Evidence for phosphorylation of rat liver glucose-regulated protein 58, GRP58/ERp57/ER-60, induced by fasting and leptin

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Abstract Glucose-regulated protein 58 (GRP58)-like immunoreactivity in rat liver obtained in the evening or after fasting underwent an electrophoretic band-shift, which disappeared after phosphatase-treatment. Since mass spectrometric analysis raised a possibility that Ser150 of GRP58 is phosphorylated, an antibody against the phosphoserine150 GRP58 was generated. Immunoreactivity to this antibody was increased in the evening and after fasting. Since GRP58 was shown to interact with signal transducer and activator of transduction 3 (STAT3), a leptin-related protein, the effect of leptin was examined. Immunoreactivity to the anti-phosphoGRP58 antibody was markedly elevated after the leptin injection, indicating that Ser150 of GRP58 is phosphorylated after fasting and leptin-treatment.
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1. Introduction

To investigate daily changes in liver function, protein expression patterns in the rat liver were analyzed by proteome analysis using one- and two-dimensional electrophoresis (1DE and 2DE), mass spectrometry and immunoblotting. Multiple spots of immunoreactivity to an anti-glucose-regulated protein 58 (GRP58) antibody were detected. GRP58 is a stress protein of about 60 k whose expression is induced in conditions such as in glucose starvation and viral infection [1,2]. It is homologous to protein disulfide isomerase (PDI) and possesses two thioredoxin domains including an active motif (CGHC) of the disulfide redox response as well as PDI [3]. Recently, it was reported that GRP58 modulates the intracellular signal transduction by interacting with signal transducer and activator of transduction 3 (STAT3) [4], a leptin-related protein, and

coexists with cytoplasmic STAT3 and the plasma membrane complexes [5,6]. The aim of this study was determine whether the multiple spots of GRP58-like immunoreactivity were caused by its phosphorylation and how starvation and leptin affected on the patterning of these spots. It was found that serine150 of GRP58 is phosphorylated by fasting and or treatment with leptin.

2. Materials and methods

2.1. Animals and liver sampling

Male Wistar strain rats, weighing 250–300 g, were used. They were housed in a room illuminated for 12 h (lights on 07:00–19:00 h) and kept at 24 ± 1 °C for at least 2 weeks before the experiments. Food (type ME, Oriental Yeast Co., Tokyo) and water were freely available. Animal care and handling were approved by the Institutional Animal Care and Use Committee of Osaka University. Animals were sacrificed by decapitation at 12:00 h [Zeitgeber time (ZT) 5], 18:00 h (ZT11), 24:00 h (ZT17) and 06:00 h (ZT23) and livers were obtained. ZT represents hours after the light turned on under a 12-h light/12-h dark cycle [7].

To determine the effect of fasting, rats were deprived of food and sacrificed at 12, 24, 36 and 48 h after which their livers were harvested and analyzed.

2.2. Two-dimensional gel electrophoresis

Immobilized pH gradient (IPG) gel strips (pH 4–7; NL, 7, 13 and 18 cm; Amersham–Pharmacia Biotech) were used as the first dimension gel of 2DE for isoelectric focusing. Liver samples were homogenized in ten volumes of 8 M urea, 60 mM DTT, 2% Chaps, and 10 mM Tris–HCl (pH 7.4), and when examined with protein staining 200 μg of protein was applied on an 18 cm gel strip and examined with mass spectrophotometry. The samples were mixed with 150 μl (for 7 cm gels) or 280 μl (for 13 cm gels) of rehydration buffer containing 7 M urea, 2 M thiourea, 2% Chaps, 10 mM DTT, 2 mM Tris (2-cyanoethyl) phosphine, 2% pharmalyte 3–10, and a trace amount of bromophenol blue, and the gels rehydrated. Isoelectric focusing was carried out on the gel strips using an electrophoretic apparatus where the voltage was increased stepwise to 4500 V for 18 cm followed by incubation at each voltage for 4–5 h. After the first dimensional electrophoresis, the strips were incubated three times with 5 ml of a solution containing 50 mM Tris–HCl (pH 6.8), 8 M urea, 2% (w/v) SDS, and 60 mM DTT for 30 min, and once with 5 ml of solution containing 50 mM Tris–HCl (pH 6.8), 8 M urea, 2 M thiourea, 20 mM DTT, 30% (w/v) glycerol, 2% (w/v) SDS, and 2 mM Tris(2-cyanoethyl)phosphine for 30 min. In the second dimensional electrophoresis, SDS–polyacrylamide gels (9–16% T/2.6% C) without a stacking gel were used as resolving gels.

