A peroxisome deficiency–induced reductive cytosol state up-regulates the brain-derived neurotrophic factor pathway

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The peroxisome is a subcellular organelle that functions in essential metabolic pathways, including biosynthesis of plasmalogens, fatty acid β-oxidation of very-long-chain fatty acids, and degradation of hydrogen peroxide. Peroxisome biogenesis disorders (PBDs) manifest as severe dysfunction in multiple organs, including the central nervous system (CNS), but the pathogenic mechanisms in PBDs are largely unknown. Because CNS integrity is coordinately established and maintained by neural cell interactions, we here investigated whether cell–cell communication is impaired and responsible for the neurological defects associated with PBDs. Results from a noncontact coculture system consisting of primary hippocampal neurons with gial cells revealed that a peroxisome-deficient astrocytic cell line secretes increased levels of brain-derived neurotrophic factor (BDNF), resulting in axonal branching of the neurons. Of note, the BDNF expression in astrocytes was not affected by defects in plasmalogens biosynthesis and peroxosomal fatty acid β-oxidation in the astrocytes. Instead, we found that cytosolic reductive states caused by a mislocalized catalase in the peroxisome-deficient cells induce the elevation in BDNF secretion. Our results suggest that peroxisome deficiency dysregulates neuronal axogenesis by causing a cytosolic reductive state in astrocytes. We conclude that astrocytic peroxisomes regulate BDNF expression and thereby support neuronal integrity and function.

The peroxisome represents a ubiquitous and essential subcellular organelle engaged in a variety of metabolic pathways, including biosynthesis of ether-phospholipids, β-oxidation of very-long-chain fatty acid (VLCFA), α-oxidation of branched-chain fatty acid, and degradation of n-aminocids (1). Among these metabolic pathways, there are a number of oxidases that use O2 to oxidize substrates and generate H2O2, such as acyl-CoA oxidases (AOX) and n-aminocid oxidases (2). Catalase is a peroxisomal tetrameric matrix enzyme that catalyzes degradation of H2O2. The physiological importance of peroxisomal metabolisms is demonstrated by severe pathological manifestations in peroxisome biogenesis disorders (PBDs). PBDs are caused by the defect of PEX genes encoding peroxisome biogenesis factors, peroxins (Pex). Zellweger spectrum disorders (ZSDs), accounting for about 80% of PBD patients, are classified into three groups according to their clinical severity: Zellweger syndrome (ZS), neonatal adrenoleukodystrophy, and infantile Refsum disease (IRD) (3). Patients with ZS, the most severe PBD, manifest severe impairment in the central nervous system (CNS), such as migration defect of cortical neurons, abnormal morphology of Purkinje cells, and dysplasia of inferior olivary nucleus (ION) (3–6). Several Pex-deficient ZS model mice show the impairments of the CNS, as
observed in ZS patients (7–10). Moreover, in the Nes-Pex5−/− mouse, the dysfunction of peroxisomes in all neural cells, including neurons, oligodendrocytes, and astrocytes, gives rise to abnormal development and aberrant morphology of CNS (11, 12). However, brain cell type–specific (i.e. projection neuron-, astroglia-, or oligodendrocyte-specific) knockout of Pex5 does not show abnormal CNS development (13, 14), suggesting that supportive effects among different brain cell types are responsible for normal development in the mutant mice (13).

Very recently, we reported that the Pex14ΔC/ΔC mouse, a ZS model mouse, shows up-regulation of brain-derived neurotrophic factor (BDNF) in the neurons of ION and the elevation of a truncated form of its receptor, TrkB-T1, on Purkinje cells in the cerebellum (10). The malformation of Purkinje cells in the Pex14ΔC/ΔC mouse is caused by a combination of elevated BDNF and prominent expression of TrkB-T1 (10). Astrocytes, the most abundant cell type in the CNS, are engaged in divergent metabolic reactions and neuronal development (15), and neuron-astrocyte interaction plays a pivotal role in CNS integrity (16). Therefore, a cell co-culture system composed of two distinct types of brain cells, including neurons and astrocytes, might serve as a potential way to address the pathogenic mechanisms underlying abnormal development of neuronal cells.

In the present study, to uncover the pathological mechanism underlying PBDs, we focused on searching for soluble factors, if any, that influence neuronal development, by using a co-culture system of primary hippocampal neurons with glial cells (17, 18). We found that peroxisome deficiency in astrocytes elevated the expression and secretion of BDNF, leading to the axonal branching of hippocampus neurons. We also show that the cytosolic reductive condition, but not the defects of peroxisomal β-oxidation and plasmalogen biosynthesis, is involved in the up-regulation of Bdnf mRNA. Therefore, these results suggest a new function of astrocytic peroxisomes in regulating BDNF expression for the neuronal integrity.

Results

Establishment of peroxisome-deficient cultured astrocytes

To identify soluble factor(s) regulating neuronal development, we attempted to establish a peroxisome-deficient RCR-1 cell line, an astrocyte-like cultured cell line derived from rat embryonic cerebellum (19). To abrogate peroxisome biogenesis in RCR-1 cells, we designed two types of dominant-negative forms of Pex proteins, including C-terminally truncated Pex5p(1–243) (20) fused to a yellow fluorescent protein Venus (21), termed Pex5p-DN, and the N-terminal soluble region (residues 1–93) of Pex14p containing the Pex5p-binding domain (22) fused to Venus, named Pex14p-DN (Fig. S1A). Both truncated forms were anticipated to target the endogenous Pex5p-cargo complex to Pex14p, resulting in perturbation of the peroxisomal matrix protein import (Fig. S1B). We established three RCR-1 cell lines, each stably expressing Pex5p-DN (RCR-1/Pex5p-DN), Pex14p-DN (RCR-1/Pex14p-DN), and Venus (RCR-1/Venus). Peroxisomal matrix protein import was assessed by immunofluorescent microscopy with antibody against peroxisome-targeting signal 1 (PTS1), which recognizes a dozen peroxisomal proteins, including AOx and trifunctional protein (23). Import of PTS1 proteins and catalase was severely impaired by the expression of the truncated mutant of Pex5p or Pex14p, but not Venus (Fig. 1, A and B). Because several PTS1 proteins are unstable in the cytosol of peroxisome biogenesis–defective cells (24, 25), fluorescent intensity of PTS1 proteins appeared to be decreased in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN cells (Fig. 1A). Subcellular fractionation analysis revealed that almost all catalase was localized in the cytosol (Fig. 1, C and D). Intraperoxisomal processing of AOx and alkylidihydroxyacetone phosphate synthase (ADAPS) was attenuated, and AOx A-chain and the larger precursor of ADAPS were detected both in cytosol and organelle fractions from RCR-1/Pex5p-DN and RCR-1/Pex14p-DN cells (Fig. 1C). To verify whether organelle-associated AOx and ADAPS precursor were transported into peroxisomal matrix, we performed protease protection analysis. Proteinase K treatment of post-nuclear supernatant (PNS) fraction leads to the marked reduction of AOx A-chain and ADAPS precursor in the organelle fraction (Fig. S1C, lanes 10 and 16), suggesting that only a part of AOx and ADAPS was translocated into the peroxisomal matrix (Fig. S1C and Fig. 1A (e and f)). In the presence of Triton X-100, AOx and ADAPS were digested by the protease treatment (Fig. S1C, lanes 5, 6, 11, 12, 17, and 18). Consistent with these results, marked reduction of plasmalogens (PlsEtn; Fig. 1E) and accumulation of VLCFA-containing phosphatidylcholine (VLCPC; Fig. 1F) were evident. Therefore, peroxisomal biogenesis and metabolism were attenuated in the RCR-1 cells stably expressing Pex5p-DN or Pex14p-DN.

