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Role of the PLC-related, catalytically inactive protein p130 in $GABA_A$ receptor function

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The protein p130 was isolated from rat brain as an inositol 1,4,5-trisphosphate-binding protein with a domain organization similar to that of phospholipase C-δ1 but lacking PLC activity. We show that p130 plays an important role in signaling by the type A receptor for γ-aminobutyric acid (GABA). Yeast two-hybrid screening identified GABARAP ($GABA_A$ receptor-associated protein), which is proposed to contribute to the sorting, targeting or clustering of $GABA_A$ receptors, as a protein that interacts with p130. Furthermore, p130 competitively inhibited the binding of the γ2 subunit of the $GABA_A$ receptor to GABARAP *in vitro*. Electrophysiological analysis revealed that the modulation of GABA-induced Cl^- current by Zn^{2+} or diazepam, both of which act at $GABA_A$ receptors containing γ subunits, is impaired in hippocampal neurons of p130 knockout mice. Moreover, behavioral analysis revealed that motor coordination was impaired and the intraperitoneal injection of diazepam induced markedly reduced sedative and antianxiety effects in the mutant mice. These results indicate that p130 is essential for the function of $GABA_A$ receptors, especially in response to the agents acting on a γ2 subunit.

Keywords: diazepam/GABA/knockout mice/p130

Introduction

D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], a product of receptor-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] by phospholipase C (PLC), plays an important role as an intracellular second messenger by mobilizing Ca^{2+} from non-mitochondrial stores (Berridge, 1993). We previously isolated two Ins(1,4,5)P_3-binding proteins with molecular masses of 130 and 85 kDa from rat brain (Kanematsu *et al*., 1992; Yoshida *et al*., 1994) with the use of an Ins(1,4,5)P_3 affinity column (Hirata *et al*., 1985, 1990). Partial amino acid sequencing revealed that the 85 kDa molecule was PLC-δ1 (Kanematsu *et al*., 1992). The Ins(1,4,5)P_3-binding protein with a molecular mass of 130 kDa, termed p130, was a previously unidentified molecule (Kanematsu *et al*., 1992; Yoshida *et al*., 1994). The predicted amino acid sequence of rat p130 shares 38.2% identity with that of rat PLC-δ1 (Kanematsu *et al*., 1996); the five identified domains of PLC-δ1 [pleckstrin homology (PH), EF-hand, putative catalytic (X and Y) and C2 domains] are all present in p130. The domain organization of p130 suggests that the protein is likely to possess a fold similar to that of PLC-δ1, a notion that is supported by the results of limited proteolysis with trypsin (Kanematsu *et al*., 2000). However, p130 exhibits some distinct characteristics. It is larger than the PLC-δ isozymes and it possesses unique regions both at the N-terminus, preceding the PH domain, and at the C-terminus. Moreover, the residues within the catalytic domain of PLC-δ that are critical for enzyme activity (His356 and Glu390) are not conserved in p130 (Kanematsu *et al*., 1996). The PH domain of p130, like that of PLC-δ1, is important for the binding of Ins(1,4,5)P_3 (Takeuchi *et al*., 1996, 1997).

To investigate the physiological functions of p130, we previously examined the possible role of the binding of inositol compounds to the PH domain of p130 (Takeuchi *et al*., 1996, 1997; Hirata *et al*., 1998; Lemmon and Ferguson, 2000). Our results suggested that the high-affinity binding of Ins(1,4,5)P_3 to the PH domain of p130 might serve to sequester Ins(1,4,5)P_3 and therefore prevent its interaction with Ins(1,4,5)P_3 receptors and metabolizing enzymes (Takeuchi *et al*., 2000).

We have now applied the yeast two-hybrid system to identify proteins that interact with p130. With the unique N-terminal region of p130 as the bait for screening a human brain cDNA library, we isolated two positive clones, one of which was shown to encode the catalytic subunit of protein phosphatase 1α (Yoshimura *et al*., 2001). Another clone was found to be GABARAP ($GABA_A$ receptor associated protein) that was identified as a molecule capable of binding the γ2 subunit of $GABA_A$ receptor and tubulin (Wang *et al*., 1999). In the present study, we studied the significance of the association of these two proteins in $GABA_A$ signaling in brain using $p130^{-/-}$ mice, and found that p130 is essential for the function of $GABA_A$ receptors that contain a γ2 subunit.

Results

Association between p130 and GABARAP and competition with $GABA_A$ receptor γ subunit

We screened a human brain cDNA library with the use of the yeast two-hybrid system. A total of 51 clones (out of