2.3. Protein staining and image analysis

After the 2DE, gels were washed for 30 min in a gel fixing solution containing 7% acetic acid and 10% methanol. For maximum sensitivity all gels were stained using SYPRORuby protein gel fluorescent stain

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(BioRad) for 3 h and rinsed in fixing solution for 60 min. Fluorescent signals were detected using a fluorescence image analyzer (Fluorimager 595; Molecular Dynamics).

2.4. 1D-PAGE and 2D-PAGE for Western blotting for analysis of GRP58

For 1D-PAGE, an equal amount of protein from each sample (10 μg) was electrophoresed on an 8% SDS–polyacrylamide gel. For the first dimension of 2D-PAGE, IPG gel strips (pH 4–7; NL; 7 and 13 cm) were used. The sample was mixed with rehydration buffer and applied on a gel (60 μg protein for a 7 cm gel and 100 μg for a 13 cm gel). The voltage for the electrophoresis was increased stepwise to 3000 V at 100 μA intervals for 3–5 h.

After samples were separated by either 1D-PAGE or 2D-PAGE, they were transferred onto a nitrocellulose membrane (Schleicher & Schuell). The membranes were blocked in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 (Tween-TBS), incubated with rabbit anti-GRP58 polyclonal antibodies (StressGen Biotechnologies) for 3 h, washed with Tween-TBS, incubated with HRP conjugated secondary antibodies (Cell Signaling Technology) against rabbit IgG for 1 h, and washed with Tween-TBS. Immunoreactivity to the anti-GRP58 antibodies was visualized using an enhanced chemiluminescence system (NEN Life Science Products).

2.5. MALDI-TOF-mass spectrometry

Gel sections from the 2-DE gels were incubated with 1 ml of 50 mM ammonium bicarbonate-50% methanol at 37 °C overnight. The solution was then removed and the gel sections incubated in a solution containing 100 μl of 10 mM DTT-100 mM ammonium bicarbonate for 60 min at 60 °C. The gels were alkylated with 100 μl of 50 mM iodoacetamide/50 mM ammonium bicarbonate for 30 min at room temperature. The gels were washed twice with distilled water and dried under a vacuum pump. The gels were then digested with 50 μl of 50 mM ammonium bicarbonate containing 10% acetonitrile and 1 pmol of trypsin (Sigma) for 16 h at 37 °C and resultant peptides eluted in a solution containing 50% acetonitrile, 50 mM ammonium bicarbonate, and 0.1% TFA. The supernatant obtained after centrifugation was concentrated to a volume below 30 μl under vacuum and desalted with ZipTip 18 (Millipore). For measurement of mass spectrometry (MS) spectra, 0.5 μl of sample solution was mixed with 0.5 μl of matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA), deposited on the target, and dried completely. MALDI-TOF-mass spectra were generated using an AXIMA-CFR instrument (Shimadzu Corp., Japan) under reflectron mode operating conditions after calibration using ACTH and bradykinin. Peptides were matched using the MASCOT database search under the following conditions: fixed modifications of carbamidomethyl (C); variable modifications of phosphorylated Ser and Thr; Taxonomy of Rattus; and a tolerance of one missed cleavage.

2.6. Alkaline phosphatase treatment

Sample was incubated with 0.5 μl alkaline phosphatase (New England BioLab) for 12 h at 4 °C. Then the sample was separated with SDS–PAGE, and immunoblotted using anti-GRP58 antibodies.

2.7. Leptin injection

For intracranial administration of leptin, a brain cannula, made of PE-10 (Clay Adams, Parsippany, NJ), was inserted into the right lateral cerebral ventricle (LCV), three days before the experiment under pentobarbital anesthesia (35 mg/kg) as previously described [8]. The effect of leptin on GRP58 was examined by injecting leptin [Sigma–Aldrich, 10 μg/20 μl of artificial cerebrospinal fluid (aCSF)] into the LCV at each time point under unanesthetic condition using the LCV cannula. For control experiment, 20 μl of aCSF was injected into the LCV. Animals were sacrificed at 0, 15, 30, 60, and 180 min after the administration of leptin or aCSF and liver samples were obtained.