Co-culture of peroxisome-deficient astrocytes with primary hippocampal neurons

To examine the effect of astrocytic peroxisome deficiency on neural development, we established a cell co-culture system (Fig. 2A). Using this co-culture system, we analyzed neuronal morphology in the early developmental stage at 2 days in vitro (DIV) (26). Neurons cultured with RCR-1/Venus as feeder cells extended a single primary axon (Fig. 2B, a and b). By contrast, neurons cultured with RCR-1/Pex5p-DN or RCR-1/Pex14p-DN as feeder cells developed aberrantly elongated collateral branches (Fig. 2B, c, d, g, and h) with tertiary and quaternary branches (Fig. 2B, e, f, i, and j, arrowheads). We also analyzed the axonal morphology with anti-Tau-1 antibody, which recognizes the primary axons (Fig. S2A, arrows). From the primary axon with higher fluorescent intensity of Tau-1 staining, axonal collaterals more frequently emerged in the neurons co-cultured with peroxisome-deficient RCR-1 cells (Fig. S2A, arrowheads). We performed statistical analyses on the morphology of the neurons. The neurons isolated from the other cells were selected, and their longest processes were considered axons. In the neurons co-cultured with RCR-1/Pex5p-DN or RCR-1/Pex14p-DN, no significant difference in the axon length was observed (Fig. 2C). By contrast, the percentages of neurons with branched axons were increased (Fig. 2D). Elongation of the collaterals was also promoted (Fig. 2E) and collaterals per 100 μm of axon length were more frequent (Fig. 2F) in the neurons co-cultured with peroxisome-deficient...
RCR-1 cells as feeder cells than in those with control feeder cells. The formation of axonal collaterals was also evoked in neurons cultured in conditioned medium (CM) that had been prepared from peroxisome-deficient astrocytes (Fig. 2, G–J). Therefore, the secretion of factor(s) required for neuronal axon development is most likely dysregulated in astrocytes defective in peroxisome biogenesis.

To investigate whether cytoskeletal structure in the neurons was affected, actin filaments and microtubules were stained with fluorescent phalloidin and antibody to βH9251-tubulin, respectively. Fluorescent phalloidin staining indicated actin cytoskeleton protruding from the axon of neurons, upon their co-culture with peroxisome-deficient RCR-1/Pex5p-DN cells, whereas there was no obvious difference in microtubule structure between normal and RCR-1/Pex5p-DN cells (Fig. S2B). Thus, reorganization of actin cytoskeleton was properly promoted in the neurons co-cultured with the RCR-1 cells defective in peroxisomal biogenesis.

Elevation of BDNF in peroxisome-deficient astrocytes

Of the molecules involved in axon development, we focused on neurotrophic factors, including nerve growth factor (NGF), BDNF, glia-derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), and NT-4 (27). We recently demonstrated that a defect of peroxisome biogenesis in a neuroblastoma cell line, SH-SY5Y, leads to the up-regulation of BDNF, but not other factors including NGF, NT-3, and NT-4 (10). To assess mRNA levels of neurotrophins, RCR-1 cells were cultured in Neurobasal medium, which was used in a co-culture system, for 2 days, and then total RNA was extracted. Similarly to SH-SY5Y cells, Bdnf mRNA, not Ngf and Nt-3 mRNA, was elevated in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN cell lines, as assessed by RT-PCR (Fig. 3A). The expression of Gdnf and Nt-4 was below detectable levels (data not shown). In real-time RT-PCR, the expression of Bdnf was 3–4-fold increased in both types of peroxisome-deficient RCR-1 cell lines compared with control RCR-1 cells (Fig. 3B). The level of secreted mature BDNF from the peroxisome-deficient astrocytes was also increased (Fig. 3C). To investigate whether the elevated BDNF induces axon branching, CM from cells expressing recombinant BDNF (rBDNF) was diluted and added to the culture medium of primary hippocampal neurons (Fig. 3D). The number of axon collaterals was increased by the addition of rBDNF in a
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A

Glia cells

Conditioned medium

Embryonic hippocampal primary neurons (E18.5)

2 DIV

Microscopy analysis

Paraffin balls

φ32 mm cover glass

B

<Primary neurons>

Venus

Pex5p-DN

Pex14p-DN

a

c

e

g

b

d

f

h

i

j

C

Axon length (µm)

n.s.

n.s.

Venus

Pex5p-DN

Pex14p-DN

coculture:

Venus

Pex5p-DN

Pex14p-DN

D

Neuron with collaterals (%)

***

**

Venus

Pex5p-DN

Pex14p-DN

coculture:

Venus

Pex5p-DN

Pex14p-DN

E

% of total neurons

Total collateral length (µm)

-40 -30 -20 -10 0 10 20 30 40

Venus

Pex5p-DN

Pex14p-DN

coculture:

Venus

Pex5p-DN

Pex14p-DN

F

Collateral number /100 µm axon

***

***

Venus

Pex5p-DN

Pex14p-DN

coculture:

Venus

Pex5p-DN

Pex14p-DN

G

Axon length (µm)

n.s.

n.s.