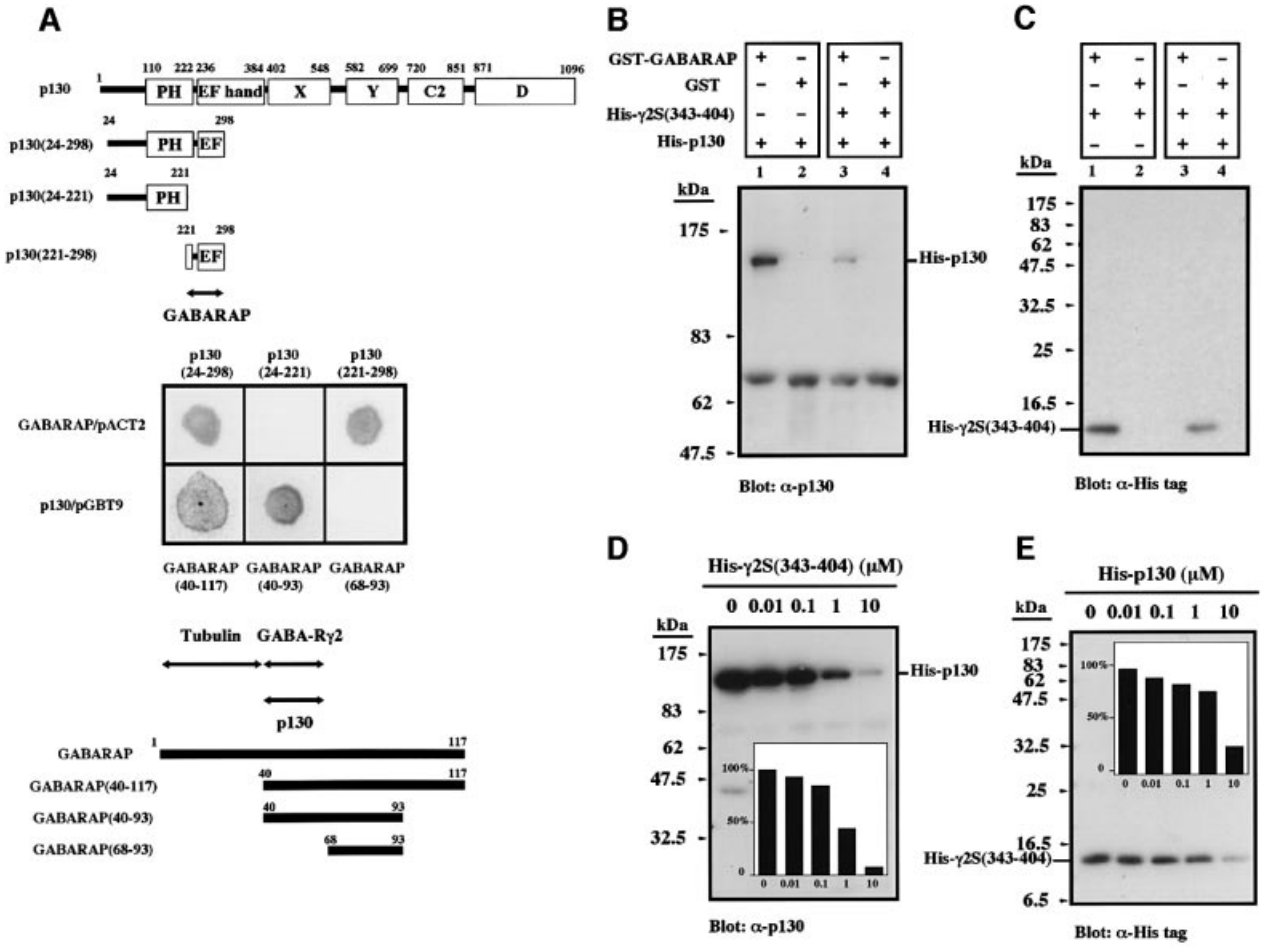

Fig. 1. Interaction of GABARAP with p130 and the γ2 subunit of the GABA$_A$ receptor. (**A**) The interactions of various truncation mutants of rat p130 (top) and of human GABARAP (bottom) with full-length GABARAP and p130, respectively, were examined by yeast two-hybrid analysis (center). Putative binding regions of both proteins are indicated by arrows. (**B** and **C**) Precipitation assays. His$_6$-p130 (B) or His$_6$-γ2S(343–404) (C) at 1 μM was incubated with GST or GST–GABARAP in the absence or presence of His$_6$-γ2S(343–404) or His$_6$-p130 at 1 μM, respectively. The band at 69 kDa in (B) is a non-specific signal attributable to the bovine serum albumin present in the assay mixture. (**D** and **E**) Competition assays. GST–GABARAP (1 μM) was incubated either with His$_6$-p130 (1 μM) and the indicated concentrations of His$_6$-γ2S(343–404) (D), or with His$_6$-γ2S(343–404) (1 μM) and the indicated concentrations of His$_6$-p130 (E). Insets show the percentage inhibition of His$_6$-p130 (D) and His$_6$-γ2S(343–404) (E) binding, as determined by densitometry.

2×10^6 examined) were detected with a plasmid encoding residues 24 to 298 of rat p130 (which includes the PH domain and a portion of the EF hand motif) as the bait (Figure 1A). Ten of these clones were found to be false positives, and the remainder were divided into two groups on the basis of restriction endonuclease mapping. Sequence determination revealed that one of the clones encoded residues 40 to 117 of GABARAP; i.e. lacking the N-terminal 39 residues of the full-length protein (Wang *et al*., 1999). To identify the regions of these two proteins responsible for their interaction, we prepared several deletion constructs of p130 and GABARAP cDNAs in the yeast vectors pGBT9 and pACT2, respectively, and examined the association of the encoded proteins in the yeast two-hybrid assay (Figure 1A). Amino acids 24 to 298 and 221 to 298, but not residues 24 to 221, of p130 bound to GABARAP, and amino acids 40 to 93, but not residues 68 to 93, of GABARAP bound to p130. These results indicated that residues 40 to 67 of GABARAP interact with residues 221 to 298 of p130. This region of GABARAP (residues 40 to 67) has also been shown to be important for association with the intracellular loop of the γ2S subunit (Arg386 to Asp403) of the GABA$_A$ receptor (Wang *et al*., 1999; Wang and Olsen, 2000), suggesting that p130 might compete with the receptor γ2 subunit for binding to GABARAP.

