipitated, the supernatants were precleared with protein A-Sepharose (Amersham Biosciences), and specific antibodies, anti-FLAG (M2), anti-Myc (9E10), or anti-Dorfin (Dorfin-30) were then added and then incubated at 4 °C with rotation. Immune complexes were then incubated with protein A-Sepharose for 3 h, collected by centrifugation, and washed four times with the lysis buffer. For protein analysis, immune complexes were dissociated by heating in SDS-PAGE sample buffer and loaded onto SDS-PAGE. The samples were separated by SDS-PAGE (12% gel or 4–12% gradient gel) and transferred onto a polyvinylidene difluoride membrane. Finally, Western blotting was performed with specific antibodies.

Immunohistochemistry—HEK293 cells grown on glass coverslips were fixed in 4% paraformaldehyde in PBS for 15 min. Then the cells were blocked for 30 min with 5% (v/v) normal goat serum in PBS, incubated for 1 h at 37 °C with anti-HA antibody (12CA5), washed with PBS, and incubated for 30 min with Alexa 496-nm anti-mouse antibodies (Molecular Probes, Inc., Eugene, OR). The coverslips were washed and mounted on slides. Fluorescence images were obtained using a fluorescence microscope (DMIRE2; Leica, Bannockburn, IL) equipped with a cooled charge-coupled device camera (CTR MIC; Leica). Pictures were taken using Leica Qfluoro software.

Pathological Studies—Pathological studies were carried out on 10% formalin-fixed, paraffin-embedded spinal cords and brain stems filed in the Department of Neurology, Nagoya University Graduate School of Medicine. The specimens were obtained at autopsy from three sporadic cases of ALS and four sporadic PD patients. The spinal cord and brain stem specimens of these ALS and PD cases were immunohistochemically stained with antibodies against Dorfin (Dorfin-41) and VCP. Dou-

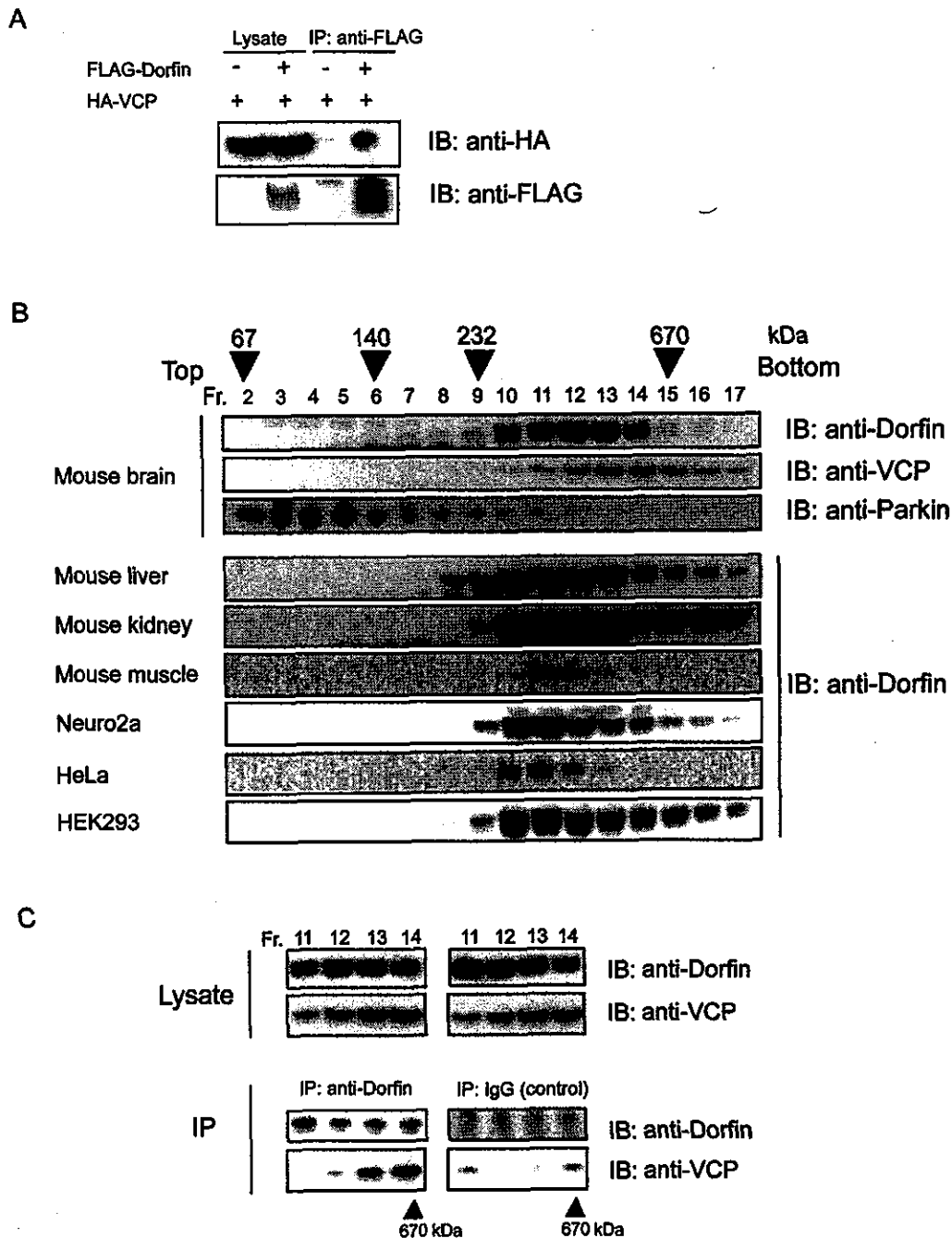


FIG. 1. *In vivo* interaction between Dorfin and VCP. A, FLAG-Dorfin and HA-VCP are co-expressed in HEK293 cells. FLAG-mock vector was used as a negative control. The amounts of HA-VCP in 10% of the lysate used are shown (*Lysate*); the rest was subjected to immunoprecipitation (*IP*) with anti-FLAG (M2) antibody. Following immunoblotting (*IB*) with anti-HA (12CA5) antibody revealed that HA-VCP was co-immunoprecipitated with FLAG-Dorfin. B, 5 mg of protein of various mouse tissues (brain, liver, kidney, and muscle) and 1 mg of protein of cultured cells (HEK293, HeLa, and Neuro2a) were each homogenized in 1 ml of PBS. Supernatants were fractionated by 10–40% glycerol gradient centrifugation followed by separation into 30 fractions using a fraction collector. Immunoblotting using anti-Dorfin, anti-VCP, and anti-Parkin antibodies was performed on the fractions (*Fr.*), including fractions 2–17. Endogenous Dorfin was co-sedimented with VCP in the fractions with a molecular mass of around 400–600 kDa. The positions of co-migrated molecular mass markers are indicated *above* the panels. C, immunoprecipitation with polyclonal anti-Dorfin antibody (anti-Dorfin-30) was performed on fractions 11–14 collected by glycerol gradient centrifugation analysis, where endogenous Dorfin was seen in B. As a negative control, immunoprecipitation with nonimmune rabbit IgG was used on the same fractions.

ble staining of identical sections was performed as described previously (7). In immunofluorescence microscopy, Alexa-488- and Alexa-546-conjugated secondary antibodies (Molecular Probes) were used. All human and animal studies described in this report were approved by the appropriate Ethics Review Committees of the Nagoya University Graduate School of Medicine.