Venus

Pex5p-DN

Pex14p-DN

CM:

Venus

Pex5p-DN

Pex14p-DN

H

Neuron with collaterals (%)

***

***

Venus

Pex5p-DN

Pex14p-DN

CM:

Venus

Pex5p-DN

Pex14p-DN

I

% of total neurons

Total collateral length (µm)

-40 -30 -20 -10 0 10 20 30 40

Venus

Pex5p-DN

Pex14p-DN

CM:

Venus

Pex5p-DN

Pex14p-DN

J

Collateral number /100 µm axon

***

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Venus

Pex5p-DN

Pex14p-DN

CM:

Venus

Pex5p-DN

Pex14p-DN
concentration-dependent manner, without elongation of the neuronal axon (Fig. 3, E–H).

BDNF positively regulates axonal outgrowth via binding to TrkB receptor on the neuronal surface (27). Knockdown of TrkB alone (Fig. 4A) affected neither axon elongation nor collateral formation under the culture conditions with CM prepared from RCR-1/Venus (Fig. 4, B–E). By contrast, in the presence of CM from peroxisome-deficient RCR-1 cells, the promotion of collateral formation, but not axon elongation, was markedly lowered in the primary neurons with a significantly reduced level of TrkB (Fig. 4, B–E). The collateral generation in the CM from peroxisome-deficient cells was also decreased by inhibiting BDNF targeting to the TrkB with the recombinant extracellular domain of the p75 neurotrophic receptor

Figure 2. Peroxisome-deficient astrocytes increase neuronal axon branching. A, schematic view of the neuron-astrocyte co-culture system. Astrocyte cells were seeded on a paraffin ball–attached coverglass and cultured for 2 days in Neurobasal medium. Primary hippocampal neurons isolated from a rat at E18.5 were seeded on glass-bottom dishes. CM and astrocytes were inverted over the primary neurons and cultured for 2 DIV. B, primary hippocampal neurons were co-cultured for 2 days with RCR-1/Venus (a and b), RCR-1/Pex5p-DN (c–f), and RCR-1/Pex14p-DN (g–j) as feeder cells. Higher-magnification images (e, f, i, and j) of boxed regions in c, d, g, and h, respectively, are shown. Arrows and arrowheads, primary axons and axonal collaterals, respectively. Scale bar, 10 µm. C–F, statistical analyses of neuronal axon length (C), percentage of neurons with axon collateral branching (D), histogram of total collateral length (E), and the number of collaterals per 100-µm axon (F) are shown (n > 40 cells; 3–4 cultures each). G–J, CM was obtained from astrocytes that had been plated at a density of 6.5 × 10^6 cells/cm^2 and cultured in Neurobasal medium for 4 days. Primary hippocampal neurons were incubated only in the collected CM for 2 DIV. Statistical analyses are shown as in C–F (n > 50 cells; 3–4 cultures each). Axon length (C and G) and number of collaterals per 100-µm axon (F and J) are represented by a set of box plots and dot plots. The mean values are shown as solid dots. n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001, by Dunnett’s test (B, C, E, and H); χ^2 test (F). Error bars, S.D.

Figure 3. Elevation of BDNF secretion from peroxisome-deficient astrocytes causes neuronal axon branching. A, mRNA levels of neurotrophins and Gapdh were assessed by RT-PCR using total RNAs from RCR-1/Venus, RCR-1/Pex5p-DN, or RCR-1/Pex14p-DN cultured for 2 days in Neurobasal medium. Gapdh was used as an internal control. B, Bdnf mRNA level was determined by real-time PCR (n = 3). C, CM containing secreted BDNF was prepared as in Fig. 2 (G–J) and concentrated by ammonium sulfate precipitation, and BDNF was detected by SDS-PAGE and immunoblot analysis with an anti-BDNF antibody. A molecular size marker in kDa is shown on the left. The BDNF band was quantified as described under “Experimental procedures” (bottom, n = 3). D–H, CM from nontransfected RCR-1 (–) cells and RCR-1 cells stably expressing rBDNF was collected. Serial 10-fold dilutions (10⁻² to 10⁻⁹) of rBDNF-containing CM were made up by adding CM from RCR-1 (–), and each was added to the culture of primary hippocampal neurons (E18.5) for 2 DIV as in Fig. 2 (A and B). D, secreted rBDNF and BDNF secreted to the CM from RCR-1 (–), RCR-1/Venus, and RCR-1/Pex14p-DN were analyzed as in C.* nonspecific band. BDNF levels relative to that in the CM from RCR-1 (–) are indicated at the bottom. The band at the lane “10⁻² dilution” was saturated, indicating that its actual level is higher than the measured value of “40”. Molecular mass markers were loaded in lane M, and their migrations were dotted at 10 and 15 kDa (dots). E–H, statistical analyses of axonal morphology were performed as in Fig. 2 (C–F) (n > 50 cells; 3–4 cultures each). Axon length (E) and number of collaterals per 100-µm axon (H) are represented by a set of box plots and dot plots. The mean values are shown as solid dots. n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001, by Dunnnett’s test (B, C, E, and H); χ^2 test (F). Error bars, S.D.
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Figure 4. TrkB-dependent axonal branching on hippocampus neurons. Primary hippocampal neurons (E18.5) were treated with a control siRNA (siControl) or siRNAs against TrkB (#1 and #2) and then cultured in CM from RCR-1/Venus or RCR-1/Pex14p-DN for 2 DIV. A, the expression level of TrkB mRNA was determined by real-time PCR (n = 3). (B–E) Statistical analyses of axonal morphology were performed as in Fig. 2 (n > 30 cells, two cultures each). Axon length (B) and number of collaterals per 100-μm axon (C) are represented by a set of box plots and dot plots. The mean values are shown as solid dots. n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001, by Dunnett’s test (A); Tukey-Kramer test (B and E); χ² test (C). Error bars, S.D.

(p75ECD-His, Fig. S3A), another receptor for BDNF (27) (Fig. S3, B–E). Taken together, our results suggested that the increase in secreted BDNF from peroxisome-deficient astrocytes gives rise to aberrant branching of neuronal axons mediated by TrkB. Because p75ECD-His also binds to other neurotrophins, such as NGF, NT-3, and NT-4, in CM (27), we cannot exclude the possibility that inhibition of axonal elongation (Fig. S3B) might be owing to the effect of p75ECD-His on these factors.