To investigate the interactions among p130, GABARAP, and the γ2 subunit of the GABA$_A$ receptor *in vitro*, we performed precipitation assays with recombinant proteins. Histidine-tagged p130 (His$_6$-p130) and a histidine-tagged fragment of γ2S [His$_6$-γ2S(343–404)] each bound to a glutathione *S*-transferase (GST) fusion protein containing full-length GABARAP but not to GST alone (Figure 1B and C). Furthermore, the presence of His$_6$-γ2S(343–404) inhibited the binding of His$_6$-p130 to GABARAP and vice versa, thus indicating that p130 and γ2S(343–404) compete for binding to GABARAP. The mutual inhibition of the binding of each of these two proteins to GABARAP was dose dependent (Figure 1D and E); the binding of His$_6$-γ2S(343–404) to GABARAP,

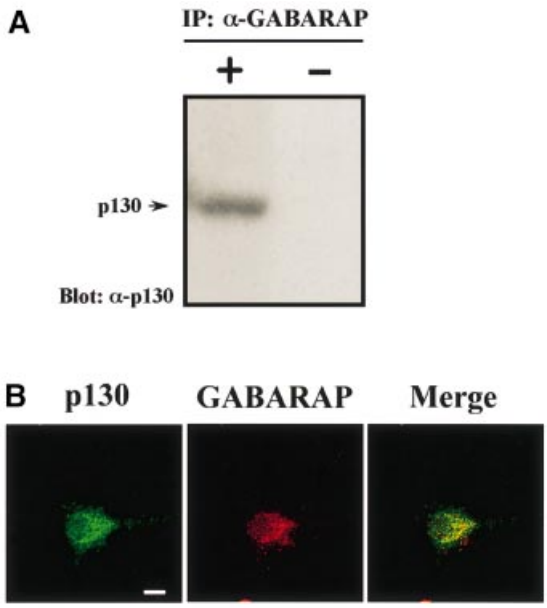

Fig. 2. Association of p130 with GABARAP in a cellular environment. (**A**) Immunoprecipitates (IP) prepared from mouse brain extract with antibodies to GABARAP (left lane), or in the absence of antibodies as a control (right lane), and protein G-conjugated beads were probed by immunoblot analysis with antibodies to p130. (**B**) Immunofluorescence localization of GABARAP (red) and p130 (green) in cultured cortical neurons using a confocal microscope. Bar: 10 μm.

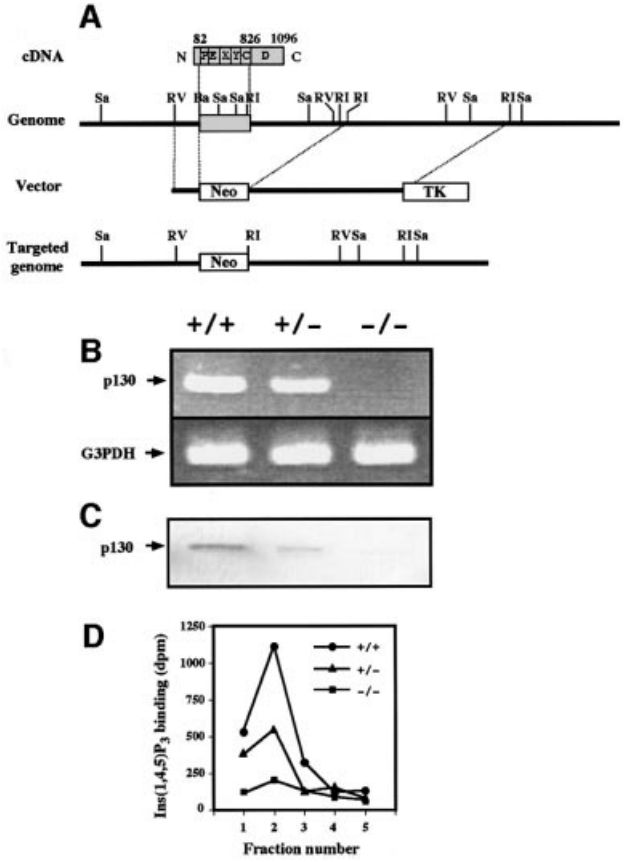

Fig. 3. Targeted disruption of the mouse p130 gene. (**A**) Targeting strategy. The localization of introns and exons of the mouse p130 gene is incomplete, but the single exon that encodes at least the PH domain, EF hand, and X, Y, and C2 domains is indicated. Numbers correspond to amino acid residues of rat p130. Sa, *Sac*I; RV, *Eco*RV; Ba, *Bam*HI; RI, *Eco*RI; Neo, neomycin resistance gene; TK, thymidine kinase. (**B**) RT–PCR analysis of total RNA from the brains of $p130^{+/+}$, $p130^{+/-}$ and $p130^{-/-}$ littermates with primers specific for p130 or glyceraldehyde-3-phosphate dehydrogenase (G3PDH). (**C**) Immunoblot analysis of cytosolic fractions of the brains of littermates with antibodies to p130 (2F9). (**D**) $Ins(1,4,5)P_3$ binding assay. Brain cytosolic fractions were applied to an $Ins(1,4,5)P_3$ affinity column, which was then washed with 0.5 M NaCl before elution with 2 M NaCl. Portions (10 μl) of eluted fractions (1 ml) were assayed for binding of $[^3H]Ins(1,4,5)P_3$, as described previously (Hirata *et al*., 1990; Kanematsu *et al*., 1992; Yoshida *et al*., 1994). Data are means of triplicates from an experiment that was repeated four times with similar results.

however, was more resistant to inhibition, indicating that the affinity of GABARAP for the γ2 subunit might be greater than its affinity for p130. Given that GABARAP also contains a tubulin binding site within its N-terminal 36 residues (Wang *et al*., 1999; Kneussel *et al*., 2000; Wang and Olsen, 2000) (Figure 1A), we examined the interaction between GABARAP and tubulin with crude extracts of COS-1^{p130} cells, a stable cell line expressing p130 (Takeuchi *et al*., 2000); both p130 and tubulin were precipitated from cell extracts with GST–GABARAP immobilized to glutathione–Sepharose beads (data not shown).

