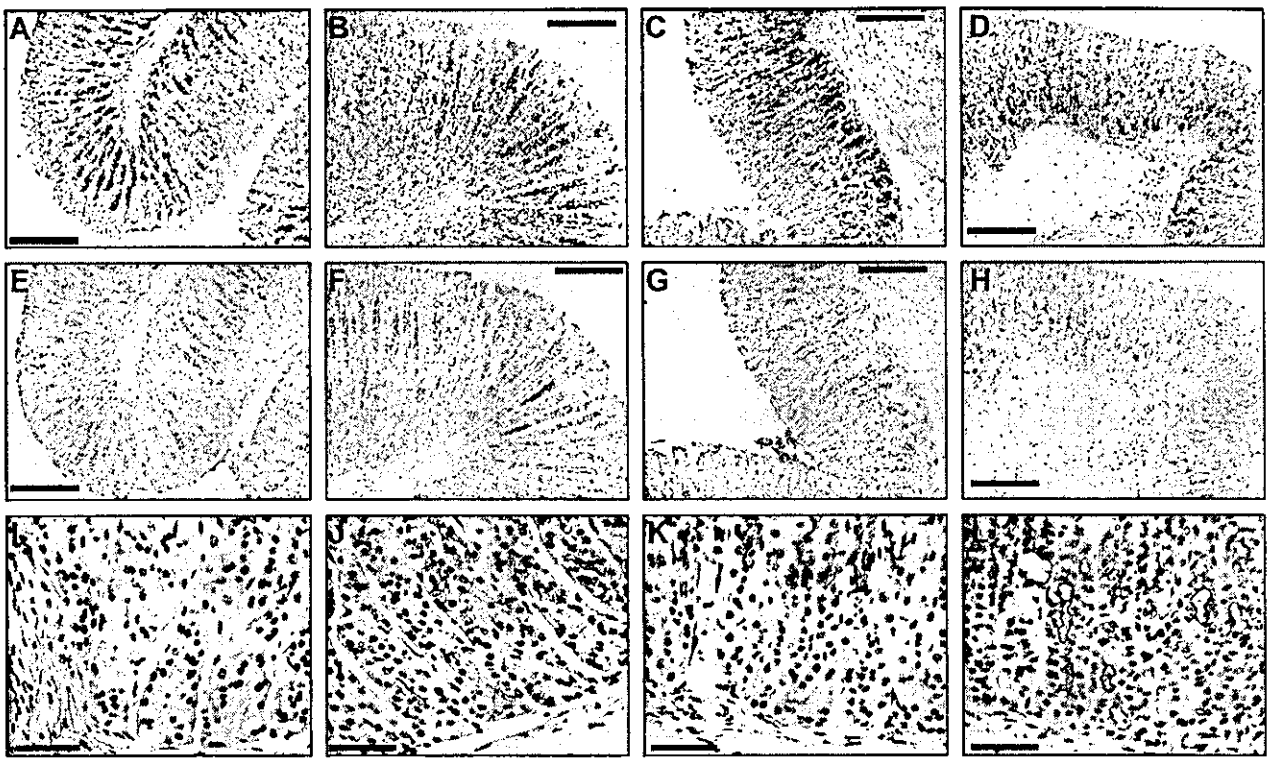

Fig. 5. Expressions of pepsinogen and type III mucin in oxyntic mucosa from 10-week-old wild-type, histamine H_2 receptor-null, gastrin receptor-null and double-null mice. Sections of oxyntic mucosa from wild-type (A, E, I), histamine H_2 receptor-null (B, F, J), gastrin receptor-null (C, G, K) and double-null (D, H, L) mice were stained with anti-pepsinogen antibody (A, B, C, D) and anti-type III mucin antibody (E, F, G, H, I, J, K, L). In I, J, K, L, type III mucin-positive cells are marked with asterisks. Scale bars, 200 μm (A, B, C, D, E, F, G, H), 50 μm (I, J, K, L).

carbachol at 1 mg/kg BW, a dose which is too high to be tolerated in measuring in vivo acid production, was examined in double-null mice. Fig. 6 shows that while gastrin receptor-null mice were responsive to both histamine and carbachol, double-null mice were unresponsive to both.

3.4. Long term follow-up of histamine H_2 receptor-null mice and double-null mice

At 6 months, while there were no changes in gastric mucosa from wild-type mice, further elongation of gastric glands was observed in histamine H_2 receptor-null mice

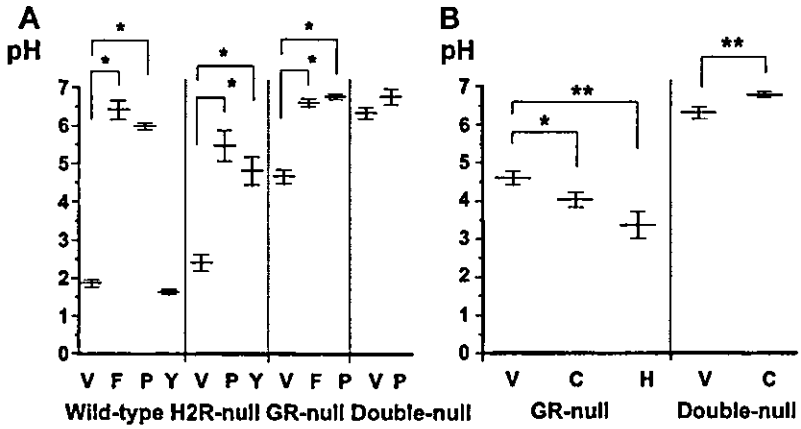

Fig. 6. Gastric pH in wild-type, histamine H2 receptor-null, gastrin receptor-null and double-null mice. Wild-type, histamine H_2 receptor-null (H2R-null), gastrin receptor-null (GR-null) and double-null (10 to 12 weeks old) mice were fasted overnight with free access to water. (A) At 1.5 h after subcutaneous injection of 0.5% methylcellulose as a vehicle (V) (n=20), 10 mg/kg BW of famotidine (F) (n=20), 10 mg/kg BW of pirenzepine (P) (n=20) or 10 mg/kg BW of YM022 (Y) (n=20), the mice were killed and their stomachs immediately excised. Gastric pH was measured using an ultra-thin pH monitor. (B) At 15 min after subcutaneous injection of vehicle (V) (n=20), 10 mg/kg BW of histamine (H) (n=20) or 1 mg/kg BW of carbachol (C) (n=20), the mice were killed and their stomachs immediately excised. Gastric pH was measured using an ultra-thin pH monitor. Data are presented as means±S.E. *P<0.001 vs. respective values.

Table 2
Stomach weight and gastric pH in aged wild-type and aged histamine H_2 receptor-null mice

	Stomach weight (g)	Fasting gastric pH
Wild-type	0.16±0.02	1.61±0.13
Histamine H_2 receptor-null	0.38±0.02[a]	2.14±0.13[b]

Stomach weight and fasting gastric pH were measured in 12-month-old wild-type and histamine H_2 receptor-null mice. Data are expressed as means±S.E. (n=10, each group).

[a] P<0.0001 vs. wild-type mice.

[b] P=0.0134 vs. wild-type mice.

(data not shown). However, the structure of gastric oxyntic mucosa from 6-month-old histamine H_2 receptor-null mice was very similar to that of mucosa from 10-week-old histamine H_2 receptor-null mice, except for the presence of cysts near the basal region. In 12-month-old histamine H_2 receptor-null mice, in addition to the marked increase in stomach weight (Table 2), oxyntic mucosal structures appeared to differ strikingly from those of wild-type and younger histamine H_2 receptor-null mice (Fig. 7B). Oxyntic mucosa from aged histamine H_2 receptor-null mice was full of cystic structures (Fig. 7B). Most gastric glands were dilated and, in addition, interstitial tissues between cysts were markedly increased (Fig. 7D), which is in sharp contrast to the findings in gastric mucosa from aged wild-type mice (Fig. 7C). Some cells lining the cysts were positive for $H^{(+)}/K^{(+)}$-ATPase, pepsinogen and HDC (Fig. 7E,F,G), indicating that the cysts were derived from dilated gastric glands. However, small portions of oxyntic mucosa remained mostly unaltered (Fig. 7H), suggesting that the program for formation of normal gastric glands is preserved in gastric mucosal stem cells. Gastric pH values in aged histamine H_2 receptor-null mice were essentially preserved (Table 2). Similar features were observed in gastric mucosae from 24-month-old histamine H_2 receptor-null mice (data not shown). Unlike histamine H_2 receptor-null mice, there were no significant differences in oxyntic mucosae between 10-week-old and 12-month-old double null mice (data not shown).