Involvement of cytosolic catalase in regulation of BDNF expression in RCR-1 cells

To investigate how deficiency in peroxisome biogenesis enhances the Bdnf expression, several peroxisomal metabolisms, including synthesis of plasmalogens and fatty acid β-oxidation, were assessed. Restoration of plasmalogens in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN by treatment with hexadecylglycerol (28) did not alter the elevated Bdnf expression (Fig. S4, A and B), suggesting that plasmalogens were not involved in the regulation of Bdnf expression. Next, the deficiency of peroxisomal fatty acid β-oxidation in RCR-1 cells was induced by knockdown of Alox, the first-step enzyme in the β-oxidation, using short hairpin RNA for the Alox gene (shAlox). Alox proteins were markedly reduced (Fig. S4C), resulting in the accumulation of VLCPC (Fig. S4D) in RCR-1 cells stably expressing shAlox. However, there is no increment of the Bdnf mRNA level in Alox-depleted cells (Fig. S4E), suggesting that the up-regulation of Bdnf mRNA level is not induced by the impaired peroxisomal fatty acid β-oxidation. Interestingly, Bdnf expression was lowered by treatment with 20 mM 3-amino-1,2,4-triazole (3AT), a catalase inhibitor, of the peroxisome-deficient RCR-1 cells, RCR-1/Pex5p-DN and RCR-1/Pex14p-DN (Fig. 5A). At 60 mM 3AT, BDNF mRNA levels in both types of peroxisome-deficient cells were further lowered to that in untreated control cells (Fig. 5A). To investigate whether catalase in peroxisome-deficient cells is involved in the elevation of Bdnf mRNA, catalase was knocked down by siRNA treatment (Fig. 5E). In peroxisome-deficient cells, although catalase expression level was reduced, it remained in the cytosolic fraction (Fig. S5, lanes 7 and 11). Real-time PCR analysis revealed that the knockdown of catalase decreased the mRNA level of Bdnf in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN (Fig. 5F). Because catalase was localized in the cytosol of peroxisome-deficient RCR-1 cells (Fig. 1, B–D), cytosolic catalase was suggested to be involved in the up-regulation of Bdnf expression. Catalase possesses PTS1-like signal, KANL at the C terminus, whose binding affinity to Pex5p is lower than canonical PTS1 signal, such as SKL (29). We generated RCR-1 cells stably expressing C-terminal ANL-deleted catalase (catalase-ΔC). RCR-1/catalase-ΔC showed the elevation of the Bdnf expression (Fig. 5G). Inactivation of catalase-ΔC by treatment with 20 mM 3AT or mutation of the active site His75 (30) to Ala (catalase-ΔC-mut) significantly reduced the Bdnf expression (Fig. 5G), implying the requirement of enzymatically active cytosolic catalase. Both catalase-ΔC and catalase-ΔC-mut were mostly localized in the cytosol, but partially detected in organelle fractions (Fig. 5, H and I). Because a catalase tetramer is preassembled in the cytosol prior to peroxisomal import, a part of both catalase-ΔC and catalase-ΔC-mut are likely to form a tetramer with an endogenous catalase monomer, being imported to peroxisomes by a "piggyback" transport mechanism (29, 31). Taken together, these results suggested that cytosolic catalase is essential for the elevation of Bdnf expression.

We recently reported that knockdown of PEX5 in a neuroblastoma cell line, SH-SY5Y, induced the up-regulation of
BDNF mRNA (10). The treatment with 3AT of siPEX5-transfected SH-SY5Y cells reduced the level of BDNF mRNA to that in control cells (Fig. S6), suggesting that the up-regulation of BDNF in peroxisome-deficient SH-SY5Y cells is induced by the cytosolically mislocalized catalase.

**Defect of peroxisome biogenesis induces cytosolic redox state**

We earlier reported that the redox state in the cytosol of peroxisome-deficient mutants is more reductive than that of WT cells and that catalase inhibitor, 3AT, induces the cytosolic oxidative state (32), both implying the involvement of cytosolic catalase in the reductive state of peroxisome-deficient mutants. The cytosolic reductive state in peroxisome-deficient mutants is also indicated by increase of the ratio of reduced glutathione (GSH) to oxidized GSH (GSSG). To examine this, we determined the levels of GSH and other reductive compounds, including NADH and NADPH, by LC coupled with tandem MS (LC-MS/MS). The cell line pex1 ZP107 is defective in peroxisomal matrix protein import (33). As compared with WT TKa cells, GSH redox index reflecting GSH redox potential, $E_{\text{GSH}}$ (34), was relatively elevated in pex1 ZP107 cells (Fig. 6A). NADH/NAD$^+$ and NADPH/NADP$^+$ levels in ZP107 cells were also higher than those in control cells (Fig. 6, B and C). To investigate the effect of restoration of peroxisome biogenesis on the reductive state in the mutant cell, we assessed the reductive metabolites in pex1 ZP107 cells complemented by stably expressing human PEX1, termed ZP107/PEX1 (33). Levels of GSH redox index, NADH/NAD$^+$, and NADPH/NADP$^+$ in ZP107/PEX1 were respectively lowered to the levels in WT TKa cells (Fig. 6A–C). To assess peroxisomal lipid metabolism, we also determined the levels of plasmalogens and VLCPC in the mutant cells. Plasmalogens were markedly decreased, and VLCPC was accumulated in pex1 ZP107 cells (Fig. 6, D and E), consistent with other PEX-deficient CHO mutant cells, including pex2 Z65 and pex19 Z65 (35). The defect of peroxisomal lipid metabolism in pex1 ZP107 cells was restored by complementation with PEX1 expression (Fig. 6, D and E). These results...
demonstrated that peroxisome biogenesis deficiency induces cytosolic reductive states.