The association between p130 and GABARAP under more physiological conditions was also examined. Immunoprecipitation analysis with brain extract revealed that complex formation between p130 and GABARAP also occurs in a cellular environment (Figure 2A). The cellular localization of each of these two proteins was characterized previously (Takeuchi *et al*., 2000; Wang and Olsen, 2000). We now show that the majority of p130 and GABARAP co-localize as punctate structures inside cultured cortical neurons by immunofluorescence analysis, suggesting the interaction *in vivo* (Figure 2B). However, p130 does not appear to be enriched at inhibitory synaptic sites (data not shown). The abundance of p130 was previously shown to be highest in brain (Matsuda *et al*., 1998), and *in situ* hybridization revealed that p130 mRNA is localized in hippocampal pyramidal cells, in dentate granule cells and the pyramidal and granule cell layers of the cerebral cortex, and in the granular cell and Purkinje cell layers and cerebellar nuclei of the cerebellum (Matsuda *et al*., 1998). This regional distribution is similar to that of $GABA_A$ receptors (Rudolph *et al*., 1999). The results obtained here, together with those cited, indicated that p130 might contribute to regulation of $GABA_A$ receptor function through its association with GABARAP.

Generation of p130 KO mice

To examine the possible role of p130 interacting with GABARAP in regulation of $GABA_A$ receptor function, we generated p130 knockout mice by replacing a fragment of the protein-coding sequence (corresponding to residues 82 to 826 of rat p130) of the p130 gene in mouse embryonic stem cells with a neomycin resistance cassette by homologous recombination (Figure 3A). The success of gene targeting was confirmed by RT–PCR (Figure 3B) and