4. Discussion

Oxyntic mucosal atrophy in double-null mice confirms the oxyntic mucosal hypertrophy observed in histamine H_2 receptor-null mice to be due to stimuli delivered via gastrin receptors. In double-null and gastrin receptor-null mice, numbers of gland cells as a whole (downward migrating cells) were decreased. However, despite gastric mucosal atrophy surface mucous cell number was moderately but significantly increased in gastrin receptor-null and double-null mice as compared with wild-type mice (Table 1). Turnover of surface mucous cells is far faster than that of downward migrating cells (Karam and Leblond, 1992, 1993a,b,c,d, 1995). Thus, it is likely that most of the increases in BrdU labeling in oxyntic mucosae in gastrin receptor-null and double-null mice are attributable to increased growth and differentiation into surface mucous cells. In the case of gastrin-null mice, the percentage of BrdU positive cells in oxyntic mucosa was not different from that in wild-type mice and there was a marked decrease in the surface mucous cells in gastrin-null mice as compared with wild-type mice (Koh et al., 1997). Thus, gastric mucosae from gastrin receptor-null and double-null mice and those from gastrin-null mice are different in terms of number of surface mucous cells. Post-translational modification of preprogastrin yields progastrin and glycine-extended gastrin as well as gastrin-17 (Dockray et al., 2001). In G-cells, gastric mucosal processing of preprogastrin yields gastrin and glycine-extended gastrin (Dockray et al., 2001). Glycine-extended gastrin reportedly has very low affinity for the gastrin receptor and has been suggested to interact with a novel receptor, which remains to be identified (Dockray et al., 2001). Thus, serum and oxyntic mucosal levels of glycine-extended gastrin may well be elevated, like those of gastrin-17, in gastrin receptor-null and double-null mice. In a study using gastrin-null mice, infusion of gastrin-17 and glycine-extended gastrin had distinct effects on gastric acid secretion, via different signal transduction pathways (Chen et al., 2000; Hollande et al., 2001; Stepan et al., 1999). Thus, the absence of glycine-extended gastrin effects in gastrin-null mice and possible hyperstimulation of the glycine-extended gastrin receptor in gastrin receptor-null mice might account for the difference in surface mucus cells in these mice. The finding of similar surface mucous cell increases in double-null mice indicates that a glycine-extended gastrin-dependent increase in surface mucous cells in the absence of gastrin receptors is not dependent on the histamine H_2 receptor. We speculate that a similar increase in surface mucous cell number in histamine H_2 receptor-null mice was caused by such a glycine-extended gastrin effect. Taken together, our results show gastrin and glycine extended-gastrin to have distinct roles in the growth of gastric mucosa.

We previously reported that maturation of the chief cell lineage was impaired in gastric mucosa from histamine H_2 receptor-null mice (Fukushima et al., 2003). In this report, mature chief cells, which we define as being positive for pepsinogen and negative for type III mucin, were present gastrin receptor-null mouse. In contrast, in histamine H_2 receptor-null mice and double-null mice expression levels of pepsinogen per cell are very low and mature chief cells were very scarce. Considering the marked difference in pH values in histamine H_2 receptor-null mice and double-null mice (Fig. 6), the difference in chief cells in these mice is not attributable to low acidity but rather to disruption of the histamine H_2 receptor itself. Genetic ablation of parietal cells with $H^{(+)}/K^{(+)}$-ATPase promoter resulted in loss of mature chief cells, which can be taken as evidence that parietal cells are involved in chief cell maturation (Canfield et al., 1996; Li et al., 1996). However, it has been suggested

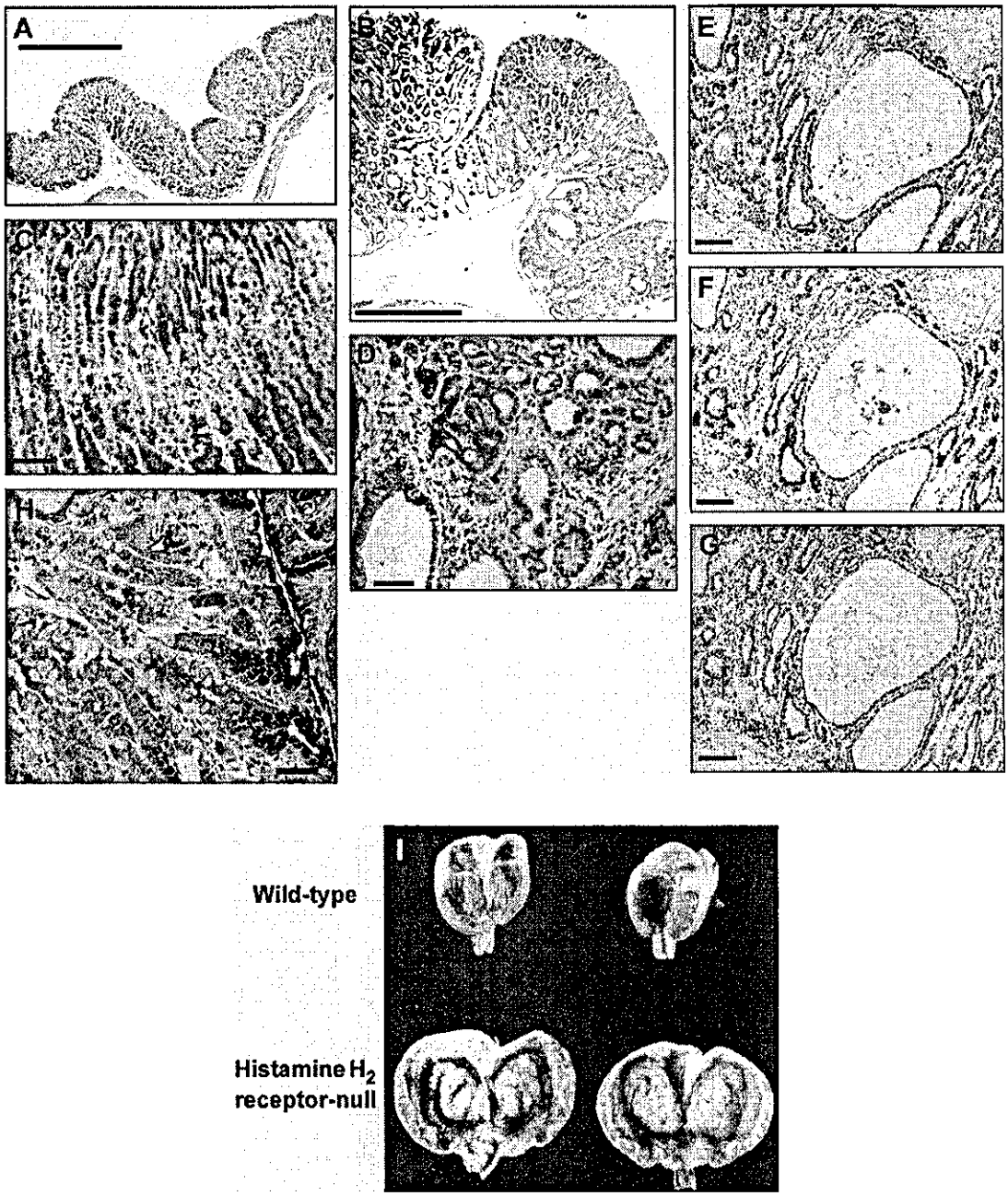

Fig. 7. Oxyntic mucosa from 12-month-old wild-type and histamine H_2 receptor-null mice. A, B, C, D, E, F, G and H Sections of oxyntic mucosa from histamine H_2 receptor-null mice (B, D, E, F, G, H) and wild-type (A, C) mice were stained with hematoxylin and eosin (A, B, C, D) and with anti-$H^{(+)}/K^{(+)}$-ATPase antibody (E), anti-pepsinogen antibody (F) or with anti-HDC antibody (G) and PAS (H). Arrows indicate interstitial cells. Scale bars, 1000 μm (A, B); 100 μm (E, F, G); 50 μm (C, D, H). (I) Macroscopic views of stomachs from wild-type and histamine H_2 receptor-null mice. The excised stomachs from 12-month-old mice were opened along the greater curvature.

that chief cell precursor cells express $H^{(+)}/K^{(+)}$-ATPase (Mutoh et al., 2002). Thus, it is likely that ablation of chief cell precursors rather than ablation of parietal cells resulted in the loss of chief cells observed in the study (Canfield et al., 1996; Li et al., 1996). In contrast, our pepsinogen and type III mucin findings show that the histamine H_2 receptor per se is involved in production and/or secretion of pepsinogen in chief cells. Thus, the histamine H_2 receptor is indispensable for chief cell maturation at least in terms of pepsinogen secretion.

Even in double-null mice, with severely impaired acid production, parietal cells and $H^{(+)}/K^{(+)}$-ATPase were present (Table 1). In addition, electron microscopic analysis of parietal cells from double-null, gastrin receptor-null and histamine H_2 receptor-null mice revealed no essential ultrastructural differences as compared to wild-type mice

(data not shown). Thus, there is no apparent structural alteration in gastric acid secretion mechanisms in double-null mice. However, gastric pH values were higher than in double-null mice than in the other three kinds of mice studied and were unresponsive even to carbachol. In histamine H_2 receptor-null mice, carbachol-induced acid production was mostly preserved (Fukushima et al., 2003; Kobayashi et al., 2000). Thus, considering the loss of the in vivo acid production response in gastrin receptor-null mice, acid production via cholinergic stimuli is largely dependent on the gastrin receptor. The finding that gastrin receptor disruption in histamine H_2 receptor-null mice, i.e. double-null mice, resulted in marked elevation of gastric pH (Fig. 6) reinforces the notion that gastrin receptors in parietal cells function in gastric acid secretion (Fukushima et al., 2003). In any case, it is noteworthy that disrupting histamine H_2 and gastrin receptors resulted in loss of response to secretagogues, even in terms of gastric pH, confirming the pivotal roles of these receptors in gastric acid production and secretion.