2.8. Anti-phosphorylated GRP58 Ser-150 antibody production

A synthetic peptide, EFKKFIpSDKDASC (corresponding to amino acid residues of GRP58 surrounding Ser150) was conjugated with Imject Maleimide activated mcKLH (Pierce), mixed with Fre-

und's complete adjuvant and injected into rabbits. The resultant polyclonal antiserum against this peptide was subjected to affinity purification and used to detect GRP58 phosphorylated on Ser150.

2.9. Dephosphorylation of proteins using hydrogen fluoride–pyridine

Dephosphorylation of proteins using hydrogen fluoride–pyridine was performed as described previously [9]. Samples were dephosphorylated using hydrogen fluoride–pyridine, neutralized with NaOH, desalinated by using a centrifugal filter (Millipore), then eluted in TNE buffer.

2.10. Immunoprecipitations

Protein samples were pre-treated with protein G–Sepharose (Amersham-Pharmacia Biotech) for 1 h at 4 °C. After centrifugation, the supernatant was incubated for 1 h at 4 °C with protein G–Sepharose that had been preincubated with 2 μg of mouse anti-Stat3 antibody (BD Biosciences Pharmingen) or mouse IgG. The Sepharose beads were washed five times with TNE buffer. Immunoprecipitated proteins were analyzed using SDS–PAGE electrophoresis.

3. Results

3.1. GRP58 identified as a protein showing daily changes in its expression in rat liver

To identify proteins showing daily changes in their expression in the rat liver, livers were sampled at 18:00 h (ZT11) and 06:00 h (ZT23) and their proteins were separated by 2DE after homogenization. The 2DE-gels were stained with SYPRO Ruby and about 1200 spots were detected in gels of pH 4–7 after a comparative analysis of the protein patterns was carried out using the image analysis software, PDQUEST. Among the proteins that showed changes in their expression patterns, a spot (shown as arrow heads in Fig. 1A) was identified that appeared to undergo daily changes in its expression pattern (high at ZT11 and low at ZT23) after quantitative analysis. Peptide mass fingerprinting of 21 fragments generated from the spot showed that they matched the amino acid sequence of GRP58 with a MASCOT score of 243, and a sequence coverage of 39%, indicating that this protein is GRP58 (Table 1).