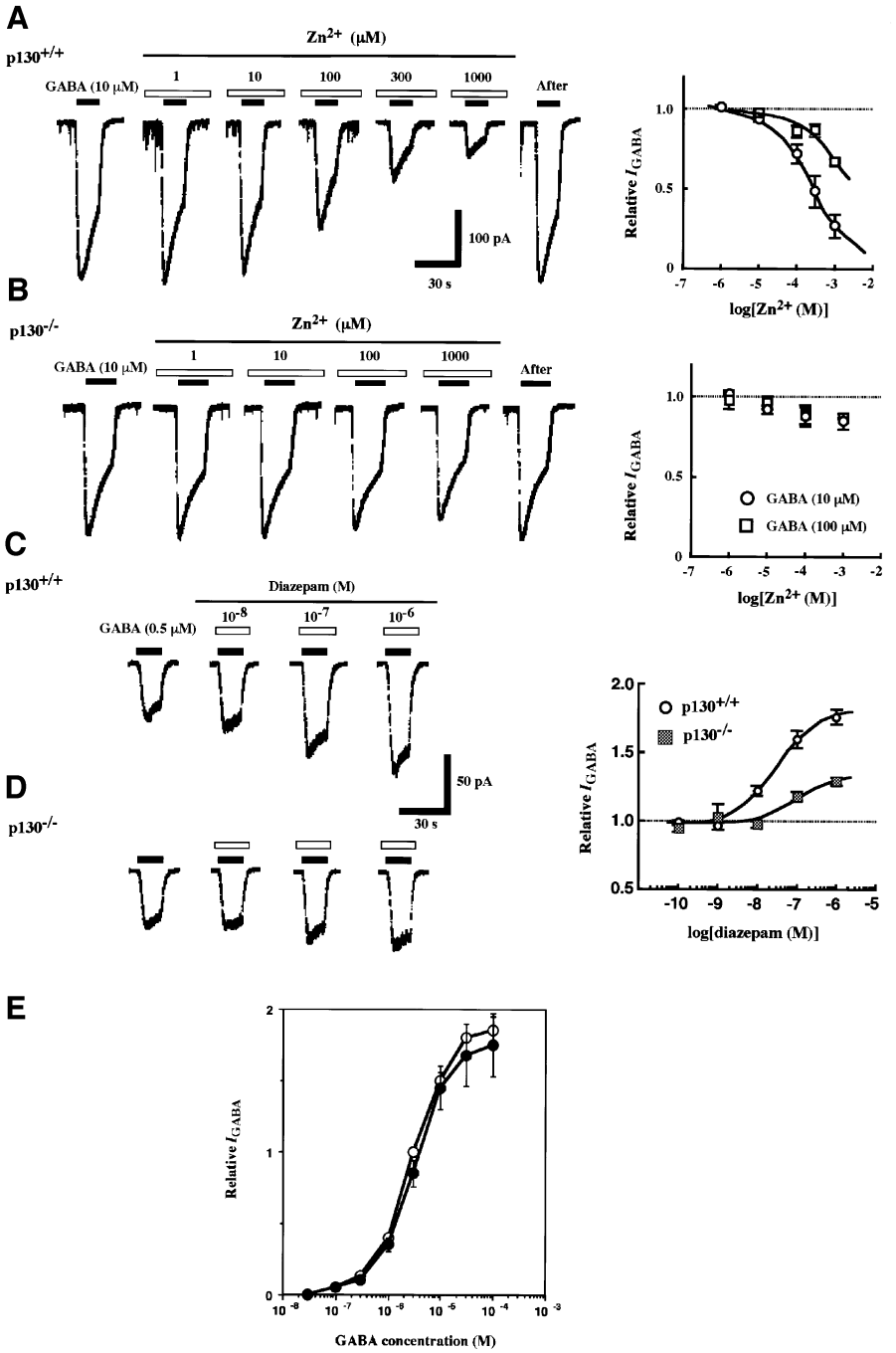

Fig. 4. Impaired signaling by the γ subunit of GABA$_A$ receptors in p130$^{-/-}$ mice. (**A** and **B**) GABA-induced inward current (I_{GABA}) in the presence of various concentrations of Zn^{2+} was measured in hippocampal CA1 cells from p130$^{+/+}$ (A) and p130$^{-/-}$ (B) mice. The intervals of GABA (10 μM) and Zn^{2+} application are indicated by solid and open bars, respectively. Graphs on the right are the dose–response relations for the effect of Zn^{2+} on I_{GABA} evoked by 10 or 100 μM GABA. Data are expressed relative to the current amplitude in the absence of Zn^{2+} and are means ± SEM of values from three to eight experiments. (**C** and **D**) Traces of I_{GABA} induced by 0.5 μM GABA in CA1 cells from p130$^{+/+}$ (C) and p130$^{-/-}$ (D) mice in the absence or presence of the indicated concentrations of diazepam. Graphs on the right are the dose–response relations for the effect of diazepam on I_{GABA} induced by 0.5 μM GABA. Data are means ± SEM of values from six to nine experiments. (**E**) Concentration–response relationships of GABA-elicited postsynaptic currents in the wild-type (open circles) and knock-out (closed circles) hippocampal CA1 neurons. All currents were normalized to those elicited in the control neuron by 3 μM GABA. Data are means ± SEM of values from five experiments.

immunoblot analysis (Figure 3C). Furthermore, assay of p130 function was performed by measurement of the binding of [^{3}H]Ins(1,4,5)P_3 to fractions of brain cytosol eluted from an Ins(1,4,5)P_3 affinity column (Hirata *et al.*, 1990; Kanematsu *et al.*, 1992; Yoshida *et al.*, 1994) (Figure 3D). Ins(1,4,5)P_3 binding activity was virtually absent, and markedly reduced in the brains of p130$^{-/-}$ and p130$^{+/-}$ mice, respectively, compared with that in the brain of wild-type animals. The mice lacking p130 appeared to grow normally and were fertile.

Electrophysiological analysis

We first examined $GABA_A$ receptor-mediated Cl^- current (I_{GABA}) in nystatin-perforated patches of hippocampal CA1 cells freshly isolated from wild-type and p130 knockout mice (Figure 4). Although the current induced by 10 μM GABA was slightly smaller in cells from $p130^{-/-}$ mice than in those from control mice (Figure 4A and B), this difference was not statistically significant. The dose–response relationship was not changed, i.e. concentrations required for half-maximal I_{GABA} were 2.8 and 2.9 μM for the control and mutant mice, respectively (Figure 4E). Moreover, the maximal GABA-induced currents in neurones derived from $p130^{-/-}$ and wild-type mice appeared to be similar (Figure 4E), suggesting no overt differences in functional cell surface-receptor number in the absence of p130.

We then examined the effect of agents that are known to modulate the function of $GABA_A$ receptors containing the γ subunit. The effect of Zn^{2+} on the current evoked by 10 or 100 μM GABA was examined first. Low concentrations (~1 μM) of Zn^{2+} inhibit I_{GABA} mediated by receptors that do not contain the γ2 subunit, whereas higher concentrations of this cation are required for inhibition at receptors that do contain this subunit (Draguhn *et al*., 1990; Gingrich and Burkat, 1998). In neurons isolated from $p130^{+/+}$ mice, Zn^{2+} reversibly blocked I_{GABA} in a dose-dependent manner (Figure 4A); the concentration of Zn^{2+} required for half-maximal inhibition of the effect of 10 μM GABA was ~210 μM, indicating that the receptors analyzed contained the γ2 subunit (Draguhn *et al*., 1990; Smart *et al*., 1991; Gingrich and Burkat, 1998). In contrast, Zn^{2+} had little effect on I_{GABA} in the hippocampal neurons from $p130^{-/-}$ mice (Figure 4B) up to a concentration of 1 mM. To investigate further $GABA_A$ receptor signaling in $p130^{-/-}$ mice, we examined the effect of the benzodiazepine (BZ) agonist diazepam, a potent modulator of the receptors containing γ subunits (Günther *et al*., 1995), on I_{GABA} in hippocampal CA1 neurons. Diazepam reversibly potentiated in a dose-dependent manner I_{GABA} elicited by 0.5 μM GABA in neurons from $p130^{+/+}$ mice (Figure 4C). In contrast, both the efficacy and potency of diazepam with regard to potentiation of I_{GABA} were markedly reduced in neurons of $p130^{-/-}$ mice (Figure 4D).

These conflicting observations on Zn^{2+} modulation and diazepam responsiveness in the I_{GABA} are therefore opposite to the results obtained from the study of recombinant receptors. In these systems, sensitivity to benzodiazepines is determined by the presence of a γ subunit in heteromeric αβγ receptors which are effectively antagonized by 50–100 μM Zn^{2+} (Smart *et al*., 1991), in contrast to the almost total insensitivity of I_{GABA} in $p130^{-/-}$ neurons. To determine if these changes in the $p130^{-/-}$ mice are accompanied by changes in receptor subunit expression, we first examined the relative abundance of α/γ subunits (BZ binding sites) and α/β subunits (GABA binding sites) (Günther *et al*., 1995) in the $GABA_A$ receptors of $p130^{+/+}$ and $p130^{-/-}$ mice, by measuring the binding of the BZ antagonist [^{3}H]Ro15–1788 and the GABA antagonist [^{3}H]SR95531 to intact hippocampal cells. Scatchard analysis of the binding data revealed no marked difference in the number of GABA binding sites or BZ binding sites between $p130^{+/+}$ and $p130^{-/-}$ mice (data not shown). This observation is in keeping with the GABA dose–response analysis (Figure 4E) that indicates no overt loss of cell surface receptor number in the $p130^{-/-}$ mice. We next examined the relative abundance of receptor subunits by immunoblotting analysis with antibodies