Recently, Ogawa et al. (2003) reported that findings in the stomachs of aged histamine H_2 receptor-null mice were compatible with Menetrier's disease. Menetrier's disease is characterized by hyperplasia of oxyntic mucosa which is attributable to hyperplasia of surface mucous cells and is often accompanied by hypoplasia of gland cells and low gastric acidity (Wolfsen et al., 1993; Yamada et al., 1995). As we previously reported, oxyntic mucosa from histamine H_2 receptor-null mice is characterized by marked hyperplasia of downward migrating cells, while hyperplasia of surface mucous cells is negligible (Fukushima et al., 2003). In 12-month-old mice, marked gastric mucosal hypertrophy was observed. However, as shown in Fig. 7, the extremely hypertrophic gastric mucosa consists of markedly elongated glands, cysts which originated from dilated gastric glands and increased interstitial tissues. The contribution of surface mucous cells is minimal. Thus, we consider it difficult to conclude that the gastric mucosal findings of aged histamine H_2 receptor-null mice are compatible with Menetrier's disease.

Rather, histological findings in aged histamine H_2 receptor-null mice can be fully explained by the findings in their 10-week-old counterparts. Oxyntic mucosal stem cells reside in the upper one-third of the mucosa away from the basal region and differentiate, growing upward or downward (Karam and Leblond, 1993a). In histamine H_2 receptor-null mice, marked hyperplasia of downward migrating cells results in unlimited movement of stem cells away from the basal region of the gastric mucosa (Fukushima et al., 2003). In addition, in the mid-portion of gastric glands both the number and mucous content of mucous neck cells are increased, which can lead to increased viscosity of the gastric juice retained in the mid-portions of gastric glands. Thus, due to this marked elongation of gastric glands together with the increased viscosity of gastric juice, gastric glands in histamine H_2 receptor-null mice would presumably be susceptible to occlusion. Once occlusion occurs, secretions from gland cells, even if impaired, promote the formation of cysts. Since gastric pH values per se are essentially preserved in histamine H_2 receptor-null mice (Fukushima et al., 2003; Kobayashi et al., 2000), leakage of contents and cystic rupture are expected to induce inflammation and an increase in interstitial tissues. Therefore, although the phenotype of stomachs from aged histamine H_2 receptor-null mice appears to be quite unusual, there is no essential difference between gastric mucosae from young and aged histamine H_2 receptor-null mice.

In conclusion, we have used double-null mice to show that (1) gastrin and histamine H_2 receptors are both essential in gastric acid production and secretion, (2) the histamine H_2 receptor plays a pivotal role in chief cell maturation, (3) gastrin gene products other than gastrin-17, such as glycine-extended gastrin, might be involved in surface mucous cell proliferation and (4) hypertrophy of gastric mucosa from histamine H_2 receptor-null mice is due to hyperstimulation of gastrin receptors via marked hypergastrinemia. Since gastric oxyntic mucosa is quite unique in that different cell types interact with each other both structurally and functionally, our murine models are potentially valuable for further analyzing differentiation of gastric mucosa and gastric acid secretion mechanisms.

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ORIGINAL ARTICLE

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Genome-wide linkage analysis of type 2 diabetes mellitus reconfirms the susceptibility locus on 11p13–p12 in Japanese

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Abstract Type 2 diabetes mellitus is a heterogeneous disorder, and the development of type 2 diabetes mellitus is associated with both insulin secretion defect and insulin resistance. The primary metabolic defect leading to type 2 diabetes mellitus has been thought to be varied among populations, especially in Japanese and Caucasians. Here, we have done the genome-wide scan for type 2 diabetes mellitus using 102 affected Japanese sib-pairs to identify the genetic factors predisposing to type 2 diabetes mellitus. Nonparametric linkage analysis showed one suggestive evidence for linkage to 11p13–p12 [*D11S905*: two-point maximum LOD score (MLS) of 2.89 and multipoint MLS of 2.32] and one nominally significant evidence for linkage to 6q15–q16 (*D6S462*: two-point MLS of 2.02). Interestingly, the 11p13–p12 region was reported to be a susceptibility locus for Japanese type 2 diabetes mellitus with suggestive evidence of linkage, and *D11S905* was within 5 cM to *D11S935* with the highest MLS in the previous linkage analysis reported. The only overlapped susceptibility region with suggestive evidence of linkage for Japanese type 2 diabetes mellitus was *D11S935–D11S905* among

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the three reports including this study. These results taken together suggest that a susceptibility gene for type 2 diabetes mellitus in Japanese will reside in 11p13–p12.

Keywords Type 2 diabetes mellitus · Japanese · Affected sib-pairs · Linkage · Chromosome 11p

Introduction

Type 2 diabetes mellitus is one of the most common diseases, and its prevalence is dramatically increasing worldwide (Zimmet et al. 2001). Type 2 diabetes mellitus is a heterogeneous disorder, and the development of type 2 diabetes mellitus is associated with both insulin secretion defect and insulin resistance. Japanese patients with type 2 diabetes mellitus were reported to be characterized by a lower body mass index (BMI) and lower fasting insulin levels than other populations (Ehm et al. 2000). Insulin secretion defect is thought to be the primary defect in Japanese (Kadowaki et al. 1984) whereas impaired insulin sensitivity is the first metabolic defect predisposing to the development of type 2 diabetes mellitus in Caucasians (Martin et al. 1992). These findings suggest that Japanese individuals with type 2 diabetes mellitus will have a different genetic risk factor, which affects the responsiveness of insulin secretion to glucose, from other populations. Therefore, we need to identify the susceptibility genes for the development of type 2 diabetes mellitus in Japanese to start a primary prevention based on genetic information and to develop the personalized medicine for type 2 diabetes mellitus in Japanese. So far, two whole-genome linkage analyses were carried out using 224 affected sib-pairs (ASPs) from 159 Japanese families (Mori et al. 2002) and 256 ASPs from 164 Japanese families (Iwasaki et al. 2003), besides the analysis of 45 ASPs from 18 Japanese American families (Ehm et al. 2000). The Japanese people may have advantages in the genetic analysis of polygenic disorders like diabetes since they are supposed to be a relatively homogeneous population. However, the two previous reports on the ASP analysis in Japanese did not give good overlapping regions, except for 6p and 2q, and it has been argued that the replication by the third panel is indispensable for genetic susceptibility loci in Japanese. Here, we have carried out the third whole-genome linkage analysis on 102 ASPs from 102 Japanese families to identify the susceptibility loci for the development of type 2 diabetes mellitus.

Subjects and methods

One hundred and two ASPs with type 2 diabetes mellitus from 102 families were collected mainly from the Kyushu region in southwestern Japan. Parents and other siblings were not available in this study. The participants were interviewed and examined and gave written informed consent. This project was approved by the ethics committees of the related institutes. The diagnosis of type 2 diabetes mellitus was made based on the American Diabetes Association's 1997 criteria (Expert

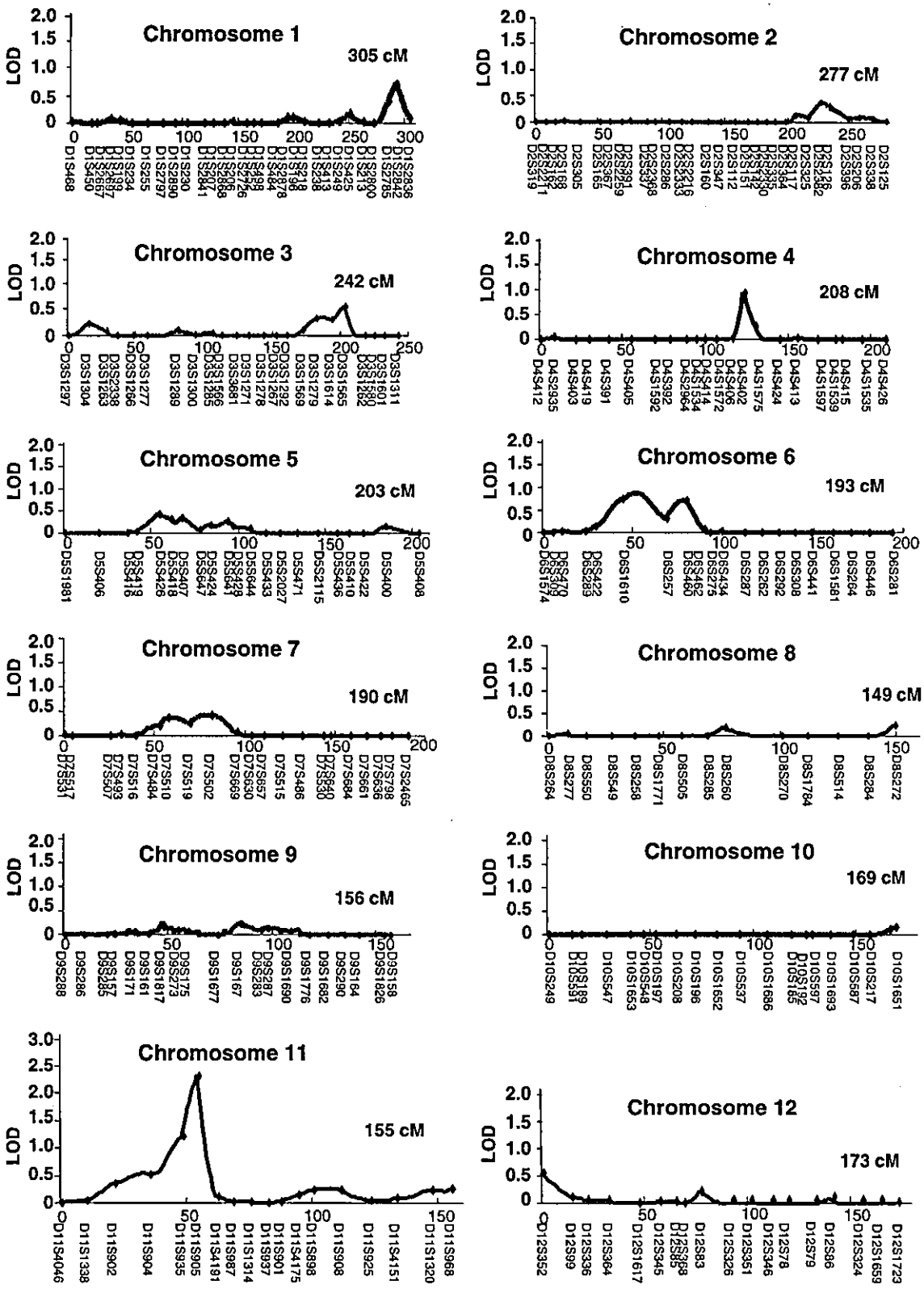

Fig. 1 Multipoint LOD score map of type 2 diabetes mellitus by linkage analysis of 382 markers in 102 affected sib-pairs. The *horizontal axis* is cM position from the p-terminal end of the chromosome