3.2. Confirmation of the protein as GRP58

To confirm that the above protein spot is actually GRP58, livers were sampled at ZT 5, 11, 17, and 23, examined by SDS–PAGE and immunoblotted using a rabbit anti-GRP58 polyclonal antibodies. However, a clear daily change in the immunoreactivity to the anti-GRP58 antibodies was not detected in 1DE (Fig. 1B), even though a clear shift in the migration of the immunoreactive band was observed at ZT11 in comparison with sample bands obtained at other time points (Fig. 1B). These results suggested that this protein might undergo protein modification such as phosphorylation. The protein was examined further using 2DE and immunoblotting with the anti-GRP58 antibodies which detected four immunoreactive spots of similar molecular sizes but with different isoelectric points (1-1, 1-2, 1-3 and 1-4 in Fig. 1C). The spot identified in Fig. 1A (arrowheads) was identical to the spot at 1-3 in Fig. 1C. Immunoreactivities of the GRP58-like immunoreactive substance (GRP58LIS) at more acidic isoelectric points (1-3 and 1-4) increased at ZT11 in comparison to those at other time points (Fig. 1C).
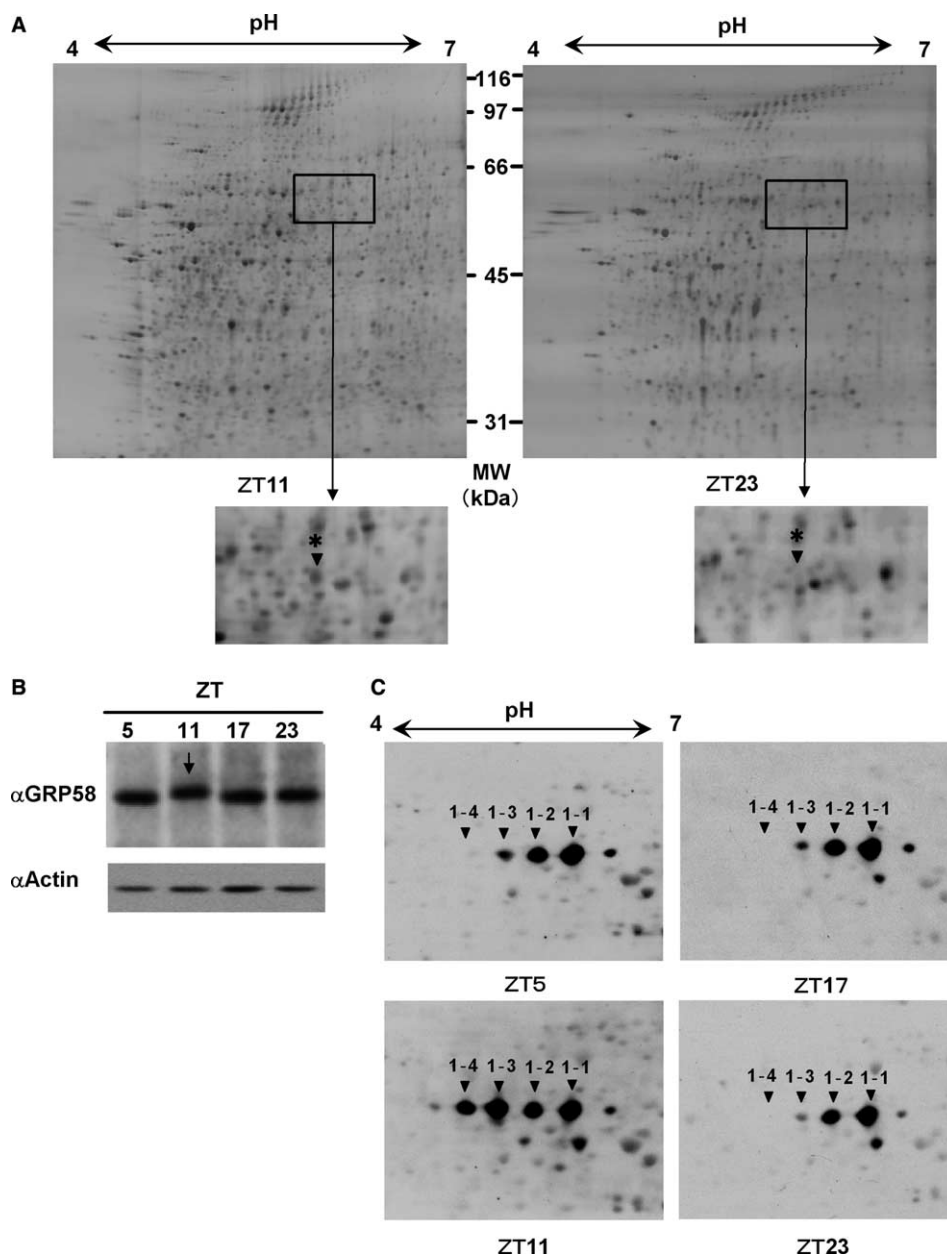
Fig. 1. Detections of proteins exhibiting daily expression changes in the liver. (A) 2DE of rat liver was sampled at ZT11 and ZT23 with isoelectric focusing showed different expression patterns for spot showed by arrowheads. (B) Immunoblotting using an anti-GRP58 antibody in 1DE. Livers were sampled at 12:00 (ZT5), 18:00 (ZT11), 24:00 (ZT17) and 06:00 (ZT23) h under 12-h light and 12-h dark period (light on at 07:00 h). Livers were homogenized, centrifuged, and supernatants were dissolved in SDS–PAGE sample buffer. Samples then underwent electrophoresis and were immunoblotted using anti-GRP58 antibodies. (C) Immunoblotting using an anti-GRP58 antibodies in 2DE. Different expression patterns were observed spots 1-1, 1-2, 1-3 and 1-4.