RESULTS

Identification of Dorfin-associated Protein in the Cells—In an effort to identify protein(s) that physically interacts with Dor-

fin in the cells, FLAG-Dorfin was expressed in HEK293 cells and then immunoprecipitated by anti-FLAG antibody. The immunoprecipitates were eluted with a FLAG peptide and then digested with Lys-C endopeptidase (*Achromobacter protease I*), and the cleaved fragments were directly analyzed using a highly sensitive “direct nanoflow LC-MS/MS” system as described under “Materials and Methods.” Following data base search, a total of 13 peptides were assigned to MS/MS spectra obtained from the LC-MS/MS analyses for the FLAG-Dorfin-

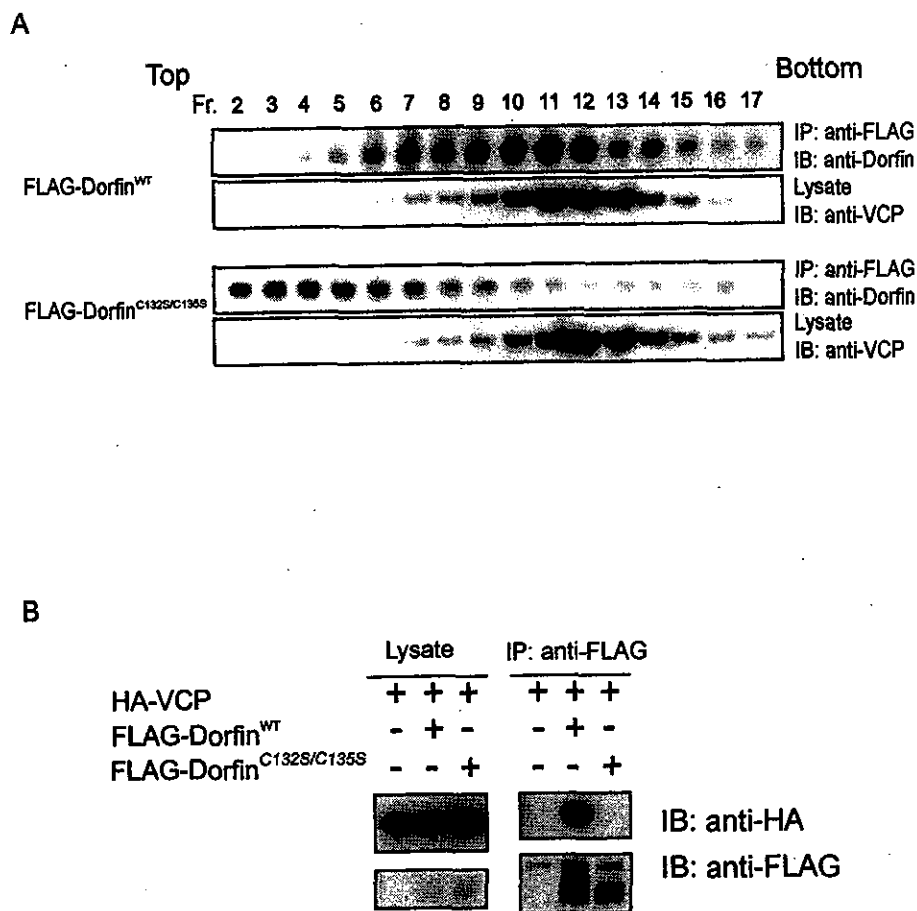


FIG. 2. Loss of physical interaction between Dorfin^{C132S/C135S} and VCP. A, transfected Dorfin^{WT}, but not Dorfin^{C132S/C135S} (*Dorfin^{C132S-C135S}*), forms a high M_r complex. Lysate of HEK293 cells overexpressed with FLAG-Dorfin^{WT} or FLAG-Dorfin^{C132S/C135S} was fractionated by 10–40% glycerol gradient centrifugation. The selected fractions (*Fr.*), fractions 2–17, were subjected to immunoprecipitation (*IP*) using anti-FLAG (M2) antibody. Immunoblotting (*IB*) with anti-Dorfin antibody revealed that exogenous FLAG-Dorfin^{WT} formed a high molecular weight complex, whose peak was at fraction 11, whereas FLAG-Dorfin^{C132S/C135S} migrated in fractions of smaller M_r (around fraction 7). Ten percent of the fractionated samples were shown as “lysate.” B, Dorfin^{WT} can interact with VCP, but Dorfin^{C132S/C135S} cannot. FLAG-Dorfin^{WT} or FLAG-Dorfin^{C132S/C135S} and HA-VCP were co-expressed in HEK293 cells. FLAG-mock vector was used as a negative control. The amounts of HA-VCP in 10% of the lysate used are shown (*Lysate*); the rest was subjected to immunoprecipitation with anti-FLAG (M2) antibody. Following immunoblotting with anti-HA (12CA5) antibody revealed that HA-VCP was co-immunoprecipitated with FLAG-Dorfin^{WT} but not with FLAG-Dorfin^{C132S/C135S}.

associated complexes. These peptide data identified nine proteins as candidates for Dorfin-associated proteins. One of these identified proteins was VCP that has been proposed to have multiple functions, such as membrane fusion or endoplasmic reticulum-associated degradation (ERAD) (18–22). In the next step, we examined the relationship between Dorfin and VCP, because the latter has been reported to be linked to various aspects of neurodegeneration (15).

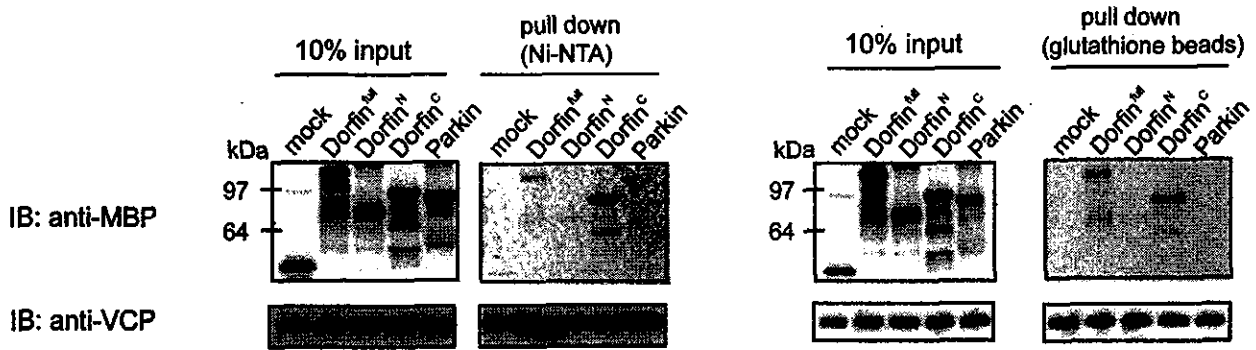
Dorfin Interacts with VCP in Vivo—To verify the interaction between Dorfin and VCP, FLAG-Dorfin and HA-VCP were transiently overexpressed in HEK 293 cells. Immunological analyses revealed that HA-VCP was co-immunoprecipitated with FLAG-Dorfin but not with FLAG-mock (Fig. 1A), confirming their physical interactions in the cells. To determine whether endogenous Dorfin forms a complex, the lysate from mouse brain homogenate was fractionated by glycerol density gradient centrifugation. Each fraction was immunoblotted with anti-Dorfin antibody. The majority of endogenous Dorfin was co-sedimented with VCP around a size of 400–600 kDa, although endogenous Parkin, which is another RING-IBR type E3 ligase (12), existed in the fractions of much lighter molecular weight (M_r) (Fig. 1B, *top panels*). Moreover, Dorfin was sedimented in the fractions of 400–600 kDa in other tissues, such as the liver, kidney, and muscle of mouse, and various