**BDNF expression is regulated by cytosolic redox state**

We next analyzed the redox states in peroxisome-deficient RCR-1 cells by LC-MS/MS. Similarly to pex1 CHO ZP107 cells, GSH redox index, NADH, and NADPH were relatively increased in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN (Fig. 7, A and B), confirming that the cellular reductive condition was induced by peroxisome deficiency. Notably, treatment with a catalase inhibitor, 3AT, decreased the reduced forms of metabolites in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN cells (Fig. 5, B–D), as shown above in CHO mutant cells (Fig. 6)(32). Therefore, cytosolic catalase is most likely responsible for the reductive condition in peroxisome-deficient cells. To investigate whether a redox state regulates the Bdnf expression, reductive state was induced in RCR-1/Venus cells by treatment with n-acetyl cysteine (NAC), a precursor of GSH. Because the treatment with NAC in Neurobasal medium was cytotoxic (data not shown), DMEM/F-12 medium supplemented with 1% FBS was used instead of Neurobasal medium. Bdnf mRNA was indeed up-regulated by the NAC treatment (Fig. 7C). LC-MS/MS analysis revealed that GSH redox index and NADH/NAD+ ratio, not NADPH/NADP+ ratio, were elevated by the NAC treatment (Fig. 7, D and E), consistent with the earlier reports describing the increase of GSH (36) and NADH/NAD+ ratio (37), not NADPH/NADP+ ratio (38). Inversely, to induce the oxidative state, the cells were treated with pyruvate that oxidizes NADH to NAD+ by the lactate dehydrogenase (LDH) pathway (39, 40). Pyruvate treatment indeed gave rise to decrease in NADH/NAD+, thereby reducing the level of BDNF expression in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN (Fig. 7, F and G). Inhibition of the complex I with rotenone showed no effect on the BDNF mRNA (Fig. 7H), suggesting that the cytosolic NADH/NAD+ ratio, not the mitochondrial ratio, regulates BDNF expression. Reducing the level of NADH by treatment with FK866, an inhibitor of nicotinamide phosphoribosyltransferase, induced the down-regulation of Bdnf mRNA (Fig. 7I). Taken together, these results suggest that Bdnf expression is regulated by the cytosolic state of the NADH/NAD+ ratio.

**Discussion**

In this report, we revealed that a defect of peroxisome biogenesis elevates the expression and secretion of BDNF in the glial cell line. Secreted BDNF leads to the axonal branching of primary hippocampal neurons. Up-regulation of BDNF is likely induced by cytosolically mislocalized catalase in a manner dependent on reductive metabolites, including GSH, NADH, and NADPH, in peroxisome-deficient RCR-1 and SH-SY5Y cells. Therefore, these results suggest that the impaired peroxisome biogenesis–dependent cytosolic redox state affects the neuronal morphology via modulating the BDNF expression in glial cells.

Redox state of peroxisome-deficient cells is more reductive than that in the normal cells, such as CHO and RCR-1 cell lines (Figs. 6 and 7), as we earlier demonstrated with a redox state probe, Redoxfluor, using a pex5 CHO mutant, ZP105 (32). This suggests that peroxisomes are also involved in the homeostasis of cellular redox state. Because the redox state becomes oxidative by the exposure of the cells to 3AT, a catalase inhibitor (32), the reductive condition of cytosol in peroxisome-deficient cells, including CHO pex1 mutant and RCR-1 stably expressing dom-

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**Figure 6. Defect of peroxisome biogenesis induces cytosolic reductive states in CHO cells.** A–C, cellular levels of GSH, GSSG, NADH, NAD+, NADPH, and NADP+ in TKa, pex1 ZP107, and ZP107/PEX1 cells were analyzed by LC-MS/MS. A, GSH redox index was calculated for each cell line, along with the differences in the redox potential of GSH (ΔE<sub>GSH</sub>) in ZP107 and ZP107/PEX1 cell lines relative to that in TKa cell line as under “Experimental procedures.” Relative levels of NADH/NAD+ (B) and NADPH/NADP+ (C) are represented by taking as 1 the levels in TKa cells (n = 3), D and E, total amounts of plasmalogens (D) and VLCPC were analyzed by LC-MS/MS (n = 3). *, p < 0.05; **, p < 0.01; ***, p < 0.001, by Tukey–Kramer test. Error bars, S.D.
NADH, NAD

dation of H2O2 by cytosolic catalase likely competes with that is detected in the cytosol (41–43). Cytosolic H2O2 is mainly in peroxisomes, whereas a part of enzymatically active catalase

together, the decrease of cytosolic H2O2 caused by mislocalized production of the reduced form of NADH and NADPH. Taken closely linked (45), the increase of GSH more likely leads to GSH. Because redox ratios of GSH, NADH, and NADPH are closely linked (45), the increase of GSH more likely leads to production of the reduced form of NADH and NADPH. Taken together, the decrease of cytosolic H2O2 caused by mislocalized catalase and the elevation of reductive compounds, including GSH, NADH, and NADPH (Figs. 6 (A–C) and 7 (A and B)), most likely induce the reduction of the cytosolic state in peroxi-

in-ant-negative forms of Pex proteins, is likely owing to the cyto-
solic catalase. Catalase is a tetrameric peroxisomal matrix enzyme that catalyzes degradation of H2O2 generated by peroxi-
osomal oxidases, including AOx and d-α-amino acid oxidase (2). The peroxisome-targeting signal of catalase, KANL, pos-
sesses a weaker binding affinity to the cytosolic receptor Pex5p (2). The peroxisome-targeting signal of catalase, KANL, pos-

ordinated BDNF pathway

Figure 7. Cytosolic reductive condition elevates Bdnf expression. A and B, cellular levels of GSH, GSSG, NADH, NAD+, NADPH, and NADP+ in RCR-1/Venus, RCR-1/Pex5p-DN, and RCR-1/Pex14p-DN were analyzed by LC-MS/MS. GSH redox index was calculated for each cell line as in Fig. 6A (A). Relative levels of NADH/NAD+ and NADPH/NADP+ are represented as taking as 1 the levels in RCR-1/Venus (B, n = 3). C–E, RCR-1/Venus cells cultured in DMEM/F-12 medium supplemented with 1% FBS were treated with 10 μM NAC for 8 h. mRNA level of Bdnf was analyzed by real-time PCR (C, n = 3). Cellular levels of GSH, GSSG, NADH, NAD+, NADPH, and NADP+ were determined by LC-MS/MS. GSH redox index (D) and relative levels of NADH/NAD+ and NADPH/NADP+ are repre-

is regulated partly, if not completely, via subcellular localization of catalase.

Very recently, we reported that peroxisomal deficiency causes the up-regulation of BDNF and a truncated form of its receptor, TrkB-T1, resulting in malformation of Purkinje cells in the cerebellum of Pex14b/−/− mouse (10). Elevation of BDNF expression is also found in a peroxisome-depleted neuroblas-
toma cell line, SH-SY5Y, and in the IO neurons of the Pex14b/−/− mouse (10). In this report, we revealed that peroxi-

ase deficiency is caused by mislocalized catalase both in a neuroblastoma cell line, SH-SY5Y, and an astrocyte-like cell line, RCR-1. Elevated level of BDNF in neuron was observed in IN of the neonatal Pex14b/−/− mouse, and the BDNF was likely to be delivered to the cerebellum though climbing fibers, resulting in the abnormal morphogenesis of Purkinje cells (10). However, the pathogenic role of BDNF elevation of astrocytes in ZSDs remains defined. Further investigation would delineate how the elevated BDNF affects peroxisome-deficient astrocytes, leading to dysmorphogenesis of CNS.