3.3. Phosphorylation of GRP58

Since horizontal movement of the protein spots in the 2DE is often observed due to changes in isoelectric point induced by protein phosphorylation, the result obtained in the experiment shown in Fig. 1C raised the possibility that phosphorylation of GRP58 might be increased at ZT11. To examine whether GRP58 was phosphorylated at ZT11, the liver sample obtained at ZT11 was treated with alkaline phosphatase and examined by immunoblotting. As seen in Fig. 2, after treatment with phosphatase, the mobility of GRP58LIS at ZT11 slightly increased in 1DE (Fig. 2A) and the immunoreactivities

of 2 spots in the more acidic side (1-3 and 1-4) decreased while the immunoreactivity of the spot at the most alkaline side (1-1) increased at ZT11 in 2DE gel (Fig. 2B). These findings suggest that GRP58 is phosphorylated at ZT11.

3.4. Effects of fasting

Since protein expression in the liver is known to be frequently affected by fasting and ZT11 is almost at the end of the resting (non-eating) period for nocturnal animals like rats, it was hypothesized that the above daily change in GRP58 mobility might be affected by fasting. Therefore, liver samples

Table 1
Mascot search results for fragments of protein spots corresponding to GRP58

Signal	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Start-end	Sequence	Spot no.			
1	1191.70	1190.69	1190.59	0.10	0	63–73	LAPEYEAAATR	1-1	1-2	1-3	1-4
2	1397.81	1396.80	1396.57	0.23	0	83–94	VDCTAVTNTCNK	1-1	1-2	1-3	1-4
3	1652.88	1651.87	1651.76	0.11	1	105–119	IFRDGEEAGAYDGPR	1-1	1-2	1-3	1-4
4	817.48	816.47	816.38	0.09	1	147–152	KFIpSDK			1-3	1-4
5	1587.91	1586.90	1586.81	0.09	1	148–161	FISDKDASVVGFFR	1-1	1-2		
6	1394.75	1393.74	1393.65	0.09	0	162–173	DLFSDGHSEFLK	1-1	1-2	1-3	
7	1607.83	1606.82	1606.74	0.08	0	259–271	DLLTAYYDVDYEK	1-1	1-2		1-4
8	1951.01	1950.00	1949.93	0.08	1	259–274	DLLTAYYDVDYEKNTK			1-3	
9	782.38	781.37	781.35	0.02	0	275–280	GSNYWR	1-1	1-2	1-3	1-4
10	1747.05	1746.04	1745.92	0.12	1	289–304	TFLDAGHKLNFAVASR	1-1	1-2	1-3	1-4
11	877.52	876.51	876.48	0.03	0	297–304	LNFAVASR	1-1	1-2	1-3	1-4
12	2733.30	2732.29	2732.40	−0.11	1	305–329	KTFSHELSDFGLESTTGEIPVVAIR	1-1	1-2	1-3	1-4
13	2605.24	2604.23	2604.31	−0.07	0	306–329	TFSHELSDFGLESTTGEIPWAIR	1-1	1-2	1-3	1-4
14	1172.61	1171.60	1171.53	0.07	0	336–344	FVMQEEFSR	1-1	1-2	1-3	1-4
15	1472.78	1471.77	1471.68	0.10	1	336–347	FVMQEEFSRDGK	1-1	1-2	1-3	
16	1529.89	1528.88	1528.77	0.12	1	352–363	FLQEYFDGNLKR	1 -1	1-2		1-4
17	1801.03	1800.02	1799.93	0.09	1	364–379	YLKSEPIPETNEGPVK			1-3	
18	1341.74	1340.73	1340.68	0.06	0	449–460	GFPTIYFSPANK	1-1	1-2	1-3	1-4
19	1469.86	1468.85	1468.77	0.08	1	449–461	GFPTIYFSPANKK	1-1	1-2	1-3	1-4
20	1363.76	1362.75	1362.71	0.04	0	472–482	ELNDLISYLQR				
21	1593.92	1592.91	1592.84	0.07	0	483–496	EATNPPIIQEEKPK	1-1	1-2	1-3	