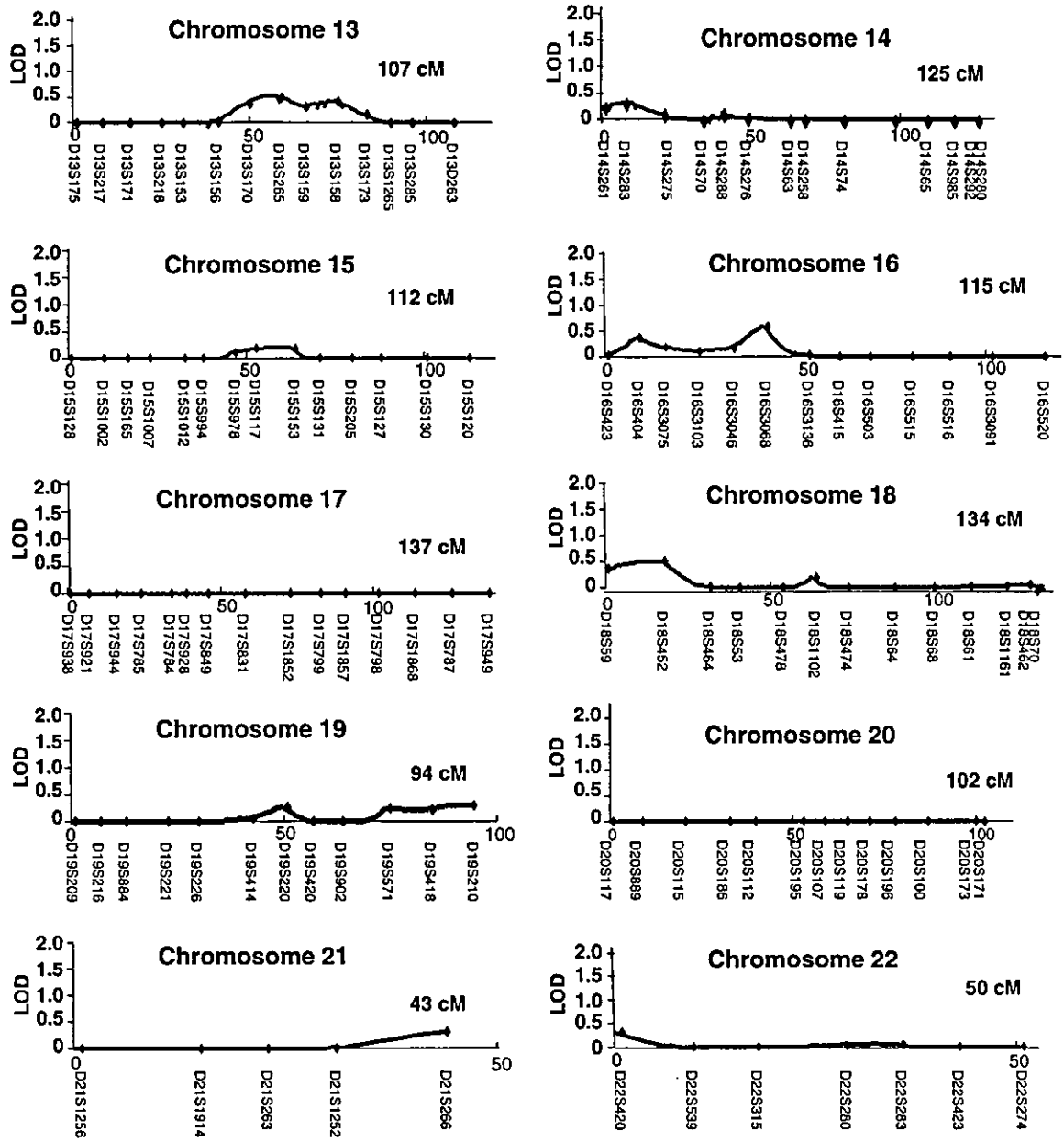

Fig. 1 (Continued)

Committee on the Diagnosis and Classification of Diabetes Mellitus 1997).

Genomic DNA was isolated from peripheral blood cells using QIAamp DNA Blood Midi Kits (Qiagen). Autosomal whole-genome screening of 382 microsatellite markers (ABI PRISM Linkage Mapping Set Version 2.5-MD10) was performed using an ABI 3730 automatic sequencer (Applied Biosytems). Analyses and assignment of the marker alleles were done with ABI PRISM GeneMapper Software Version 3.0, and 376 markers were available for the linkage analysis. Nonparametric two-point and multipoint linkage analyses were performed with the MAPMAKER/SIBS program (Kruglyak and Lander 1995), as described (Sakai et al. 2001). Heterozygosities of the markers were estimated

Table 1 Results of linkage analysis of type 2 diabetes mellitus and markers showing evidence of linkage

Marker	cM[a]	Analysis	MLS	*P*
D6S462	89	Two-point	2.02	0.0097
D11S905	54	Two-point	2.89	0.0013
		Multipoint	2.32	0.0048

[a]The distance of the marker form the p-terminal end of the chromosome in cM

with Merlin program (Abecasis et al. 2002) for all individuals.

Results and discussion

Whole autosomal genome linkage analysis using the ASP method with 382 microsatellite markers was carried out on 102 Japanese ASPs with type 2 diabetes mellitus. In this study, the average heterozygosity of the markers used was 0.72. Multipoint linkage analysis at all autosomal chromosomes using the MAPMAKER/SIBS program revealed only one region on chromosome 11p where the MLS was > 1 (Fig. 1). The highest multipoint MLS was 2.32 ($P=0.0048$) at *D11S905* (Fig. 1, Table 1). On the other hand, two-point linkage analysis revealed two markers, *D11S905* (MLS = 2.89, $P=0.0013$) and *D6S462* (MLS = 2.02, $P=0.0097$), with evidence of linkage to type 2 diabetes mellitus (Table 1). Although the heterozygosity of *D11S905* was 0.30 in this study, it was 0.75 and 0.60 in our two reports using ASPs (Sakai et al. 2001; Aoki et al. 2004), indicating that *D11S905* itself will be useful in the genetic analysis in terms of heterozygosity in the Japanese population, and particular alleles of *D11S905* might be associated with type 2 diabetes mellitus. The 11p13–p12 region was reported to be linked to Japanese type 2 diabetes mellitus specifically, in which multipoint analysis showed the highest MLS of 3.08 near *D11S935* (Mori et al. 2002). The distance between *D11S905* and *D11S935* is about 5 cM. These findings together suggest that the 11p13–p12 region will be a susceptibility region for Japanese type 2 diabetes mellitus.

In addition to *D11S905*, one nominally significant evidence of linkage was detected at *D6S462* (MLS of 2.02) by two-point analysis. However, the multipoint MLS at *D6S462* was 0.08, and two other reports did not show evidence of linkage to this region (Mori et al. 2002; Iwasaki et al. 2003), suggesting that 6q15–q16 might not be a susceptibility region for type 2 diabetes mellitus. Two susceptibility regions for type 2 diabetes mellitus in Japanese, chromosome 2 (236.8 cM) and chromosome 6 (42.2 cM), were reported to be overlapped between the two previous linkage studies (Iwasaki et al. 2003). However, the MLS at these two loci were < 1 (Iwasaki et al. 2003). Among the three reports including this study, the overlapped susceptibility region with suggestive evidence of linkage for Japanese type 2 diabetes mellitus was *D11S935–D11S905* only.

In conclusion, we have reconfirmed that the evidence of linkage for type 2 diabetes mellitus in Japanese to 11p13–p12 and 11p13–p12 will be a promising region for future studies on identification of susceptibility genes for type 2 diabetes mellitus in Japanese.

Acknowledgements We thank all patients for participating in this study, T. Amano in Kyushu University and T. Baba in SRL, Inc., for collecting the samples, and A. Murakami, E. Yachi, K. Ohki, and T. Fujimoto for technical assistance. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of Pharmaceuticals and Medical Devices Agency (PMDA).