The Bdnf gene consists of nine exons, a common protein encoded by exon 9 and eight 5’ noncoding exons (exons 1–8) (50). Transcription of the gene yields BDNF transcripts containing one of the eight 5’ exons linked to exon 9 or the 5’ extended coding exon (exon 9A). These splicing variants are
**Peroxisome-deficient reductive state abrogates BDNF pathway**

thought to have different promoters and be independently regulated (50, 51). It remains unknown yet which promoter of the BDNF transcript variant is up-regulated in the peroxisome-deficient cells. The ectopically expressed cytosolic catalase (catalase–ΔC) elevates the Bdnf expression in RCR-1 cells (Fig. 5G). However, the elevated Bdnf level in RCR-1/catalase–ΔC cells is much less than that in RCR-1/Pex5p-DN and RCR-1/Pex14p-DN (Fig. 3B), thereby raising a possibility that another factor(s) is involved in the elevation of Bdnf transcript. Further investigation is required to address the precise mechanisms underlying the up-regulation of Bdnf expression in peroxisome-deficient cells.

In this report, we demonstrated that the cytosolic reductive states induced by mislocalized catalase elevated the Bdnf expression. Cytosolic catalase is observed in the skin fibroblasts from patients with IRD and less severe ZSDs, whereas punctate structures of PTS1 proteins are discernible (52). These cells showed that abnormalities of peroxisomal metabolisms, such as accumulation of VLCFA and defect of plasmaglan biosynthesis, were only partial (53). LC-MS/MS analysis also revealed a slight reduction of the plasmalogen level but unaltered VLCPC level in the fibroblasts from a patient with IRD.3 Therefore, a cytosolic reductive state induced by mislocalized catalase is a common phenotype in all cells from patients with ZSDs, including IRD, regardless of their severity. Better understanding of the effect of cytosolic reductive condition on the cellular functions should open a way to the elucidation of pathological mechanisms underlying ZSDs, besides the elevation of BDNF expression.

**Experimental procedures**

**Antibodies and reagents**

Mouse monoclonal antibodies to α-tubulin and lactate dehydrogenase A (LDHA) were purchased from BD Biosciences and AbFrontier (Seoul, Korea), respectively. Mouse monoclonal antibodies to FLAG (M2) and Tau-1 (PC1C6) were from Sigma. Rabbit anti-BDNF antibody (N-20) was from Santa Cruz Biotechnology, Inc. (Dallas, TX). We used rabbit antisera to PTS1 peptide (23), rat AOx (25), rat catalase (25), mouse ADAPS (54), and a C-terminal 19-amino acid sequence of rat Pex14p (Pex14pC) (55). Guinea pig anti-Pex14p antiserum (22) was also used.

Predesigned siRNA for rat catalase (SASI_Rn01_00053417), N-acetyl cysteine, and rotenone were purchased from Sigma. Rabbit anti-BDNF antibody (N-20) was from Santa Cruz Biotechnology, Inc. (Dallas, TX). We used rabbit antisera to PTS1 peptide (23), rat AOx (25), rat catalase (25), mouse ADAPS (54), and a C-terminal 19-amino acid sequence of rat Pex14p (Pex14pC) (55). Guinea pig anti-Pex14p antiserum (22) was also used.

Predesigned siRNA for catalase (SASI_Rn01_00053417), N-acetyl cysteine, and rotenone were purchased from Sigma. The siRNA for PEX5 (5'-UUUAGCUCAGACACCGC- AAUG-3' and 5'-CAUUUGCCGAAGGUCUGGACUAAA-3') was from Invitrogen. FK-866 was from AdipoGen (San Diego, CA).

**Plasmids**

cDNAs, each encoding Chinese hamster Pex5p, rat Pex14p, and Venus, were amplified by PCR from pcDNAZeol/His-CIPEX5-HA (56), pHis-RP14 (22), and pCS2/Venus (21), respectively. The cDNA encoding a fusion of amino acid sequence at 1–243 of Chinese hamster Pex5p with Venus was generated by overlap extension PCR (57) and ligated into the BamHI-NotI sites of pcDNA3.1/Zeo(+)-vector, yielding pcDNAZeol/PEX5-DN-Venus. To generate pcDNAZeol/Pex14-DN-Venus, a fusion of amino acid sequence at 1–93 of rat Pex14p with Venus was also amplified and ligated into BamHI-NotI sites. cDNA encoding rat BDNF (58) was ligated into the BamHI-NotI sites of pcDNA3.1/Zeo(+) vector. The p75ECD amino acid sequence at 1–747 was ligated into the HindIII-XhoI sites of pSecTag2/Hygro C vector (Invitrogen), yielding pSecTag2/p75ECD-His (10). Vector for the expression of short hairpin RNA was constructed as follows. Briefly, cDNA encoding EGF fused with a nuclear localization signal (NLS) of SV40 T antigen protein (TagNLS-EGFP) (59) was inserted into NotI-Xbal sites of pcDNA3.1/Zeo(+) vector, yielding pcDNAZeol-NLS-EGFP. A knockdown cassette based on the mouse miR155 (60) was produced by a fill-in reaction of KOD-FX (Toyobo) using synthetic oligonucleotides: miR155_top, 5'-CCCAAGCTTGTCAGCCTGGAGCTGCAG- TGTATGCTAGGACGTAGATCGTCATGCAGACGACGACGGG-AACAG-3' and miR155_bottom, 5'-CGGATCTCGAGGCCATTGTTCCATGTGAGTGCTAGTAACAGGCCCTT- GTTGGCCTGAGGACCCATC-3'. A synthesized double-strand knockdown cassette was ligated into HindIII-BamHI sites of pcDNA-Zeo-NLS-EGFP, termed pmiR155-NLS-EGFP. Predesigned BLOCK-IT miRNA sequences targeting rat AOx (Rmi662893; Invitrogen) were inserted into BsmBI sites of the knockdown cassette of pmiR155-NLS-EGFP. cDNA encoding human catalase was cloned into BamHI (blunted)-NotI sites of pcDNA3.1 Zeo-FLAG vector (56), yielding pcDNAZeol/FL-catalase. A Nhel-NotI fragment of pcDNAZeol/FL-catalase was cloned into Nhel-NotI sites of pIRESpuro3 vector (Clontech, Mountain View, CA), generating pIRE/FL-catalase. H75A and ΔANL mutations were introduced into FL-catalase by site-directed mutagenesis.