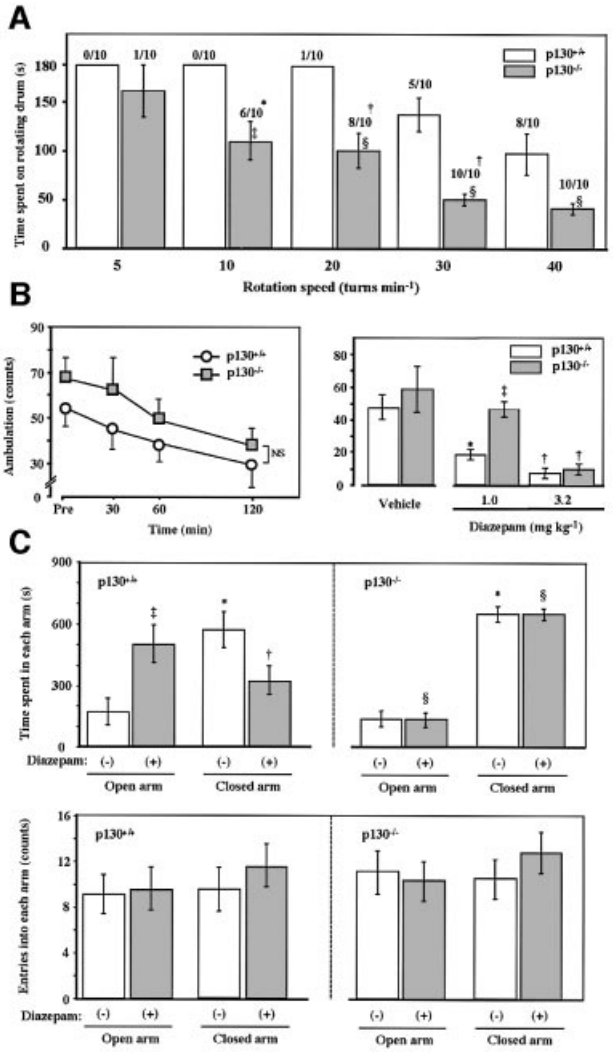

Fig. 5. Behavioral analysis of p130 knockout mice. (**A**) Rotarod test. Ten mice each of the $p130^{+/+}$ and $p130^{-/-}$ genotypes were examined for 3 min. The number of animals that fell from the rotating drum at the indicated speeds during the period of examination is shown at the top of each column. $^*p$ <0.05, $^\dagger p$ <0.01, versus the corresponding value for wild-type mice. Times spent on the rotating drum are presented as means ± SEM for all mice examined. $^\ddagger p$ <0.05, $^\S p$ <0.01 versus the corresponding value for wild-type mice. (**B**) Hall's open field test. Mice were placed in the ambulation chamber equipped with an infrared beam to count the number of crosses over 3 min. The left panel shows the number of crosses during 3 min beginning at the indicated time after placing the animals in the chamber. The right panel shows the number of crosses over 3 min, beginning 30 min after the intraperitoneal administration of diazepam or vehicle (0.3% carboxymethylcellulose). Data are means ± SEM of values from five mice per genotype. $^*p$ <0.05, $^\dagger p$ <0.01, versus the corresponding value for vehicle-treated mice; $^\ddagger p$ <0.01 versus the corresponding value for wild-type mice. (**C**) Elevated plus-maze test. The test was performed 30 min after the intraperitoneal administration of diazepam (1 mg/kg) (solid bars) or vehicle (open bars). Data are means ± SEM of the time spent in the open (upper panel), and the number of entries into the closed arms (lower panel) of the maze for seven $p130^{+/+}$ mice and six $p130^{-/-}$ mice. $^*p$ <0.01, versus the corresponding value for the open arm; $^\dagger p$ <0.05, $^\ddagger p$ <0.01, versus the corresponding value for vehicle-treated mice; $^\S p$ <0.01, versus the corresponding value for wild-type mice.

specific for α1, for β2 and β3, for γ1 and γ3, for γ2, or for δ subunits. No differences in the abundance of these subunits in both hippocampal and cortex cells were apparent between $p130^{+/+}$ and $p130^{-/-}$ mice (data not shown). Thus, the expression of the major $GABA_A$ receptor subunits in hippocampal and cortex neurons does not appear to be affected by the lack of p130.

Given the predominantly intracellular localization of p130, this may suggest a key role for this protein in $GABA_A$ receptor assembly.