Appendix

Collaborating groups listed in alphabetic order: Nobuyuki Abe (Abe Diabetes Clinic), Katsumi Eguchi (Nagasaki University), Nobuaki Fujio (Beppu Medical Center), Masafumi Haji (Kyushu Rosai Hospital), Mine Harada (Kyushu University), Sinsuke Hiramatsu (Kyushu Medical Center), Mitsuo Iida (Kyushu University), Minako Imamura (Kyushu University), Hidehiro Ishii (Kitakyushu Municipal Medical Center), Eiji Kawasaki (Nagasaki University), Kunihisa Kobayashi (Kyushu University), Ichiro Komiya (University of the Ryukyus), Shiori Kondo (Matsuyama Red Cross Hospital), Yasuko Kono (Imamura Hospital), Nobuyuki Koriyama (Kagoshima University), Minoru Kuriyama (Social Health Insurance Inatsuki Hospital), Kazunari Matsumoto (Sasebo Central Hospital), Kazuo Mimura (Fukuoka Medical Association Hospital), Isao Morimoto (Inoue Hospital), Mieko Nakayama (Kyushu University), Shyoichi Natori (Aso Iizuka Hospital), Yosuke Okada (University of Occupational & Environmental Health), Haruka Sasaki (Fukuoka University), Naotaka Sekiguchi (Kyushu University), Yasunori Sera (Sasebo Central Hospital), Michio Shimabukuro (University of the Ryukyus), Yuji Tajiri (Fukuoka Medical Association Hospital), Chuwa Tei (Kagoshima University), Maiko Tsuda (Kitakyushu Municipal Medical Center), Takero Uemura (Yatsushiro General Hospital), Eiji Kawasaki (Nagasaki University).

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Research report

Differential regulation of the regulatory subunits for phosphatidylinositol 3-kinase in response to motor nerve injury

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Abstract

Type Ia phosphatidylinositol 3-kinase (PI3K) generates lipid products that operate as one of major second messengers following activation of tyrosine kinase receptors. PI3K is a heterodimer composed of a 110-kDa catalytic subunit and a regulatory subunit. In this study, we determined the expression of mRNA for the regulatory subunits after injury of rat hypoglossal nerves. In situ hybridization histochemistry revealed that the expression of PI3K regulatory subunit α isoforms (p85α, p55α, and p50α) was significantly enhanced in injured motor neurons, whereas other regulatory subunits such as p85β or p55γ were not detected. Of the α isoforms, the greatest increase was observed in p55α mRNA levels, while there were smaller increases in p85α and p50α mRNA expression. These results were confirmed by RT-PCR analysis. Further immunohistochemical analysis also confirmed the increased level of p55α protein in injured motor neurons. Taken together with the previously reported induction of the p110α catalytic subunit in injured neurons, these results suggest that PI3K, consisting of p55α and p110α, plays a crucial role in the process of nerve regeneration.

Theme: Development and regeneration
Topic: Regeneration

Keywords: Nerve injury; Hypoglossal nerve; PI3K; Phosphoinositide; Regulatory subunit; Rat

1. Introduction

Previous work has shown that a peripheral nerve injury induces an organized expression of a wide range of molecules belonging to intracellular signaling pathways. For instance, Akt/Protein kinase B (PKB), Shc, 14-3-3, extracellular signaling kinase 1 (ERK1), ERK kinase 1 (MEK1), and JAK2/3 were all up-regulated in regenerating motor neurons [21,28–30,35,43]. Of these molecules, we have shown the functional consequences of Akt-mediated signaling pathways during the regeneration of injured motor neurons [28]. Adenoviral gene transfer of constitutively activated Akt could rescue injured motor neurons in vivo. In addition, Akt promotes axonal elongation of injured adult rat hypoglossal nerves [28]. It was therefore concluded that Akt is one of the key signaling molecules during nerve regeneration. The well-established signaling molecule of Akt activation is phosphatidylinositol 3-kinase (PI3K) [3,4]. In response to growth factor stimulation, PI3K can phosphorylate phosphoinositides at the D-3 position of the inositol ring [42]. This lipid by-product of PI3K recruits the PH domain of Akt to the cell membrane, enables Akt kinase PDK1 to phosphorylate Akt, and thereby activates Akt [4]. In addition to the survival activity of PI3K via Akt, PI3K is involved in various cellular responses, including protein synthesis, glucose uptake, proliferation, membrane ruffling, receptor internalization,

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and chemotaxis [4], indicating that some might play an important role during nerve regeneration.

On the basis of substrate specificity, PI3K can be categorized into three classes (I, II, and III). Among those subclasses, Class I PI3K plays a major role in generating the lipid product PI(3,4,5)P_3 following activation of receptor tyrosine kinases [34]. Class I PI3K is a heterodimer composed of a 110-kDa catalytic subunit and a regulatory subunit. The primary role of the regulatory subunit of PI3K is to recognize an upstream signal such as tyrosine kinase receptor and Gab-1 [13]. To date, five mammalian PI3K regulatory subunit isoforms have been identified, including two full-length versions of 85-kDa proteins (p85α and p85β), two 55-kDa proteins (p55α and p55γ), and one 50-kDa protein p50α [39]. The alpha isoforms, p85α, p55α, and p50α, are splice variants derived from a single gene, while the isoforms p85β and p55γ originate from different genes [8]. However, the biological significance of this diversity is poorly understood even though all five isoforms are abundantly expressed in neurons of the rat brain [16,33]. Although the expression of mRNA for the PI3K catalytic subunit p110α is enhanced in injured motor neurons [19], the expression of any PI3K regulatory subunits following nerve injury has yet to be determined. In this study, we have focused on the members belonging to Class I because Class I PI3K is the primary group for the production of PI(3,4,5)P_3, which is important for the various intracellular signaling in vivo. We demonstrated that the expression of Class I PI3K regulatory subunits is differentially regulated in injured motor neurons, suggesting there are functional differences between the regulatory subunits during nerve regeneration.

2. Materials and methods

2.1. Animals and surgery

Adult male Wistar rats weighing 150–200 g (6–8 weeks old, total number of animal: 55) were anesthetized with pentobarbital (45 mg/kg). They were placed in a supine position and their right hypoglossal nerves were carefully exposed. The nerve was then cut was with a pair of scissors just proximal to its bifurcation at the hyoid bone.

2.2. In situ hybridization

The techniques used were essentially as described previously [29]. Animals (three rats at each time point) were decapitated 1, 3, 5, 7, 14, 28, and 49 days after surgery under deeply anesthesia (diethyl ether). Their brains were quickly removed and frozen on powdered dry ice. The 16-μm-thick sections were cut on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane coated slides, and stored at −80 °C for later use. The following cDNA fragments were used as probes: common (1582-2170 of the accession number D64045); p85α (1-311 of the accession number D64045); p55α (-146-96 of the accession number D64048); p50α (-167-18 of the accession number D78486); p85β (-36-310 of the accession number D64046); and p55γ (97-547 of the accession number D64047) [33]. The antisense RNA probe for the common recognizes common regions of p85α, p55α, and p50α. The antisense RNA probes for p85α, p55α, p50α, p85β, and p55γ recognize their specific regions. The sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 min, washed in PB, treated with 10 μg/ml proteinase K in 50 mM Tris–HCl and 5 mM EDTA for 10 min, and then returned to the fixative solution. After washing in distilled water, the sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, rinsed with PB, dehydrated in an ascending ethanol series (70%, 95% and 100%), defatted in chloroform, rinsed in ethanol, and then air dried. The [α-^{35}S] UTP-labeled antisense RNA probes were prepared by in vitro transcription of each cDNA using T3 or T7 RNA polymerase (Promega, Madison, WI, USA) and [α-^{35}S] UTP. The labeled probes (5×10^5 cpm/μl) in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl, 5 mM EDTA, 10 mM PB, 10% dextran sulfate, 1×Denhardt's solution, 0.2% sarcosyl, 500 μg/μl yeast transfer RNA, and 200 μg/ml salmon sperm DNA) were denatured for two min in boiling water, quenched on ice, and placed on the sections. Hybridization was performed overnight in a humid chamber at 55 °C. Hybridized sections were briefly rinsed five times in saline sodium citrate buffer (SSC) and 1% 2-mercaptoethanol at 55 °C, and then washed in 50% deionized formamide, 2×SSC, and 10% 2-mercaptoethanol (high stringency buffer) for 30 min at 65 °C. After rinsing the sections in RNase buffer (0.5 M NaCl, 10 mM Tris–HCl, and 1 mM EDTA), they were treated with 1 μg/ml RNase-A in RNase buffer for 30 min at 37 °C and washed again in RNase buffer. Sections were then incubated in high stringency buffer again as described above, rinsed with 2×SSC and 0.1×SSC for 10 min each at room temperature, dehydrated in an ascending ethanol series, and then air dried. Sections were then exposed to X-ray film for 2 weeks and then dipped in Kodak NTB2 emulsion diluted 6:4 in water. Sections were then exposed for 5–6 weeks at 4 °C, developed in Kodak D-19 developer, counterstained with thionine, dehydrated in a graded series of ethanol to xylene, and coverslips mounted before examination by a microscope.

2.3. Relative quantification of mRNA

The relative optical density of signals on the X-ray film was measured using an Image Analysis System (NIH Image, National Institute of Health, USA). The defined area occupied by autoradiographic grains in the hypoglossal nuclei was measured bilaterally on the X-ray film using an image analyzer. The measured background value for cerebellar white matter was subtracted from the value for

hypoglossal nuclei, and the difference defined 'the optical density unit.' The optical density units from the right (injured) and the left (control) hypoglossal nuclei on the identical section were determined. For statistical analysis, at least four sections from three rats per time point were studied. Statistically significant difference was assessed by paired *t*-test.