cultured cells including Neuro2a, HeLa, and HEK293 cells (Fig. 1B, *bottom panels*). To determine whether endogenous Dorfin interacts with VCP, immunoprecipitation using polyclonal anti-Dorfin antibody (Dorfin-30) was performed on the fractions shown in Fig. 1B, *top panels*. Endogenous VCP was co-immunoprecipitated with endogenous Dorfin in the fractions of high M_r (fractions (*Fr.*) 13 and 14). No apparent band was observed when precipitated with rabbit IgG (Fig. 1C).

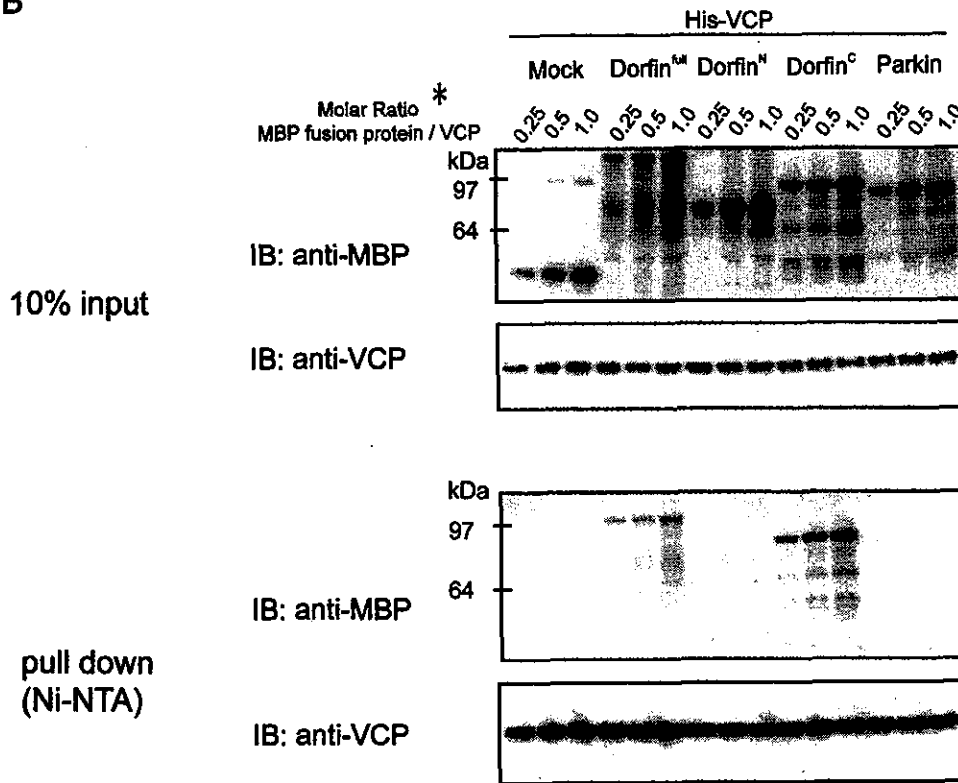
Mutations of RING Finger Domain of Dorfin Results in Loss of Dorfin-VCP Interactions—Next, we examined whether transfected Dorfin (FLAG-Dorfin^{WT}) and its RING mutant (FLAG-Dorfin^{C132S/C135S}), in which the two Cys residues at positions 132 and 135 within the RING finger domain were substituted for Ser residues, form a complex. The results showed overexpression of FLAG-Dorfin^{WT} in high molecular fractions (*Fr.* in Fig. 2), whose peak was between fractions 10 and 12, whereas overexpressed FLAG-Dorfin^{C132S/C135S} did not consist of high molecular weight complex. Overexpression of FLAG-Dorfin^{WT} or FLAG-Dorfin^{C132S/C135S} did not change the sedimentation pattern of VCP (Fig. 2A). Furthermore, immunoprecipitation analysis showed that FLAG-Dorfin^{WT}, but not FLAG-Dorfin^{C132S/C135S}, could interact with HA-VCP in HEK293 cells (Fig. 2B).

Dorfin Interacts with VCP in Vitro—To confirm the direct

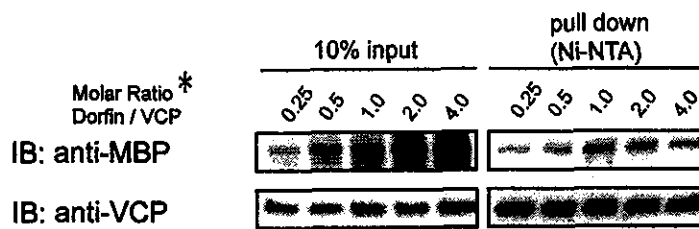
A



B



C



D

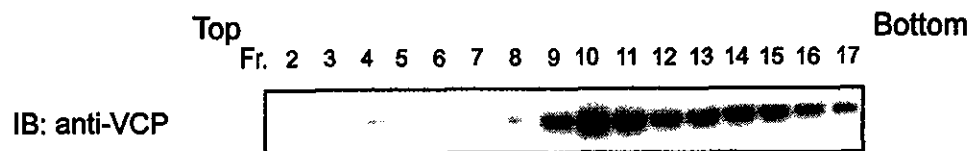


FIG. 3. *In vitro* interaction between Dorfin and VCP. A, recombinant His- or GST-VCP protein was incubated with MBP-mock, MBP-Dorfin^{full}, MBP-Dorfin^N, MBP-Dorfin^C, and MBP-Parkin proteins *in vitro*. Two μ g of His- or GST-VCP proteins and MBP fusion proteins at similar molar concentrations to VCP proteins were used for the assays. The amounts of MBP fusion and GST fusion Dorfin derivatives and His-VCP in 10% of the samples used are shown (10% input). NTA, nitrilotriacetic acid. IB, immunoblot. B, 2 μ g of His-VCP was incubated with MBP-mock,