Behavioral analysis

$GABA_A$ receptors are molecular substrates for the regulation of anxiety, as well as in sedation and motor coordination (Menard and Treit, 1999; Rudolph *et al*., 1999). We therefore compared the performance of $p130^{-/-}$ and wild-type mice in several behavioral tests. The rotarod test of motor coordination (Rudolph *et al*., 1999) revealed that such coordination was impaired in $p130^{-/-}$ mice, as reflected by both the incidence of falls and the time period before the first fall (Figure 5A). Hall's open field test of motor activity (Rudolph *et al*., 1999) also revealed that the ambulation counts of $p130^{-/-}$ mice were consistently greater than those of control mice, although the difference was not statistically significant (Figure 5B). The intraperitoneal injection of diazepam at a dose of 1 mg/kg of body mass did not induce a sedative effect in the p130 knockout mice, whereas it was clearly observed with the control mice, which is consistent with the reduced responsiveness to diazepam in I_{GABA}, but a higher dose of diazepam (3.2 mg/kg) induced sedation in mice of both genotypes (Figure 5B). Finally, we examined the effects of intraperitoneal injection of diazepam, whose anxiolytic actions are mediated by the γ subunit of $GABA_A$ receptors (Braestrup and Nielsen, 1980), on the behavior of $p130^{+/+}$ and $p130^{-/-}$ mice in the elevated plus-maze test, which provides a measure of anxiety (Rudolph *et al*., 1999). No significant difference in the time spent in the open or closed arms of the maze was apparent between $p130^{+/+}$ and $p130^{-/-}$ mice injected with vehicle. Injection of wild-type mice with diazepam (1 mg/kg) resulted in a marked increase in the time spent in the open arm, indicative of the anti-anxiety effect of this drug (Figure 5C). In contrast, the time spent by p130 knockout mice in the open or closed arms of the maze was not affected by injection of diazepam. The number of entries into the open or closed arms of the maze was not affected by diazepam administration in mice of either genotype, indicating that this dose of the drug did not impair spontaneous movements. However, injection of diazepam at a dose of 3.2 mg/kg severely reduced the spontaneous movements, similar to those seen in Hall's open field test, in mice of either genotype (data not shown). Our electrophysiological and behavioral studies thus indicated that $GABA_A$ receptor signaling through the γ subunit is impaired in $p130^{-/-}$ mice.

Discussion

This study was initiated by the finding that p130 interacts with GABARAP in a competitive manner with the γ2 subunit of $GABA_A$ receptors. To examine the significance of this interaction, we established gene-targeted mice devoid of p130. Interestingly, we found that there was no dramatic differences in the amplitude of I_{GABA} or GABA sensitivity in hippocampal neurons between the control and mutant mice. However, these mice showed impairments of $GABA_A$ receptor modulation by both Zn^{2+} modulation and benzodiazepines. Both of these pharmacological markers have been shown to be controlled by the presence of γ subunits in heteromeric α/β/γ receptors, as defined by recombinant and gene targeting studies (Günther *et al*., 1995; Gingrich and Burkat, 1998). Behavioral performance such as measured in ambulation and anxiety models showed no apparent difference in both genotypes of mice. Only the rotarod test showed a defect in the mutant mice, which might be caused by the accumulation of small (statistically insignificant) changes in I_{GABA} at several sites required for motor coordination.

Further analysis of the $p130^{-/-}$ mice revealed that there were no changes in the total number of binding sites for benzodiazepines and also that total levels of the receptor α1, β2–3, γ1–3 and δ subunits remained unaltered. Moreover, receptor density in the membranes of both strains of mice appeared to be equivalent. *In vivo*, most $GABA_A$ receptors are believed to be pentamers of α/β/γ subunits in a suggested ratio of 2:2:1 (Tretter *et al*., 1997). Benzodiazepines are believed to act at the interface of α/γ subunits, while Zn^{2+} acts at sites within β subunits. Therefore, one plausible mechanism behind the pharmacological changes in $p130^{-/-}$ mice is that this protein plays a role in the assembly of heteromeric receptors, perhaps by controlling the number and type of subunits recruited into functional pentamers. A role for p130 in receptor assembly was first indicated by the results of our yeast two-hybrid screen in which we isolated GABARAP. This protein also binds the γ2 subunit of $GABA_A$ receptors, microtubules and *N*-ethylmaleimide-sensitive factor (NSF) (Wang *et al*., 1999; Wang and Olsen, 2000; Kittler *et al*., 2001). GABARAP is homologous to GATE-16, which has been demonstrated to facilitate vesicular transport within the *trans*-Golgi network. Like GATE-16, recent high resolution studies have revealed that GABARAP is enriched on intracellular membranes, including the *trans*-Golgi network (Kittler *et al*., 2001). The predominant intracellular localization of p130 and its co-localization with GABARAP suggests that p130 may participate in $GABA_A$ receptor membrane trafficking or receptor assembly. For instance, the competitive binding of p130 and GABARAP for the receptor γ2 subunit may ensure that only mature α/β/γ receptor pentamers are transported to the cell surface. Therefore, p130 may play a central role in controlling the number and/or types of γ2 subunit that assemble into functional α/β/γ receptors, with consequent effects on receptor pharmacology. Alternatively, if GABARAP participates in the endocytic pathway as suggested for its homolog in yeast (Okazaki *et al*., 2000), proteolysis of incorrectly folded γ2 subunits by such organelles might be enhanced by p130. Clearly more experimental evidence is required to examine the role of p130 in $GABA_A$ receptor biosynthesis and membrane transport.

Our yeast two-hybrid analysis also revealed that p130 interacts with the catalytic subunit of protein phosphatase 1 (Yoshimura *et al*., 2001). Oligomeric receptor-associated ion channels in neurons are regulated by phosphorylation and dephosphorylation of various subunits (Swope *et al*.,

1999); this is indeed the case for the $GABA_A$ receptor (Brandon *et al*., 2000; Moss and Smart, 2001). Receptor phosphorylation or dephosphorylation requires that the relevant protein kinase or phosphatase be targeted to the receptor. For example, Yotiao (Westphal *et al*., 1999) and spinophilin (neurabin-II) (Yan *et al*., 1999) are thought to mediate the targeting of PP1 to NMDA (*N*-methyl-D-aspartate)- and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-sensitive glutamate receptors, respectively. It is thus possible that p130 performs an analogous function for $GABA_A$ receptors.