2.4. RT-PCR analysis

Operations were performed on adult male Wistar rats weighing 150–200g (6–8 weeks old) and killed on post-operative day 7 under deeply anesthesia. Reverse transcription–polymerase chain reaction (RT-PCR) was conducted as described by Honma et al. [12] with a slight modification. The total RNA derived from hypoglossal nuclei on either side was isolated and purified using the acid guanidine isothiocyanate/phenol/chloroform extraction (AGPC) method [5]. Aliquots from the RT reaction were used for PCR amplification using primer pairs for the ubiquitously expressed glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal control. Activating transcription factor-3 (ATF-3), which is induced after a hypoglossal axotomy, was used as a positive control. The amplifying cycle counts were 34 for the detection of PI3K regulatory subunits except p55γ, 32 for the detection of p55γ and ATF-3, and 20 for the detection of GAPDH.

Specific primers for the detection of mRNA for each PI3K regulatory subunit were used for RT-PCR. The primers used were as follows: for p85α, 668–688 and 1218–1237 of D64045; for p55α, 15–34 and 408–427 of D64048; for p50α, 1–20 and 318–337 of D78486; for p85β, 383–402 and 1034–1053 of D64046; for p55γ, 7–28 and 423–442 of D64047; for GAPDH, 836–855 and 1149–1168 of NM017008; and for ATF-3, 165–187 and 688–708 of M63282. The reaction products were separated electrophoretically on a 1% agarose gel and visualized by staining with ethidium bromide.

2.5. Immunohistochemistry

Animals were perfused at postoperative day 7. The brains were quickly removed and fixed in a 4% paraformaldehyde solution. Brains were postfixed in the same fixative for 3 days, and then immersed in phosphate-buffered saline (PBS) containing 25% sucrose before being sectioned. The 20-μm-thick sections were cut on a cryostat and floated in 6-well cell culture dishes containing PBS. The primary antibody against the N-terminal SH2 region of rat p85α, which recognizes all three α-isoforms of the PI3K regulatory subunit, was purchased from UBI (Lake Placid, NY, USA) [38]. A p55α-specific antibody was raised and characterized as previously described [15,16,33]. The primary antibodies were used at a dilution of 1:500. The sections were covered with 5% normal goat serum in PBS for 30 min and incubated with the primary antibodies over two nights. After being washed in PBS, the sections were subsequently incubated with biotinylated goat anti-rabbit IgG antibody (Vector Laboratories, USA) at a dilution of 1:200 for 2 h, washed with PBS, and treated with an avidin–biotin complex mixture (Vector Laboratories). The sections were washed with PBS and finally reacted with 3,3-diaminobenzidine tetrahydrochloride and hydrogen peroxide to reveal a brown reaction product. For a histochemical specificity, we have also examined those processes without the primary antibody, and in this control the staining was negative.

3. Results

We performed in situ hybridization to evaluate the changes in expression of mRNA for the regulatory subunit

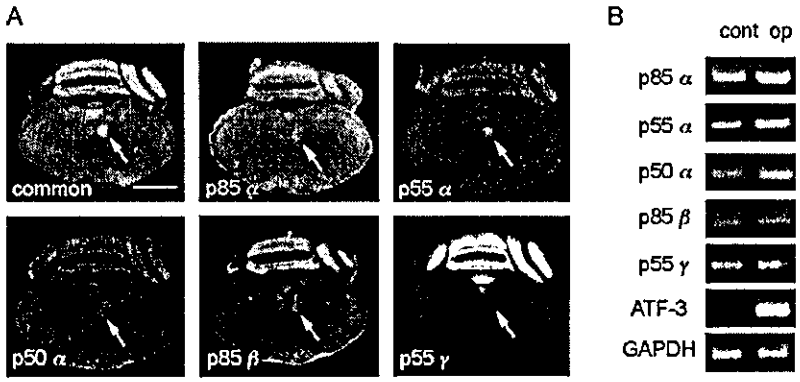

Fig. 1. Expression of mRNA for the PI3K regulatory subunit family members (p85α, p55α, p50α, p85β, and p55γ) after nerve injury. Film autoradiography of hybridized tissue sections for PI3K regulatory subunit family members 7 days after hypoglossal nerve transection (A). Only the common p85α, p55α, and p50α probes exhibited increased levels of mRNA on the operated side (right; arrows). The common sequence was a region common to all three α-isoforms. Scale bar=3 mm. Expression of mRNA for the PI3K regulatory subunit family members (p85α, p55α, p50α, p85β, and p55γ) in nerve injured hypoglossal nucleus determined by RT-PCR (B). In hypoglossal nuclei, p85α, p55α, and p50α mRNA level was higher on the injured sides compared with the uninjured side. However, the expression of p55γ and p85β on the injured side was weaker in hypoglossal nuclei. The expression of GAPDH mRNA was used as an internal control. ATF-3 mRNA expression was used as a positive control.

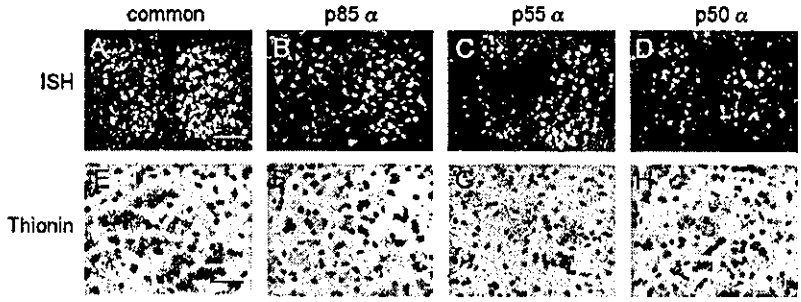

Fig. 2. Expression of mRNA for common (A and E), p85α (B and F), p55α (C and G), and p50α (D and H) in the hypoglossal nucleus 7 days after a hypoglossal nerve transection (right). Emulsion autoradiography of hybridized tissue sections for the PI3K regulatory subunit family members (common, p85α, p55α, and p50α) (A–D). Scale bars=0.2 mm. High-power bright field photographs counterstained with thionine showed that the hybridization signal (accumulation of silver grains) was found on injured motor neurons, but not on glial cells (E–H). Scale bars=50 μm.

of PI3K during nerve regeneration. A marked increase in the mRNA for the common region of the α-isoforms was observed in the autoradiographic film (Fig. 1A). As the sequence is common to all three α-isoforms, we next determined the expression of each isoform using isoform-specific RNA probes. mRNA for the three α-isoforms (p85α, p55α, and p50α) was up-regulated after nerve injury, whereas the levels of mRNA for the β-and γ-isoforms (p85β and p55γ) did not exhibit any changes. The most marked increases were observed in p55α mRNA expression (Fig. 1A), while p85α and p50α mRNA levels showed smaller increases (Fig. 1A). To confirm these increases, we measured the changes in mRNA for the PI3K regulatory subunit by RT-PCR (Fig. 1B). The increase in ATF-3 mRNA was measured as a positive control. The results obtained confirmed those attained by in situ hybridization. The expression of α-isoform mRNA was up-regulated in injured hypoglossal nucleus, whereas β and γ-isoform mRNA levels were not altered. The increase in p55α mRNA expression was the most marked (Fig. 1B).

Emulsion autoradiography was also used to determine the cell types expressing elevated levels of mRNA for all three α-isoforms (Fig. 2A–D). Counterstaining with thionine revealed an accumulation of hybridization signal in the large cells of the hypoglossal nucleus, suggesting that the mRNA for the three α-isoforms increased in injured hypoglossal motor neurons but not in the surrounding glial cells (Fig. 2E–H).

A semiquantitative analysis of the film autoradiogram showed that there were significant increases in the levels of mRNA for p85α, p55α, and p50α 1–14 days after nerve injury (Fig. 3). One day after nerve transection, there were slight increases in the levels of p50α mRNA in the ipsilateral hypoglossal nucleus and marked increases to a peak level after 5 days. The hybridization signal then gradually returned to control levels over the following 3 weeks. The hybridization signal for p55α mRNA in the ipsilateral hypoglossal nucleus increased after 1 day of nerve resection and increased markedly to a peak level after 7 days. The hybridization signal then gradually returned to control levels over the following 3 weeks. Of the three α-isoforms, p55α mRNA exhibited the greatest increases. The increase in p85α mRNA appeared relatively late and peaked 7 days after nerve injury. There was a tendency that those mRNA levels were slightly increased in the contralateral hypoglossal nuclei.

To determine the changes in subunit protein expression, we used two antibodies that can detect either common α-

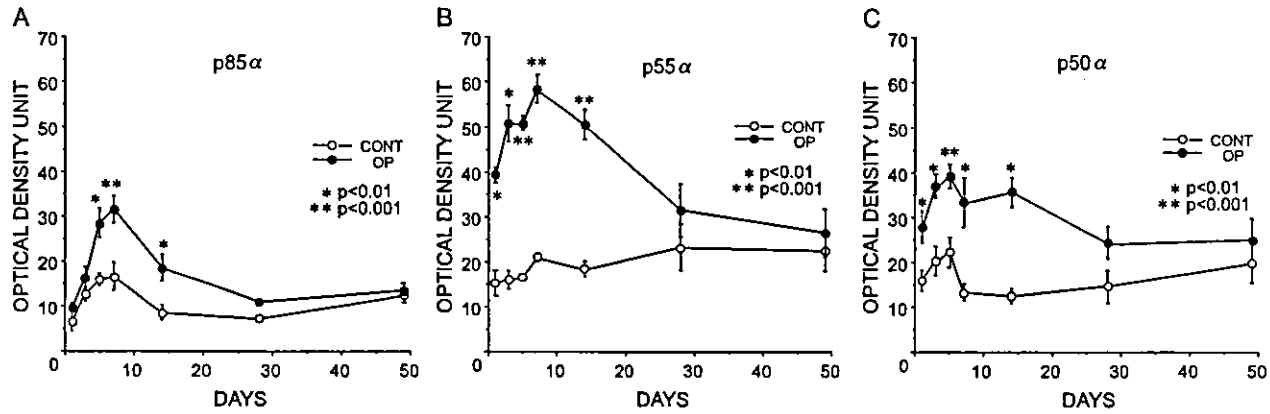

Fig. 3. Semiquantification of mRNA levels for the PI3K regulatory subunit α isoforms (p85α, p55α, and p50α) in both operated side (solid circle) and the contralateral control side (open circle). Each point shows the average intensity of positive signals and its S.E. as determined from the X-ray film autoradiograms. Statistically significant differences were determined by paired *t*-tests; $*p<0.01$ and $**p<0.001$).