binding between Dorfin and VCP and to determine the exact portion of Dorfin that interacts with VCP *in vitro*, we performed pull-down assays using recombinant proteins. Recombinant MBP-Dorfin or its deletion mutants (*i.e.* MBP-Dorfin^N and MBP-Dorfin^C) and the same molar of recombinant His-VCP or GST-VCP were mixed and incubated for 1 h at 4 °C. MBP-mock protein was used as a negative control in these experiments. A small portion of MBP-Dorfin^{full} or Dorfin^C (C-terminal substrate-recognizing domain) bound to both His-VCP and GST-VCP, whereas MBP-mock, MBP-Dorfin^N (N-terminal RING-IBR domain), and MBP-Parkin did not bind to His-VCP or GST-VCP (Fig. 3A). We next determined the number of Dorfins that bind one hexamer of VCP. To investigate this issue, we incubated His-VCP with increasing amounts of MBP-Dorfin^{full}, MBP-Dorfin^N, MBP-Dorfin^C, MBP-mock, or MBP-Parkin. As shown in Fig. 3B, the amount of binding portion of MBP-Dorfin^{full} and -Dorfin^C pulled down with His-VCP was not saturated below the even molar ratio. The pull-down experiments using excess amounts of MBP-Dorfin^{full} revealed that MBP-Dorfin^{full} was saturated at the even molar ratio (Fig. 3C). As reported previously (15), recombinant His-VCP sedimented in high molecular weight fractions, indicating that it formed a hexamer *in vitro* (Fig. 3D). These findings indicated that six Dorfin molecules were likely bind to a VCP complex *in vitro*.

Subcellular Localization of Dorfin and VCP in HEK293 Cells—In previous studies, we showed that exogenous and endogenous Dorfin resided perinuclearly and was colocalized with Vimentin in cultured cells treated with a proteasome inhibitor (4). The staining patterns of Dorfin were indistinguishable from those of the aggresome, namely a pericentriolar, membrane-free, cytoplasmic inclusion containing misfolded ubiquitylated proteins packed in a cage of intermediate filaments (4). VCP immunostaining was also observed throughout aggresomes in cultured neuronal cells when induced by treatment with a proteasome inhibitor (15). In order to examine the subcellular localization of Dorfin and VCP, GFP-Dorfin and HA-VCP were co-expressed in HEK293 cells. Without proteasome treatment, GFP-Dorfin-expressing cells showed granular fluorescence in the cytosol, and the HA-VCP-expressing cells showed diffuse and uniform cytoplasmic staining (Fig. 4A). Treatment with MG132 (1 μM, 16 h) resulted in accumulation of both GFP-Dorfin and HA-VCP and perinuclear colocalization as a clear large protein aggregate that mimics aggresomes (Fig. 4B).

Colocalization of Dorfin and VCP in the Affected Neurons of ALS and PD—In previous studies, immunostaining of Dorfin and VCP was independently noted in LBs of PD, and the peripheral staining pattern of both proteins in LBs was similar (7, 23). To confirm the immunoreactivities of Dorfin and VCP in the affected neurons in ALS and PD, we performed a double-labeling immunofluorescence study using a rabbit polyclonal anti-Dorfin antibody (Dorfin-41) and a mouse monoclonal VCP antibody on the postmortem samples of ALS and PD. In the ALS spinal cords, both proteins were colocalized in the LB-like inclusions (Fig. 5, A–F). The margin of LBs in PD was intensely immunostained for Dorfin and VCP, and merged images confirmed their strong colocalization (Fig. 5, G–L). Dorfin and VCP were also positive in Lewy neurites in the affected neurons of PD (Fig. 5, M–O).

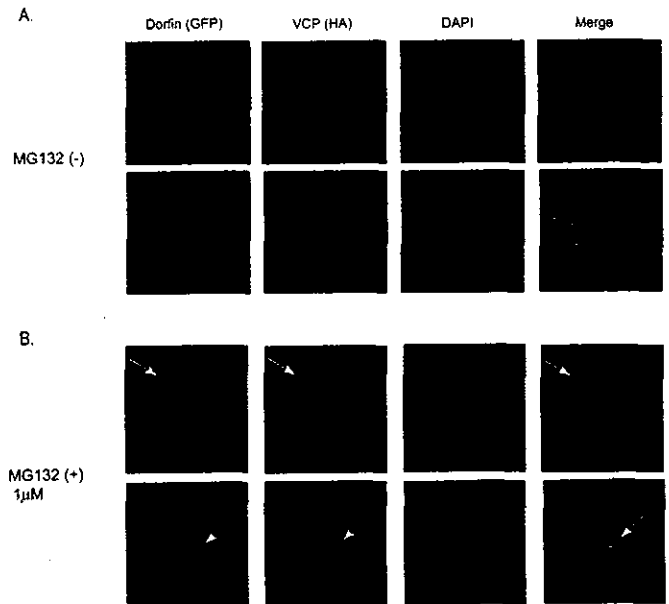


FIG. 4. Subcellular localization of GFP-Dorfin and HA-VCP in HEK293 cells treated or untreated with a proteasome inhibitor. GFP-Dorfin and HA-VCP were co-expressed transiently in HEK 293 cells. Cells were treated with (B) or without (A) 1 μM MG132 for 16 h. HA-VCP was stained with anti-monoclonal HA antibody (12CA5). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Without the treatment of MG132, GFP-Dorfin was spread through the cytosol, and it appeared like small aggregations. HA-VCP was also seen mainly in the cytosol and partly colocalized with GFP-Dorfin (A). After treatment with 1 μM MG132 for 16 h, both GFP-Dorfin and HA-VCP showed perinuclear accumulation and colocalization and appeared as clear large protein aggregates (B; arrows).

Dorfin Ubiquitylates Mutant SOD1 *In Vivo*—Unlike the wild-type form, mutant SOD1 proteins are rapidly degraded by the ubiquitin-proteasome system. Consistent with our previous results (5), SOD1^{G93A} and SOD1^{G85R} were polyubiquitylated, and co-expression with FLAG-Dorfin^{WT} enhanced polyubiquitylation of these mutant SOD1s compared with co-expression with FLAG-BAP, a negative control construct (Fig. 6A). Boiling with 1% SDS-containing buffer did not change the level of ubiquitylated mutant SOD1, indicating that mutant SOD1 itself was ubiquitylated by Dorfin (Fig. 6B). We also performed the same *in vivo* ubiquitylation assay using Neuro2a cells to examine for E3 activity of Dorfin in neuronal cells. The enhanced polyubiquitylation of these mutant SOD1s by Dorfin was observed in Neuro2a cells as well as in HEK293 cells (Fig. 6C). FLAG-Dorfin^{C132S/C135S} did not enhance polyubiquitylation of mutant SOD1s, indicating that this RING finger mutant form was functionally inactive (Fig. 6D).