**Cell culture**

RCR-1 cells were purchased from RIKEN Cell Bank. RCR-1 cells were cultured under 5% CO2 at 37 °C in DMEM (Invitrogen)/F-12 medium (Invitrogen) supplemented with 10% FBS. CHO cell lines, CHO-K1, TKa (61), and pex1 ZP107 (33), were cultured in F-12 medium containing 10% FBS. SH-SY5Y cells (Human Science Research Resources Bank, Osaka, Japan) were cultured in DMEM supplemented with 10% FBS. DNA and siRNA transfections were performed with Lipofectamine 2000 (Invitrogen) to RCR-1 cells, Lipofectamine (Invitrogen) to CHO-K1 cells, and Lipofectamine RNAiMAX (Invitrogen) to SH-SY5Y cells according to the manufacturer’s instructions (62). Stable transformants were isolated by selection with 200 µg/ml Zeocin™ (Invitrogen), 1 µg/ml puromycin (Sigma), or 1.2 µg/ml hygromycin (Sigma). For analyses of mRNA and metabolites, RCR-1 cells were cultured in Neurobasal medium containing B27 supplement (Invitrogen) and 0.5 mM L-glutamine unless otherwise described.

**Primary culture of hippocampus neurons**

RCR-1 cells were plated on the paraffin ball-attached coverglass at 4.0 × 10^5 cells/cm². One day after plating, the culture

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3 Y. Abe, M. Honsho, and Y. Fuji, unpublished data.
medium was replaced with Neurobasal medium, and the cells were incubated for 2 days.

Primary hippocampal neurons were prepared from Wistar rat embryos (embryonic day 18.5 (E18.5)). Briefly, hippocampi were excised into small pieces and dissociated with 15 units of papain (Worthington) in dissociation solutions (0.2 mg/ml l-cysteine, 0.2 mg/ml BSA, and 10 mg/ml glucose) and 0.01% DNase I (Sigma). Cells were separated by gentle trituration passes using a 10-ml pipette and were passed through a 70-μm cell strainer (BD Biosciences) to remove large debris. Cells were plated on poly-l-lysine (Sigma)- and laminin (Sigma)-coated plates in Neurobasal medium containing B27 supplement and 0.5 mM l-glutamine. Cell density was 1.0 × 10⁵ cells/cm² for morphological analysis of axonal development. After 4 h, the culture medium was replaced with the CM derived from RCR-1 cell cultures. RCR-1 cells on a coverglass were overlaid on primary neurons and cultured for 2 DIV (see Fig. 2A). Neuronal morphologies were observed using an inverted Axiovert 200M phase-contrast microscope (Carl Zeiss, Oberkochen, Germany) or AF 6000LX microscope (Leica, Wetzlar, Germany). The length of axons was measured by ImageJ software (National Institutes of Health, Bethesda, MD).

To investigate the potential effect of the RCR-1 cell-derived CM on morphology of primary neurons, RCR-1 cells were plated at a density of 6.5 × 10⁴ cells/cm² and cultured for 4 days in Neurobasal medium, of which resulting CM was then collected. Primary hippocampal neurons were incubated in the collected CM for 2 DIV. CM containing rBDNF was obtained from RCR-1 cells stably expressing rBDNF, serially diluted with the CM from nontransfected RCR-1 cells, and used for the assay.

For the siRNA transfection, primary neurons of 5 × 10⁶ cells were transfected with 5 μM dsRNA using Amaxa Nucleofector (Lonza, Basel, Switzerland). The siRNA sequences used were as follows: TrkB #1, 5′-UUUCAGCCAACACUUGGAUGUCUC-3′ and 5′-GAGACAUUCCAGUUGCAAGAA-3′; TrkB #2, 5′-UGUACUGGACUCAUUGCAGGC-3′ and 5′-GCAUACGUGAGUCCAAGUCA-3′.

**Purification of p75ECD-His**

A stable transformant of CHO-K1 cells expressing p75ECD-His was cultured in serum-free F-12 medium for 3 days. The cell culture medium was collected and centrifuged to remove floating cells. The resulting supernatant fraction was mixed and incubated with Ni-NTA–agarose beads (Qiagen, Hilden, Germany) for 4 h. The p75ECD-His–bound beads were washed six times with purification buffer (50 mM Hapes-KOH, pH 7.4, 150 mM NaCl, 20 mM imidazole, and 10% glycerol), followed by elution with the purification buffer containing 250 mM imidazole. The eluent was loaded onto PD10 column (GE Healthcare) in suspension buffer (50 mM Hapes-KOH, pH 7.4, 150 mM NaCl, and 10% glycerol) and then concentrated by ultrafiltration in an Amicon Ultra-15 (10,000 molecular weight cutoff; Millipore, Billerica, MA). Purified p75ECD-His was added to the primary neuron cell culture at 1.0 μg/ml.

**Lipid extraction**

Total cellular lipids were extracted by the Bligh and Dyer method (63). Briefly, cells were detached from culture plates by incubation with trypsin and suspended in PBS. Protein concentration was determined by the bicinchoninic acid method (Thermo Fisher Scientific). Cell suspensions containing 50 μg of total cellular proteins were dissolved in methanol/chloroform/water at 2:1:0.8 (v/v/v), and then 50 pmol of 1-heptadecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL), 1,2-didodecanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids), and 1,2-didodecanoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids) were added as internal standards. After incubation for 5 min at room temperature, 1 ml each of water and chloroform was added, and the samples were then centrifuged at 2,000 rpm for 5 min in Himac CF-16RX (Hitachi Koki, Tokyo, Japan) to collect the lower organic phase. To re-extract lipids from the water phase, 1 ml of chloroform was added. The combined organic phase was evaporated under a nitrogen stream, and the extracted lipids were dissolved in methanol.