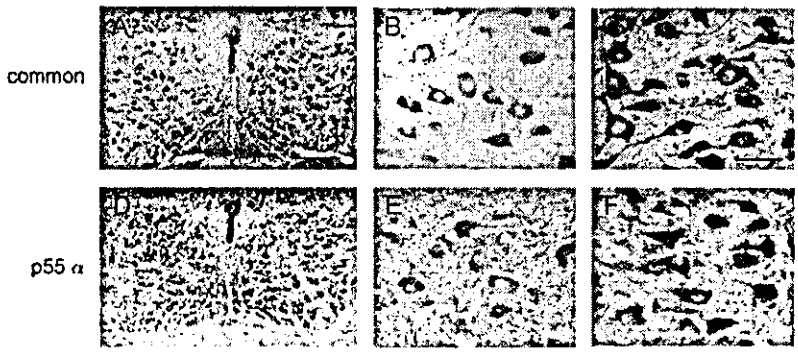

Fig. 4. Immunohistochemical detection of the α isoforms of the regulatory subunit for PI3K (common) and p55α in rat hypoglossal nuclei 7 days after nerve injury (operated side is on the right). Enhanced expression of the three α isoforms (common) and p55α were observed in injured hypoglossal nucleus (A and D). A high-power magnification also showed the three α isoforms (common) and p55α immunoreactivity in injured hypoglossal nucleus (C and F), whereas the intensity of staining in noninjured hypoglossal nucleus was weak (B and E). Scale bar=0.2 mm (A and D), and 50 μm (B, C, E and F).

subunits or specifically detect p55α subunits on the tissue sections. The specificity of these antibodies has been described previously [33,38]. Although the antibody, which detects the common N-terminal SH2 region of p85α revealed cytoplasmic staining of hypoglossal motor neurons in the noninjured nucleus, the staining was substantially greater in injured hypoglossal nucleus (Fig. 4A and C). Similarly, immunohistochemistry using the specific anti-p55α antibody revealed increased levels of p55α protein in the cytoplasm of injured hypoglossal neurons (Fig. 4D and F). These results clearly demonstrate that both the mRNA and protein of p55α were up-regulated in injured hypoglossal neurons but not in glial cells.

4. Discussion

In the present study, we determined whether the expression of PI3K regulatory subunits increased following motor nerve injury. Of the five isoforms examined, the mRNAs for the α isoforms of PI3K regulatory subunits (p85α, p55α, and p50α) are up-regulated in injured motor neurons. These isoforms have been shown to bind to p110α catalytic subunits via the p110 binding domains common to all three isoforms and thereby enhance p110α kinase activity. Since a previous report has shown that p110α expression is induced in injured motor neurons [19], a PI3K heterodimer composed of a p110α catalytic subunit and α isoforms of a regulatory subunit may play a key role in motor neuron regeneration. However, the expression profiles of all five regulatory subunits varied during nerve regeneration. The basal level of p85α mRNA is relatively high even in uninjured hypoglossal motor neurons and the up-regulation is relatively small. Similarly, moderate p110α mRNA hybridization signals were seen in the uninjured side (data not shown). These results suggest that p85α coupled with p110α are crucial in processes such as the maintenance of neuronal metabolism in normal neurons, as well as its role in neuronal regeneration. In contrast, almost no p55α and p50α expression was observed in noninjured motor neurons, but there is a substantial up-regulation of p55α mRNA and protein in response to nerve injury. Of all the PI3K regulatory subunits, p55α may be the most important in neuronal regeneration, although we need knockout or knock down strategy to conclude this. In terms of those mRNAs induction periods, Ito et al. [19] mentioned that the increased expression of p110α mRNA was seen during 2 weeks after nerve crush, but in our model the alteration of p110 mRNA was observed during 28 days after nerve cut (data not shown). This discrepancy would be due to the methods used (cut versus crush). Therefore, it seems that the mRNA alterations seen in p110 and p55α are in parallel. Our previous morphological study [26] demonstrated that outgrowth of thin regenerating axons into the frontal area of the tongue was firstly observed at 14 postoperative days, and presynaptic formation of neuromuscular junction (NMJ) was observed from 21 postoperative days. Under electron microscopic observation, reconstruction of new NMJs was observed within the interval between 21 and 28 days. These observations suggest that the alteration periods of p110 and p55α mRNAs are well corresponding to that of NMJ recovery. This may also support the involvement of PI3K in nerve regeneration.

There was a tendency that some mRNAs were slightly increased in the contralateral hypoglossal nuclei. Following peripheral nerve lesions, there are well-documented events that affect the contralateral nonlesioned structures. It is unclear whether these serve a biological purpose, but the existence of these effects implies the presence of unrecognized signaling mechanisms such as growth factors form the target organ, in this case from tongue [23].

PI3K has been shown to be involved in various cellular phenomena. In neuronal cells especially, previous studies have implicated PI3K activation in the regulation of neurite elongation [20], neuronal migration [2], synaptic plasticity [27], receptor internalization [44,45], and retrograde transport [24], in addition to mediating survival [28]. Although PI3K/Akt-induced survival activity might be one of its key biological functions in injured motor neurons [28], more recent papers have focused on its role in the regulation of cell

morphology or motility via cytoskeletal reorganization [6,7]. Such papers have shown that local activation of PI3K is crucial for the induction of correct cytoskeletal changes. In *Dictyostelium* cells or neutrophils, PI3K generates $PI(3,4)P_2$ and/or $PI(3,4,5)P_3$ at the leading edge of migrating cells in response to a gradient of chemoattractants, and PI3K localization to such a restricted region of the cells might be required for chemotaxis [9,14,40,41]. Similarly, the localization and activation of PI3K at the tip of the growth cones is necessary for determining the neuronal polarity during the specification and elongation of hippocampal axons [32]. Although the reason why such local activation of PI3K occurs remains unclear, p110 binding protein might be involved in determining the localization of p110 following factor stimulation. Here we found that of the PI3K regulatory subunits, p55α expression was the most prominent in response to nerve injury. Interestingly, p55α and p55γ contain a unique 34-amino-acid sequence in their N-terminus, which is not found in p85α or p50α. A previous biochemical study has shown that p55α exhibits strong and specific binding activity with tubulin via its unique sequence, although it is unknown whether this binding is direct [17]. Of the regulatory isoform overexpressing cells, insulin stimulated α/β tubulin-associated PI3K activity only in p55α overexpressing cells. In addition, p55α, but not p85α or p50α, was present in a purified microtubule assembly from rat brain [17]. Hence, injury-induced p55α in regenerating motor neurons might be a candidate molecule for the recruitment of PI3K to the correct site on the neuron, maybe to the microtubules. It is well known that reorganization of the cytoskeleton, including both actin filaments and microtubules, might be required for growing axon tips [25]. The regulation of actin dynamics may be a target for PI3K in regenerating axons, as PI3K activation could be involved in the regulation of actin filament polymerization by activating the Rho GTPase family [10]. However, PI3K might also have an affect on process formation or the maintenance of neurites via control of the microtubule network [22,31]. Thus, the induction of p55α in injured motor neurons (the present study) and its binding activity with tubulin [17] suggests that PI3K activity may be involved in the modification of the microtubule itself or its related molecules rather than actin dynamics during motor nerve regeneration.

Another specific function of p55α is also likely. Recent papers have shown that each isoform has a specific role in signal transduction activated by individual receptor tyrosine kinases [18,37]. For example, Inukai showed that each regulatory subunit exhibited a different response in both its association with the receptor tyrosine kinase complex, and signal transduction to their effectors [18]. These phenomena remind us that p55α is a more effective signal transducer than other isoforms, at least, in injured motor neurons. For motor neurons, GDNF would be the most effective survival promoting factor, and its signal transducing receptor, RET, was markedly up-regulated in injured motor neurons [36]. Of the α isoforms, at least p85α can bind GDNF-stimulated RET receptor complexes via a Gab-1/2 adaptor protein, and thereby transmit the evoked signal to the p110-Akt cascade [1,11]. However, whether other isoforms are more efficient than p85α in response to GDNF is still unclear. Such an isoform-specific response to GDNF and other neurotrophic factors should be investigated in further studies.

In summary, we have shown that there are increased levels of the PI3K regulatory subunit p55α in motor neurons following a nerve injury, although its precise role in signal transduction within injured neurons remains unclear. Further study is needed to elucidate its biological role in regenerating neurons.