VCP^{K524A} Suppresses the E3 Activity of Dorfin—VCP has two ATPase binding domains (D1 and D2). A D2 domain mutant, VCP^{K524A}, induces cytoplasmic vacuoles, which mimics vacuole formation seen in the affected neurons in various neurodegenerative diseases (11, 15). The D2 domain represents the major ATPase activity and is essential for VCP function (11). The ATPase activity of VCP^{K524A} is much lower than that of VCP^{WT}, and VCP^{K524A} caused accumulation of polyubiquitylated proteins in the nuclear and membrane fractions together with elevation of ER stress marker proteins due to ERAD

MBP-Dorfin^{full}, MBP-Dorfin^N, MBP-Dorfin^C, and MBP-Parkin with increasing amounts (molar ratio to VCP: 0.25, 0.5, and 1.0). The amounts of MBP fusion Dorfin derivatives and His-VCP in 10% of the samples used are shown (10% input). C, 2 μg of His-VCP was incubated with MBP-Dorfin^{full} with increasing amounts (molar ratio to VCP: 0.25, 0.5, 1, 2, and 4). The amounts of MBP-Dorfin^{full} and His-VCP in 10% of the samples used are shown (10% input). D, His-VCP protein (0.5 μg) was fractionated by 10–40% glycerol gradient centrifugation followed by separation into 30 fractions using a fraction collector. Immunoblotting using anti-VCP antibody was performed on the selected fractions (fractions 2–17). *, The molar ratio was calculated by the amount of VCP monomers, not VCP complexes.

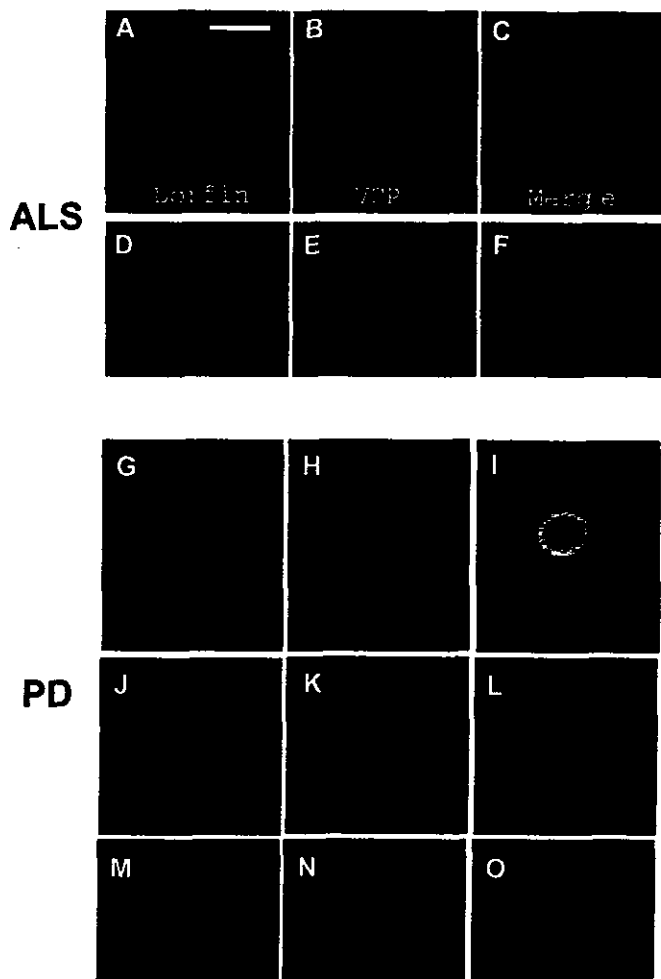


FIG. 5. Colocalization of Dorfin-41 immunoreactivity with VCP in neuronal inclusions in ALS and PD. Sections were doubly labeled with anti-Dorfin-41 antiserum and monoclonal VCP antibody and analyzed with a laser-scanning confocal microscope. The *left panels (green)* correspond to Dorfin, *middle panels (red)* correspond to VCP, and *right panels* correspond to merged images; structures in *yellow* indicate colocalization. Colocalization of Dorfin and VCP is seen in LB-like inclusions in motor neurons of the spinal cord of ALS (A–F). Dorfin is also colocalized with VCP in the margin of LBs (G–I), premature LBs (J–L), and Lewy neurites (M–O) in the nigral neurons of PD. Scale bars, 20 μm (A–L) and 10 μm (M–O).

inhibition, whereas its expression level, localization, and complex formation were indistinguishable from those of VCP^{WT} (11). In order to examine the functional effect of VCP on Dorfin, VCP^{WT}, VCP^{K524A}, or LacZ was co-expressed with SOD1^{G85R}, FLAG-Dorfin, and HA-Ub in HEK293 cells. Co-expression with VCP^{K524A} showed a marked decline of polyubiquitylation of SOD1^{G85R} compared with co-expression with VCP^{WT} or LacZ (Fig. 7A, *top* and *middle*). Since Dorfin physically interacts with mutant SOD1s (5), we next investigated whether this decline of polyubiquitylation of SOD1^{G85R} was mediated by reduced affinity between SOD1^{G85R} and Dorfin. Immunoprecipitation by anti-FLAG antibody showed that VCP^{K524A} did not change affinity between SOD1^{G85R} and Dorfin (Fig. 7A, *bottom*). Neither VCP^{WT} nor VCP^{K524A} changed the level of polyubiquitylation protein in the total lysate (Fig. 7B). To clarify whether this negative effect of VCP^{K524A} is specific for Dorfin, we assessed the autoubiquitylation of FLAG-Parkin in the presence of VCP^{WT}, VCP^{K524A}, or LacZ. Co-expression of VCP^{K524A} did not decrease autoubiquitylation of FLAG-Parkin compared with co-expression of LacZ or VCP^{WT} (Fig. 7C). We performed the same experiments using Neuro2a cells to see whether VCP^{K524A} suppress the E3 activity of Dorfin in neu-

ronal cells. The marked decline of polyubiquitylation of SOD1^{G85R} by VCP^{K524A} expression was also seen in Neuro2a cells (Fig. 7D).

DISCUSSION

UBIs in the affected neurons are histopathological hallmarks in various neurodegenerative disorders (8). Dorfin is an E3 ligase, which can ubiquitylate mutant SOD1s and synphilin-1 (5, 24). These substrates and Dorfin were identified in UBIs in various neurodegenerative diseases, such as LB-like inclusions in ALS and LBs in PD and dementia with Lewy bodies (7). This finding suggests that Dorfin may play a crucial role in the process of generating inclusions in the affected neurons. In the present study, we identified VCP as one of the Dorfin-associated proteins using mass spectrometry, and VCP-Dorfin physical interaction was confirmed by an immunoprecipitation experiment using FLAG-Dorfin and HA-VCP overexpressed in HEK293 cells (Fig. 1A). VCP is an essential and highly conserved protein of the AAA-ATPase family, which is considered to have diverse cellular functions, such as membrane fusion (25–27), nuclear trafficking (28), cell proliferation (29, 30), and the ERAD pathway (18–22). Many reports have implied that VCP is involved in the pathogenesis of various neuromuscular diseases. VCP has been implicated as a factor that modifies the progress of polyglutamine-induced neuronal cell death (15). In addition, histopathological studies revealed positive staining for VCP in UBIs in PD and ALS with dementia (23). VCP is also associated with MJD protein/ataxin-3, in which abnormal expansion of polyglutamine tracts causes Machado-Joseph disease/spinocerebellar ataxia type 3 (31). VCP is also required for the degradation of ataxin-3 in collaboration with E4B/Ufd2a, a ubiquitin chain assembly factor (E4) (32). Recent studies have indicated that missense mutations in the VCP gene cause inclusion body myopathy associated with Paget's disease of bone and frontotemporal dementia, which is characterized by the presence of vacuoles in the cytoplasm in muscle fibers (33).