**Extraction of hydrophilic metabolites**

Cells were collected in 1 ml of ice-cold methanol and lysed by freeze-thawing two times. Raffinose (50 pmol) and GSH ethyl ester (GSHee, 50 pmol) were added as internal standards. After centrifugation at 21,000 × g for 5 min at 4°C, the supernatant fraction was evaporated at room temperature under nitrogen for 6 h and dissolved in water.

**LC-MS/MS**

LC-MS/MS analysis of phospholipids was performed as described (35) using a 4000 Q-TRAP quadrupole linear ion trap hybrid mass spectrometer (AB Scieix, Foster City, CA) with an ACQUITY UPLC System (Waters, Milford, MA).

For the analysis of hydrophilic metabolites, a 10-μl aliquot was separated at 30°C by step gradient elution with mobile phases A (water, 0.1% formic acid, and 0.028% ammonia) and B (20% acetonitrile, 0.1% formic acid, and 0.028% ammonia) at the following ratios: 95:5 (for 0–5 min), 0:100 (5–25 min), 0:100 (25–30 min), and 95:5 (30–40 min), with a flow rate of 50 μl/min. The ion transitions at m/z 308.1 > +179.0, +613.3 > +355.0, +336.1 > +207.1, −661.9 > −79.0, −661.9 > −540.0, −743.9 > −79.0, −741.9 > −619.8, and +503.1 > −179.0 for GSH, GSSG, GSHee, NADH, NAD⁺, NADPH, NADP⁺, and raffinose, respectively, were used in the multiple-reaction-monitoring mode. The data were analyzed and quantified using Analyst software (AB Scieix). The GSH redox index was calculated as (ratio of GSH content in the extract from the subject relative to that from control cell)²/(ratio of GSSG content in the extract from the subject relative to that from control cell).

The ΔE_GSH values representing the difference in redox potential of GSH compared with control cells (34) are calculated as follows: ΔE_GSH = −RT/2Fln(GSH redox index), where R is the gas constant, T is absolute temperature (K), and F is the Faraday constant.
Peroxisome-deficient reductive state abrogates BDNF pathway

RT-PCR and real-time RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and first-strand cDNA was synthesized by a PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). The expression level of neurotrophin mRNAs was assessed by RT-PCR using the respective sets of primers listed in Table S1. Quantitative real-time RT-PCR was performed with SYBR Premix Ex TaqII (Takara Bio) using an Mx3000P QPCR system (Agilent Technologies, Santa Clara, CA). Several sets of primers used are listed in Table S2.

Immunofluorescent microscopy

Cultured cells were fixed with 4% paraformaldehyde in PBS, pH 7.4, for 15 min at room temperature (64). Peroxisomes were visualized by indirect immunofluorescence staining with the indicated antibodies as described (65). Antigen-antibody complexes were detected with goat anti-mouse and anti-rabbit IgG conjugated to Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen). Phalloidin-TRITC (Sigma) was used for the staining of F-actin. Images were obtained using a laser-scanning confocal microscope (LSM 710 with Axio Observer.Z1; Carl Zeiss).

Immunoblotting

Immunoblotting was performed as described (66). Precision Plus Protein All Blue standards (Bio-Rad) were used as molecular size markers. Immunoblots were developed with ECL prime reagent (GE Healthcare), and immunoreactive bands were detected by X-ray film (GE Healthcare) or an LAS-4000 Mini luminescent image analyzer (Fuji Film, Tokyo, Japan). The band intensities were quantified by Image J software (National Institutes of Health) or Image Gauge software (Fuji Film).

Catalase latency

Catalase latency was evaluated as described (25, 43). In brief, trypsinized cells were washed and suspended at 10⁶ cells/ml in 0.25 M sucrose and 10 mM Hepes-KOH, pH 7.4. The cells were treated with 10 μg/ml digitonin (Wako, Tokyo, Japan) or 1% Triton X-100. After detergent treatment, 20 μl of cell suspensions were added to 200 μl of H₂O₂ solution (20 mM imidazole-HCl, pH 7.0, 0.25 M sucrose, 0.1% BSA, and 0.01% H₂O₂) and further incubated for 15 min on ice. After incubation, the catalase reaction was halted by the addition of 20 μl Ti(SO₄)₂ solution (2.0 M H₂SO₄ and 1.25% Ti(SO₄)₂). The concentration of residual H₂O₂ was determined by absorbance at 410 nm of lo fH₂O₂ solution (20 mM imidazole-HCl, pH 7.0, 0.25 M sucrose, and 1.25% Ti(SO₄)₂ solution). The concentration after the enzymatic reaction. One unit of activity was defined as the amount of enzyme causing the destruction of 90% of the substrate in 1 min in a volume of 50 μl under assay conditions (41).

Subcellular fractionation and protease protection assay

RCR-1 cells were homogenized with a 27-gauge needle syringe in 500 μl of homogenizing buffer (10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 2 μg/ml protease inhibitor mixture (Peptide Institute, Osaka, Japan) including antipain, leupeptin, and aprotinin, and 1 mM phenylmethylsulfonyl fluoride (Nacalai, Kyoto, Japan)) and were centrifuged at 600 × g for 5 min at 4 °C. The PNS fraction was ultracentrifuged at 100,000 × g for 30 min to obtain the cytosolic fraction and organelle fraction. Each fraction was analyzed by SDS-PAGE and immunoblotting.

The protease protection assay was performed as described (65, 67). Briefly, PNS fractions from each type of RCR-1 cells (4 × 10⁵ cells) were treated with 80 μg/ml Proteinase K (Sigma) for 30 min on ice in the absence or presence of 0.2% Triton X-100. The reaction was terminated with 1 mM phenylmethylsulfonyl fluoride, and the reaction mixture was separated by ultracentrifugation at 100,000 × g for 30 min at 4 °C. Each fraction was analyzed by SDS-PAGE and immunoblotting.

Statistical analysis

Statistical analysis was performed using R software. All Student’s t tests used were one-tailed. A p value of <0.05 was considered statistically significant. Data are shown as means ± S.D. unless otherwise described.

Data availability

All data described are contained within the article and supporting information.

Author contributions—Y. A., M. Honsho, M. O., Y. S., T. Y., and Y. F. conceptualization; Y. A., R. K., and Y. I. data curation; R. K. writing—original draft; M. Honsho supervision; M. Honsho and Y. F. writing—review and editing; R. K., M. F., K. F., M. Hirokane, T. Y., and Y. F. resources; T. M., Y. I., M. F., M. O., Y. S., and T. Y. methodology; Y. F. project administration.

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