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ARTICLE

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Inhibition of endogenous SHIP2 ameliorates insulin resistance caused by chronic insulin treatment in 3T3-L1 adipocytes

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Abstract *Aims/hypothesis:* SHIP2 is a physiologically important negative regulator of insulin signalling hydrolysing the PI3-kinase product, PI(3,4,5)P3, which also has an impact on insulin resistance. In the present study, we examined the effect of inhibiting the endogenous SHIP2 function on the insulin resistance caused by chronic insulin treatment. *Methods:* The endogenous function of SHIP2 was inhibited by expressing a catalytically inactive SHIP2 (ΔIP-SHIP), and compared with the effect of treatments designed to restore the levels of IRS-1 in insulin signalling systems of 3T3-L1 adipocytes. *Results:* Chronic insulin treatment induced the large (86%) down-regulation of IRS-1 and the modest (36%) up-regulation of SHIP2. Subsequent stimulation by insulin of Akt phosphorylation, PKCλ activity, and 2-deoxyglucose (2-DOG) uptake was markedly decreased by the chronic insulin treatment. Coincubation with the mTOR inhibitor, rapamycin, effectively inhibited the proteosomal degradation of IRS-1 caused by the chronic insulin treatment. Although the coincubation with rapamycin and advanced overexpression of IRS-1 effectively ameliorated subsequent insulin-induced phosphorylation of Akt, insulin stimulation of PKCλ activity and 2-DOG uptake was partly restored by these treatments. Similarly, expression of ΔIP-SHIP2 effectively ameliorated the insulin-induced phosphorylation of Akt without affecting the amount of IRS-1. Furthermore, the decreased insulin-induced PKCλ activity and 2-DOG uptake following chronic insulin treatment were ameliorated by the expression of ΔIP-SHIP2 more effectively than by the treatment with rapamycin. *Conclusions/interpretation:* Our results indicate that the inhibition of endogenous SHIP2 is effective in improving the state of insulin resistance caused by chronic insulin treatment.

Keywords Akt · Glucose uptake · Insulin · Insulin resistance · PKCλ · SHIP2

Abbreviations 2-DOG: 2-deoxyglucose · Glut4: glucose transporter 4 · IRS-1: insulin receptor substrate-1 · mTOR: mammalian target of rapamycin · PDGF: platelet-derived growth factor · PI3-kinase: phosphatidylinositol 3-kinase · PI(3,4)P2: phosphatidylinositol 3,4-bisphosphate · PI(3,4,5)P3: phosphatidylinositol 3,4,5-triphosphate · PKC: protein kinase C · SHIP2: SH2-containing inositol 5′-phosphatase 2

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Introduction

The activation of phosphatidylinositol 3-kinase (PI3-kinase) is known to be important to the various metabolic actions of insulin [1–4]. PI(3,4,5)P3 produced by activated PI3-kinase is thought to function as a key lipid second messenger in insulin signalling to further downstream molecules [3–5]. We and others identified SH2-containing inositol 5′-phosphatase 2 (SHIP2) as a lipid phosphatase possessing 5′-phosphatase activity to hydrolyse PI(3,4,5)P3 to PI(3,4)P2 [6, 7]. Previous reports have indicated that overexpression of SHIP2 inhibits insulin-induced glucose uptake and glycogen synthesis via its 5′-phosphatase activity in 3T3-L1 adipocytes and L6 myocytes [8, 9]. Targeted

disruption of the SHIP2 gene in mice increased sensitivity to insulin without affecting other biological systems [10]. These findings indicate that SHIP2 is a physiologically important negative regulator that is relatively specific to insulin signalling. In addition, expression of SHIP2 protein is enhanced in the skeletal muscle and fat tissue of diabetic db/db mice [11]. Treatment with the insulin-senzsitizing thiazolidinedione, rosiglitazone, lowered the elevated levels of SHIP2 in the db/db mice [11]. Furthermore, a deletion in the 3′ untranslated region within the motifs implicated in the control of protein synthesis leading to the possible increase in expression of SHIP2 protein was identified in the UK and Belgian population of individuals with type 2 diabetes [12]. Therefore, SHIP2 is implicated in insulin resistance as a cause of type 2 diabetes in addition to the physiological importance in insulin signalling. Based on these findings, inhibition of endogenous SHIP2 function may be a target for ameliorating insulin signalling in the state of insulin resistance.

Hyperinsulinaemia is a hallmark of insulin resistance [13–15]. Chronic hyperinsulinaemia causes a desensitization to subsequent insulin responses, which appears to be part of the vicious cycle involved in the pathogenesis of type 2 diabetes [16–18]. In this regard, chronic treatment with insulin is known to facilitate the proteosomal degradation of IRS-1 leading to the down-regulation of insulin signalling at IRS-1 in 3T3-L1 adipocytes [17–19]. However, it is unknown whether SHIP2 is also involved in the resistance caused by chronic exposure to insulin. In the present study, the change in SHIP2 expression following chronic insulin treatment was investigated in 3T3-L1 adipocytes. In addition, the effect of inhibition of endogenous SHIP2 function using adenovirus-mediated gene transfer of a dominant-negative SHIP2 (ΔIP-SHIP2) on the possible amelioration of decreased insulin signalling caused by the chronic insulin treatment was investigated. The down-regulation of insulin signalling at the level of IRS-1 caused by the chronic insulin treatment can be ameliorated by pretreatment with rapamycin, which is an inhibitor of mTOR-dependent proteosomal degradation of IRS-1 [20, 21]. Alternatively, the decrease of IRS-1 can be prevented by overexpression of IRS-1 through adenovirus-mediated gene transfer [22]. Finally, the effects of the amelioration at the level of IRS-1 and SHIP2 on the chronic insulin treatment-induced down-regulation of insulin signalling were compared.

Materials and methods

Materials Human crystal insulin was provided by Novo Nordisk Pharmaceutical (Copenhagen, Denmark). [γ-^{32}P] ATP (111 TBq/mmol) and 2-[^{3}H]deoxyglucose (DOG; 3,330 GBq/mmol) were purchased from NEN Life Science Products (Boston, MA, USA). The two polyclonal anti-SHIP2 antibodies were described previously [7]. A polyclonal anti-PKCλ antibody was kindly provided by Dr W. Ogawa (Kobe University, Japan) [22]. A monoclonal anti-phosphotyrosine antibody (PY99) was from Transduction Laboratories (Lexington, KY, USA). A polyclonal anti-Thr^{308} phospho-specific Akt antibody, a polyclonal anti-Ser^{473} phospho-specific Akt antibody, and a monoclonal anti-PKCλ antibody were from Cell Signalling (Beverly, MA, USA). A polyclonal anti-Akt antibody and a polyclonal anti-Glut4 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A polyclonal IRS-1 antibody and a polyclonal anti-PDGF β receptor antibody were from Upstate Biotechnology (Lake Placid, NY, USA). Enhanced chemiluminescence reagents were from Amersham Pharmacia Biotech (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM) vitamin mixtures, and MEM amino acid solutions were from Gibco BRL Japan (Tokyo, Japan). All other reagents were of analytical grade and purchased from Sigma Chemical (St Louis, MO, USA) or Wako Pure Chemical Industries (Osaka, Japan).

Construction of adenoviral vectors A cDNA encoding a phosphatidylinositol 5′-phosphatase-defective mutant of SHIP2 (ΔIP-SHIP2) containing Pro^{687} to Ala, Asp^{691} to Ala, and Arg^{692} to Gly changes was subcloned into the vector pAxCAwt, and transferred to recombinant adenovirus by homologous recombination utilizing an Adenovirus Expression Vector Kit (Takara Biomedicals, Tokyo, Japan) as described previously [8]. The adenoviral vector encoding IRS-1 was also described previously [23].

Cell culture and infections with adenovirus 3T3-L1 fibroblasts were grown and passaged in DMEM supplemented with 10% donor calf serum. Cells at 2–3 days postconfluence were used for differentiation. The differentiation medium contained 10% fetal bovine serum (FBS), 250 nmol/l dexamethasone, 0.5 mmol/l isobutyl methylxanthine, and 500 nmol/l insulin. After 3 days, the differentiation medium was replaced with postdifferentiation medium containing 10% FBS and 500 nmol/l insulin. After 3 more days, the postdifferentiation medium was replaced with DMEM supplemented with 10% FBS. ΔIP-SHIP2 and IRS-1 were transiently expressed in differentiated 3T3-L1 adipocytes by means of adenovirus-mediated gene transfer. A multiplicity of infection (m.o.i.) of 10–40 pfu/cell was used to infect 3T3-L1 adipocytes in DMEM containing 2% FBS, with the virus being left on the cells for 16 h prior to removal. Subsequent experiments were conducted 24–48 h after initial addition of the virus [8]. The efficiency of adenovirus-mediated gene transfer of ΔIP-SHIP2 and IRS-1 was approximately 95%.

Measurements of PI(3,4,5)P3 and PI(3,4)P2 levels in vivo The same numbers of 3T3-L1 adipocytes transfected with LacZ or ΔIP-SHIP2 were starved of phosphate overnight in phosphate-free DMEM (Life Technology), then starved of serum for 3 h. [^{32}P]Orthophosphate (3.7 MBq/ml) was added, and the cells were cultured for an additional 2 h. Following the labelling period, the cells were incubated with or without 1 μmol/l insulin for 15 min. The reaction was terminated by washing once with ice-cold PBS, followed by the addition of methanol and 1 N HCl (1:1). The labelling of the cells with [^{32}P]orthophosphate was con-