Our results showed that endogenous Dorfin formed a 400–600-kDa complex in various tissues and various cultured cells (Fig. 1B). Dorfin is a ~91-kDa protein; therefore, this high M_r complex should include Dorfin-associated proteins, although the possibility that Dorfin itself oligomerizes in the cell cannot be excluded. Glycerol gradient centrifugation analysis and immunoprecipitation experiments in the present study showed that endogenous Dorfin interacted with endogenous VCP in a complex of approximately 600 kDa, possibly including a Dorfin molecule and a hexameric form of VCP (Fig. 1C).

The first RING mutant of Dorfin, in which Cys at positions 132 and 135 changed to Ser, was prepared. This mutant Dorfin, Dorfin^{C132S/C135S}, could not ubiquitylate mutant SOD1s (Fig. 6D). Glycerol gradient centrifugation analysis revealed that Dorfin^{C132S/C135S} did not form a high M_r complex, whereas exogenous wild type Dorfin (Dorfin^{WT}) formed a high M_r complex similar to endogenous Dorfin (Fig. 2A). Furthermore, an immunoprecipitation experiment using Dorfin^{WT} and Dorfin^{C132S/C135S} revealed that Dorfin^{WT} could interact with VCP, whereas Dorfin^{C132S/C135S} could not (Fig. 2B).

Our *in vitro* study using recombinant proteins showed that full-length (MBP-Dorfin^{full}) and the C terminus of Dorfin (MBP-Dorfin^C) directly interacted with VCP, whereas the MBP-Dorfin^N mutant, containing the entire RING finger domain (amino acid residues 1–367), did not bind to VCP (Fig. 3A). This finding was unexpected, since *in vivo* binding analysis suggested that Dorfin could interact with VCP at the RING finger domain. It is plausible that certain structural changes in Dorfin^{C132S/C135S} might render the C-terminal VCP-binding portion incapable of accessing VCP molecules. This may explain the result that Dorfin^{C132S/C135S} did not form a high M_r complex.

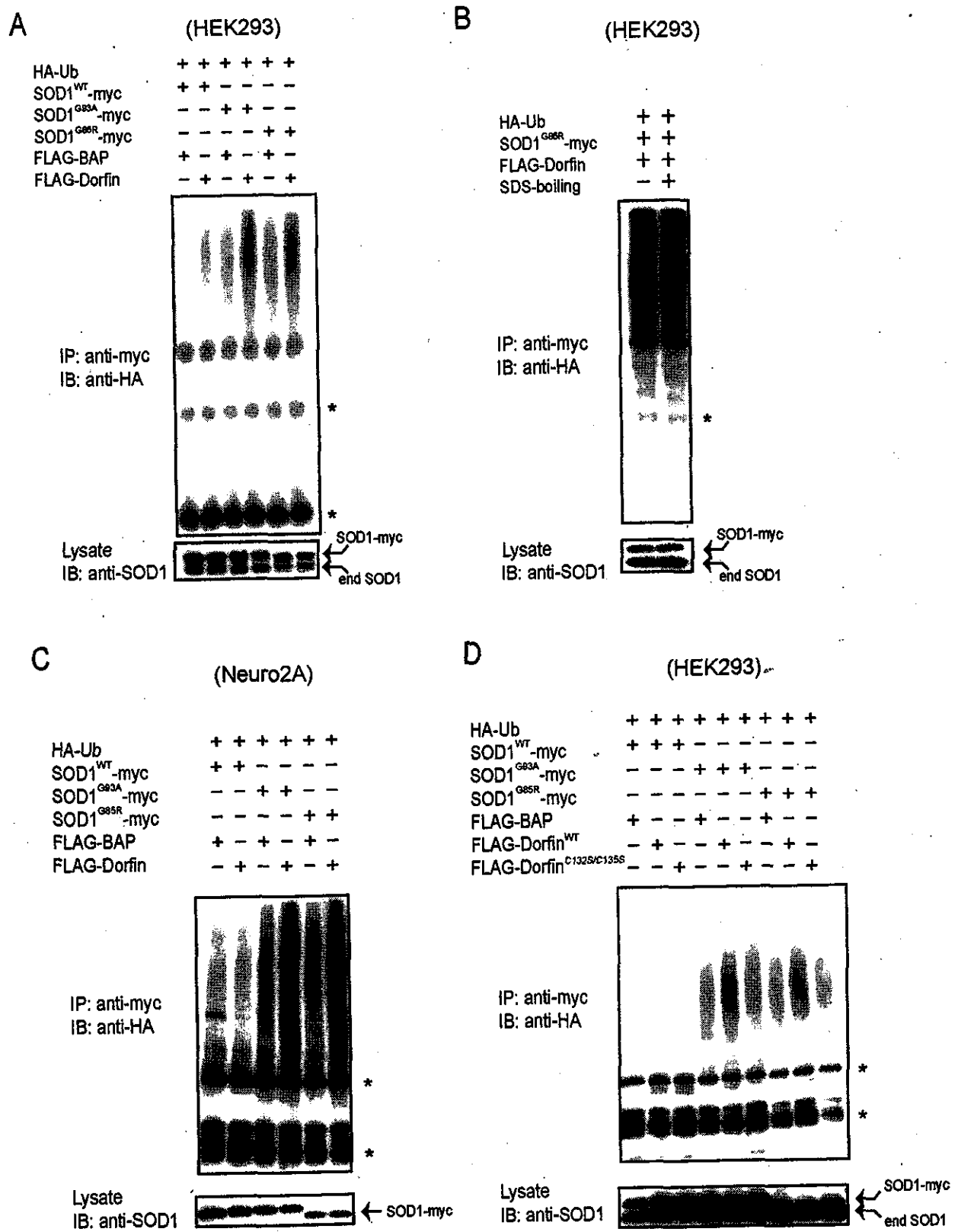


Fig. 6. Dorfin ubiquitylates mutant SOD1s *in vivo*. A, increased ubiquitylation of mutant SOD1 proteins by overexpression of Dorfin. HEK293 cells were co-transfected with SOD1^{WT}-Myc, SOD1^{G83A}-Myc, or SOD1^{G85R}-Myc and HA-Ub with or without FLAG-Dorfin. FLAG-bovine alkaline phosphatase (BAP) was used as a negative control. Immunoprecipitation (IP) was performed with Myc antibody (9E10). IB, immunoblotting. B, SDS boiling was performed prior to immunoprecipitation. To examine covalently ubiquitylated molecules, the cell lysate was boiled with the buffer containing 1% SDS for 5 min. Immunoprecipitation with Myc antibody (9E10) showed that the SDS-boiling procedure did not change polyubiquitylation level of SOD1^{G85R}-Myc by Dorfin. C, increased ubiquitylation of mutant SOD1 proteins by overexpression of Dorfin in Neuro2a cells. The same *in vivo* ubiquitylation assay as in A was performed using Neuro2a cells. D, Dorfin^{C132S/C135S} (Dorfin^{C132S/C135S}) did not have E3 activity on mutant SOD1. HEK293 cells were co-transfected with SOD1^{WT}-Myc, SOD1^{G83A}-Myc, or SOD1^{G85R}-Myc and HA-Ub with FLAG-Dorfin^{WT} or FLAG-Dorfin^{C132S/C135S}. The asterisks indicate IgG light and heavy chains.