The human p130 gene has been identified and termed PLC-L (Kohno *et al*., 1995), and genes (~70%) homologous to PLC-L have been isolated from mouse and human and termed PLC-L2 (Kikuno *et al*., 1999; Otsuki *et al*., 1999). Like rat p130 (Kanematsu *et al*., 1992; Yoshida *et al*., 1994), the native or recombinant proteins encoded by these genes do not exhibit PLC activity (Kohno *et al*., 1995; Kikuno *et al*., 1999; Otsuki *et al*., 1999). The presence of a similar gene in *Caenorhabditis elegans* suggests that this PLC-related, catalytically-inactive protein (here designated PRIP) family diverged from other PLC families early during evolution (Koyanagi *et al*., 1998). The PRIP family consists of at least two types of protein: type 1 is represented by p130 (Kanematsu *et al*., 1992; Yoshida *et al*., 1994; Kohno *et al*., 1995) and type 2 by PLC-L2 (Kikuno *et al*., 1999; Otsuki *et al*., 1999). It remains to be determined whether the type 2 proteins are also able to bind GABARAP and the catalytic subunit of protein phosphatase 1.

Finally, our studies have identified a novel role for p130 in the functional expression of $GABA_A$ receptors. The mechanism behind this remains to be determined, but as p130 is found in intracellular compartments, this strongly suggests that this protein participates in receptor assembly and transport of mature receptors to the plasma membrane.

Materials and methods

Yeast two-hybrid analysis

The *Xho*I–*Sma*I fragment (nucleotides 535–1359) of plasmid pcMT3, which contains the rat p130 cDNA (Kanematsu *et al*., 1996), was introduced into the yeast vector pGBT9 (*Sal*I and blunt-ended *Pst*I sites) to yield the bait plasmid encoding p130(24–298). Yeast cells were co-transformed with the bait plasmid and a human brain cDNA library in pACT2 (Clontech). Positive clones whose products interacted with p130 in both histidine and β-galactosidase assays were identified.

In vitro precipitation assay

The cDNAs for full-length GABARAP and the intracellular loop (residues 343–404) of the γ2S subunit of the $GABA_A$ receptor were amplified by RT–PCR with total RNA from human brain as template. The resulting GABARAP and γ2S subunit cDNAs were introduced into the *Bam*HI–*Eco*RI sites of pGEX-2T (Pharmacia) and the *Ecl*136II–*Sal*I sites of pETHis_6-30 [pET11 (Novagen) with an added sequence for a histidine tag], respectively. The recombinant proteins were expressed in *Escherichia coli* and purified by affinity chromatography. Recombinant His_6-p130 was prepared with a baculovirus expression system (Kanematsu *et al*., 2000; Takeuchi *et al*., 2000). Incubation of p130 or γ2S recombinant proteins with GST–GABARAP or GST alone for 1 h at 4°C was followed by the addition of glutathione–Sepharose 4B (Amersham Pharmacia Biotech.). Proteins that bound to the beads were analyzed by SDS–PAGE, followed by an immunoblot analysis with a mouse monoclonal antibody to p130 (2F9) (Yoshida *et al*., 1994), or antibodies to the His_6 tag (Qiagen).

Immunofluorescence analysis

Cortical neurons isolated from embryonic day 18 embryo were maintained for 21 days in medium that inhibits the growth of glia. The cultured cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and incubated first with 10% horse serum and then with goat antibody to GABARAP (Santa Cruz Biotech), and rabbit polyclonal antibody to p130. The cells were then exposed to Texas Red-conjugated donkey antibodies (red) to goat immunoglobulin and FITC-conjugated donkey antibodies (green) to rabbit immunoglobulin, and then viewed with a confocal microscope.

Generation of p130 knockout mice

Mouse p130 genomic DNA was isolated from a 129/Sv DNA library (Stratagene) by screening with the *Xho*I–*Sac*I fragment of pcMT3 (Kanematsu *et al*., 1992). The targeting vector was constructed by inserting a blunt-ended *Eco*RV–*Bam*HI fragment (1.1 kb) and an *Eco*RI–*Eco*RI fragment (6.3 kb) of the mouse p130 gene into the blunt-ended *Hin*dIII site and *Eco*RI site, respectively, of PGKNeo/PGKTK pBluescriptII (Stratagene). Four positive clones (out of a total of 288 clones analyzed) were obtained, and two of these clones (EW3 and EW17) were injected into C57/BL6 blastocysts. Chimeric males derived from each clone were crossed with C57/BL6 females. The resulting heterozygous mice were intercrossed to obtain wild-type ($p130^{+/+}$), heterozygous mutant ($p130^{+/-}$), and homozygous mutant ($p130^{-/-}$) mice. Animal experiments were reviewed by the Committee of the Ethics on Animal Experiments in the Faculty of Medical Science, Kyushu University and carried out under the control of the Guideline for Animal Experiments in the Faculty of Medical Science, Kyushu University and The Law (No. 105) and Notification (No. 6) of the Government.

Electrophysiological analysis

Hippocampal CA1 cells were dissociated without the use of enzymes from the brains of $p130^{+/+}$ and $p130^{-/-}$ 10- to 14-day-old mice. Electrophysiological measurements were performed with nystatin-perforated membrane patches at a holding potential of –60 mV under voltage-clamp conditions, as described previously (Rhee *et al*., 1999).

Behavioral analysis

The rotarod test, Hall's open field test, and the elevated plus-maze test were performed to provide measures of motor coordination, sedation and anxiolytic activity, respectively (Rudolph *et al*., 1999). Mice ($p130^{+/+}$ and $p130^{-/-}$) were subjected to the tests at 8 to 11 weeks of age. Statistical analysis was performed using the Mann–Whitney *U* test.

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