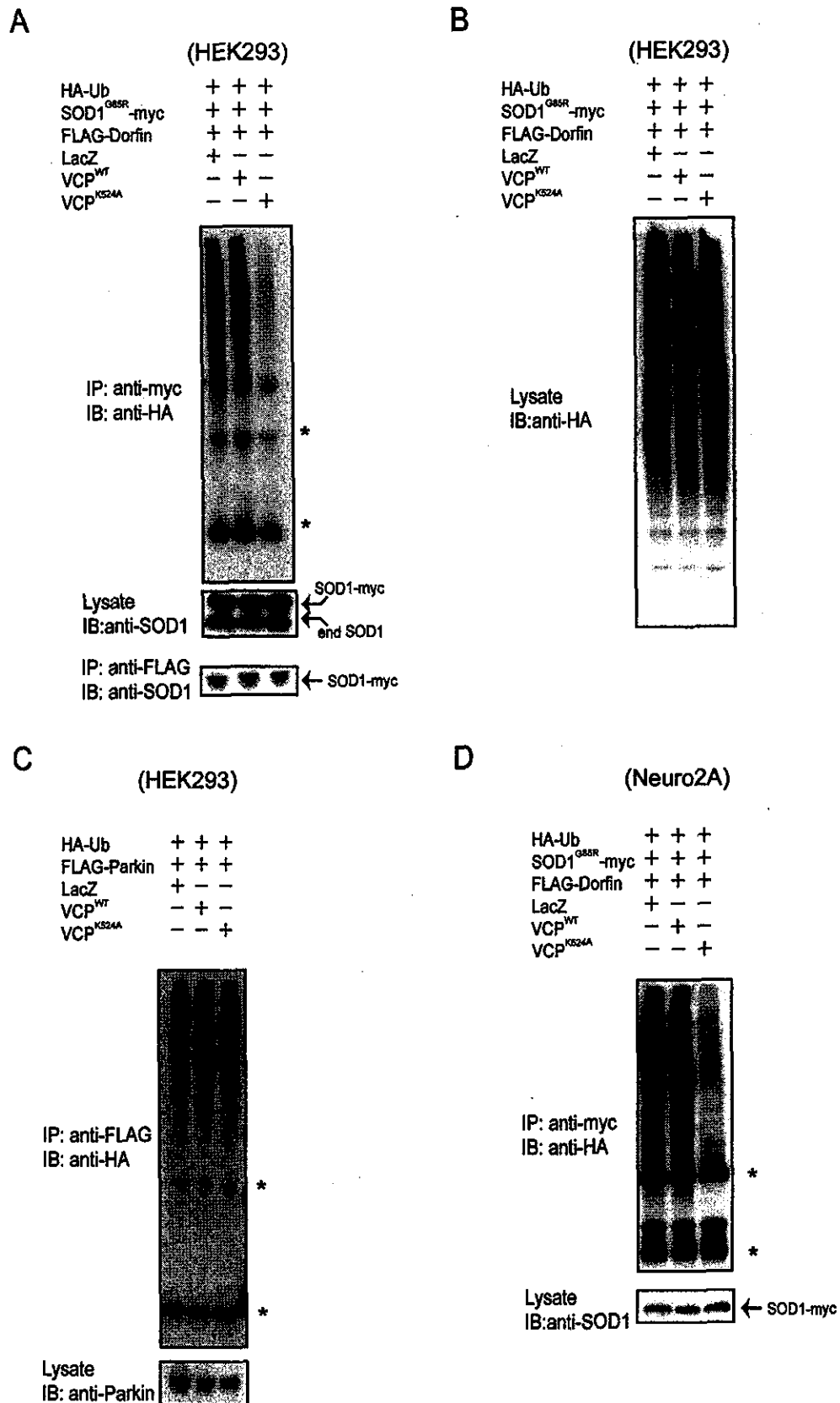


FIG. 7. A dominant negative mutant of VCP, VCP^{K524A} inhibits the E3 ubiquitin ligase activity of Dorfin. *A*, inhibition of dominant negative form mutant VCP^{K524A} on the E3 ubiquitin ligase activity of Dorfin. HEK293 cells were co-transfected with SOD1^{G85R}-Myc, HA-Ub, FLAG-Dorfin, and VCP^{WT}, VCP^{K524A}, or LacZ. Immunoprecipitation (IP) was performed with Myc antibody (9E10) and FLAG antibody (M2). *IB*, immunoblotting. *B*, neither VCP^{WT} nor VCP^{K524A} changed the level of total polyubiquitylated protein in the cell lysate. Ten percent of the volume of HEK293 cells used in *A* was subjected to immunoblotting using anti-HA (12CA5) antibody. *C*, autoubiquitylation of FLAG-Parkin was not influenced by the dominant negative form VCP^{K524A}. HEK293 cells were co-transfected with FLAG-Parkin, HA-Ub, and VCP^{WT}, VCP^{K524A}, or LacZ. Immunoprecipitation with FLAG antibody (M2) was performed. *D*, inhibition of VCP^{K524A} on E3 ubiquitin ligase activity of Dorfin in Neuro2a cells. Neuro2a cells were co-transfected with SOD1^{G85R}-Myc, HA-Ub, FLAG-Dorfin, and VCP^{WT}, VCP^{K524A}, or LacZ. Immunoprecipitation was performed using Myc antibody (9E10) and FLAG antibody (M2). The asterisks indicate IgG light and heavy chains.

The amount of Dorfin bound with VCP was saturated at even molar ratio *in vitro* (Fig. 3, B and C). Since VCP exists as a homohexamers (Fig. 3D), the *in vivo* observed size of ~600 kDa appears to be too small for the Dorfin-VCP complex if one VCP molecule binds to more than one Dorfin as shown in *in vitro* experiments. However, it is noteworthy that the size of molecules estimated by glycerol density gradient centrifugation analysis used in this study is not accurate and sufficient to discuss the molecular interaction of Dorfin and VCP in the cells. To date, various adaptor proteins, with which VCP forms multiprotein complexes, have been identified, such as Npl4, Ufd1 (18, 20), Ufd2 (34), Ufd3 (35), p47 (36), or SVIP (37). Although our *in vitro* study showed direct physical interaction between Dorfin and VCP, the environment with those adaptor proteins might reflect *in vivo* conditions. This also may explain the apparent discrepancy of the Dorfin-VCP binding fashions between *in vivo* and *in vitro* analyses.

Treatment with a proteasomal inhibitor causes the translocation of endogenous VCP and Dorfin to the aggresome in cultured cells (4, 15). Our results showed that these two proteins indeed colocalized perinuclearly in the aggresome following treatment with a proteasomal inhibitor (Fig. 4). Furthermore, we were able to demonstrate both Dorfin and VCP immunoreactivities in LB-like inclusions in ALS and LBs in PD (Fig. 5). In the majority of LBs, indistinguishable peripheral staining patterns were observed with both anti-Dorfin and anti-VCP antibodies. These results confirmed that both Dorfin and VCP are associated with the formation processes of aggresomes and inclusion bodies through physical interaction.

We showed here that co-expression of VCP^{K524A} resulted in a marked decrease of ubiquitylation activity of Dorfin compared with co-expression of VCP^{WT} or control. On the other hand, VCP^{K524A} failed to decrease autoubiquitylation activity of Parkin. VCP^{K524A} did not change the level of polyubiquitylated protein accumulation in the cell lysate in this study (Fig. 7). Knockdown experiments using the RNA interference technique showed accumulation of polyubiquitylated proteins (38). Combined with the observation that inhibition of VCP did not decrease the general accumulation of polyubiquitylated proteins, our results indicated that the E3 regulation function of VCP may be specific to certain E3 ubiquitin ligases such as Dorfin. VCP is an abundant protein that accounts for more than 1% of protein in the cell cytosol and is known to have various chaperone-like activities (39); therefore, it may function as a scaffold protein on the E3 activity of Dorfin. The localization of Dorfin and VCP in UBIs in various neurodegenerative disorders indicates the involvement of these proteins in the quality control system for abnormal proteins accumulated in the affected neurons in neurodegenerative disorders.

Since the unfolded protein response and ERAD are dynamic responses required for the coordinated disposal of misfolded proteins (40), the ERAD pathway can be critical for the etiology of neuronal cell death caused by various unfolded proteins. VCP is required for multiple aspects of the ERAD system by recognition of polyubiquitylated proteins and translocations to the 26 S proteasome for processive degradation through the VCP-Npl4-Ufd1 complex (18, 41). Our results suggest the involvement of Dorfin in the ERAD system, which is related to the pathogenesis of neurodegenerative disorders, such as PD or Alzheimer's disease. Further study including Dorfin knockout and/or knockdown models should examine the pathophysiology

of Dorfin in association with the ERAD pathway or other cellular functions. Such studies should enhance our understanding of the pathogenetic role of Dorfin in neurodegenerative disorders.

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WIDE RANGE OF LINEAGES OF CELLS EXPRESSING NERVE GROWTH FACTOR mRNA IN THE NERVE LESIONS OF PATIENTS WITH VASCULITIC NEUROPATHY: AN IMPLICATION OF ENDONEURIAL MACROPHAGE FOR NERVE REGENERATION

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Abstract—*In situ* localization of nerve growth factor (NGF) mRNA was examined in the nerve lesions of patients with vasculitic neuropathy. Double labeling of *in situ* hybridization for NGF mRNA and immunohistochemistry for cell markers showed that NGF mRNA was expressed in a wide range of lineages of cells: Schwann cells, infiltrating macrophages, T cells and perivascular cells. Round-shaped macrophages with early-phase features expressed high levels of NGF mRNA, in contrast to late-phase polymorphic macrophages, which expressed low levels of NGF mRNA. NGF mRNA was also expressed universally in T cells with various cell surface markers. Epineurial macrophages surrounding vasculitic lesions and endoneurial T cells expressed high levels of NGF mRNA in the damaged nerves. Moreover, the extent of endoneurial NGF expression level in macrophages was closely related to the degree of axonal regeneration. These results suggest that NGF is expressed in a wide range of lineages of cells but is differentially expressed spatially in vasculitic nerve lesions, and that the expressed NGF, particularly in macrophages, may play an important role in the nerve regeneration process. © 2004 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: nerve growth factor, macrophages, T cells, Schwann cells, vasculitic neuropathy.

Numerous neurotrophic factors and cytokines are produced in nerve lesions of various neuropathies. They form a multi-factorial regulation network in lesions in the acute and chronic phase and function in nerve regeneration. Cell-type-specific trophic activity and production of neurotrophic factors have been reported (Snider and Wright, 1996; Molliver et al., 1997). We have previously demonstrated that the neurotrophic factors and cytokines are up- or down-regulated temporally in the nerve lesions in human neuropathies as well as in rodent neuropathic models (Sobue et al., 1988, 1998; Ito et al., 1998).

Among these factors, nerve growth factor (NGF) is considered to play an important role in recovery of neuropathy. NGF was found as a protein influencing nerve growth in the 1950s, and is known to affect sensory neurons of dorsal root

ganglia (DRG), autonomic sympathetic neurons, and basal forebrain cholinergic neurons (Levi-Montalcini et al., 1996). NGF and other related neurotrophic factors structurally similar to NGF form a family of neurotrophins that bind to their cognate specific high-affinity receptors, which contain a tyrosine kinase domain, and also bind to low-affinity receptor p75 (Hantzopoulos et al., 1994). NGF binds to its high-affinity receptor TrkA, which interacts with p75 as a positive modulator for myelination, in order to promote neurotrophic effects by activating signaling pathways initiated by tyrosine kinase activity (Chao and Hempstead, 1995; Cosgaya et al., 2002).

NGF has a neurotrophic effect on neuropathies in animal and explant models caused by physical and chemical damage (Miyata et al., 1986; Hayakawa et al., 1998; Fischer et al., 2001). When NGF is administered prior to experimental neuropathy caused by axotomy, NGF promotes survival and regeneration of neurons (Rich et al., 1987; Apfel et al., 1992). NGF expression in the lesions of experimental neuropathy shows a two-peaked up-regulation pattern in the cut-crush mouse model (Heumann et al., 1987; Ito et al., 1998). This expression pattern is caused by infiltrating cells at the first peak, and then by lesioned Schwann cells at the second peak. TrkA and p75 are also up-regulated and maintained after nerve crush injury (Greenson et al., 1992; Yamamoto et al., 1998a).

The histopathological expression profile of NGF in the lesions of human neuropathies is not yet fully understood. In nerve lesions of human neuropathies, various types of pathology including inflammatory, ischemic, and metabolic changes, are present in different phases. In necrotizing vasculitic neuropathies, nerve pathology consists almost exclusively of axonal degeneration with Wallerian degeneration due to ischemic damages (Hawke et al., 1991; Hattori et al., 1999). We previously reported that NGF mRNA expression was increased in nerve lesions of vasculitic neuropathies, and that the increase was well correlated with the extent of invasion of macrophages and T cells (Yamamoto et al., 2001). Investigation of the histopathological distribution of NGF mRNA will help to advance our understanding of the mechanism of nerve regeneration by NGF, and be of importance from the point of view of clinical therapeutic implications. In this study we examined the histopathological localization of NGF mRNA in nerves of patients with vasculitic neuropathies using double staining consisting of *in situ* NGF hybridization and immunohistochemistry against various types of cell-specific markers, and here we report those re-

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Abbreviations: CT, computed tomography; DRG, dorsal root ganglion; MRI, magnetic resonance imaging; NGF, nerve growth factor.