MALDI-TOF-mass spectra were obtained using an AXIMA-CFR instrument (Shimadzu Corp., Japan). The MASCOT data base search identified Spot1-3 as a glucose-regulated protein of 58 kDa. Twenty-one peaks were matched to GRP58 with a score of 243 and a sequence coverage of 39%. The following conditions were used for searching the database: fixed modifications of carbamidomethyl (C); variable modifications included phosphorylated Ser and Thr; Taxonomy: Rattus; peptide tolerance: ±0.3 Da (Table 1).
Mr(expt), expected molecular mass; Mr(calc), calculated molecular mass.

were obtained from rats at 0 h and after 12, 24, 36 and 48 h of food deprivation. Mobility of GRP58LIS on 1DE gel was decreased when the liver samples were obtained after 12- and 24-h fasting in comparison to those obtained at the other time points (Fig. 2C). When these samples were analyzed on 2DE gels, it was observed that after 12- and 24-h fasting, the immunoreactivities of GRP58LIS at more acidic side (1-3 and 1-4 in Fig. 2D) were elevated but that those at 36- and 48-h of fasting were decreased (Fig. 2D). These findings indicate that phosphorylation of GRP58 in the liver is induced by fasting.

3.5. Effect of intracranial injection of leptin

Recently, it was suggested that GRP58 interacts with STAT3 to modulate intracellular signal transduction [4–6]. Since STAT3 was suggested to be involved in the intracellular signal transduction of leptin and a functional leptin receptor exists in the brain [10], the effect of intracranial injection of leptin on the mobility of GRP58 was examined. Three hours after leptin (Sigma–Aldrich, 10 µg/20 µl of a CSF) was injected into LCV using the LCV cannulae under un-anesthetic condition, the mobility of GRP58LIS from the liver was clearly shifted in 1DE (Fig. 2E) and the immunoreactivities of GRP58LIS with acidic isoelectric points (1-3 and 1-4 in Fig. 2D) increased in 2DE.

3.6. Phosphorylation site of GRP58 induced by fasting and leptin

To identify the phosphorylation site(s) of GRP58 after 12-h fasting, peptide mass finger printing was carried out on the four spots of GRP58LIS (1-1, 1-2, 1-3, 1-4 in Fig. 2D). The mass spectrometry data showed that a fragment of 1587.91 Da (sequence of GRP58 between amino acids (aa) 148–161; FISDKDASVVGFFR in Table 1) was present in spots 1-1 and 1-2 but not in spots 1-3 and 1-4. Since phosphorylated fragment was sometimes hardly detected with MALDI-

TOF-MS, it was possible that this fragment was phosphorylated in spots 1-3 and 1-4. Furthermore, the molecular mass of the first half fragment (corresponding to KFISDK) was 80 Da higher than that of the calculated molecular mass of the fragment, indicating that it was phosphorylated on one of the internal serine residues, most probably serine 150. It was also possible that unphosphorylated KFISDK (736 Da) might not be detected because of the lower mass than detecting threshold MALDI-TOF-MS. In order to obtain further support for the phosphorylation of this residue after fasting and leptin injection, we conducted a search using NetPhos which allows sequence- and structure-based prediction of eukaryotic protein phosphorylation sites [11]. NetPhos predicted with strong probability that the serine residue at aa 150 of GRP58 would be phosphorylated. To test this possibility, a rabbit polyclonal antibodies against the sequence from 144 to 156 of phosphorylated GRP58 (EFKKFIpSDK-DASC)-conjugated with KLH was generated. This anti-phosphoGRP58 antibody was first used to examine the phosphorylation states of Ser-150 in the liver after 12-h fasting. Fasting for 12 h induced a band shift of GRP58LIS (Fig. 3Aa) and increased the immunoreactivity of a band migrating at 60 kDa to anti-phosphoGRP58 antibody in 1DE (Fig. 3Ab). Furthermore, when anti-phosphoGRP58 antibody was preabsorbed with the antigen (EFKKFIpSDKDASC), immunoreactivity to the anti-phosphoGRP58 antibody disappeared in 1DE (Fig. 3Ac). Immunoreactivity to this anti-phosphoGRP58 antibody was observed only in the spots corresponding to 1-3 and 1-4 in 2DE (Fig. 3B). In controls, anti-phosphoGRP58 was preabsorbed to the antigen (EFKKFIpSDK-DASC), which eliminated all immunoreactivity to anti-phosphoGRP58 in 2DE (data not shown). Three hours after interacranial injection of leptin immunoreactivity to anti-phosphoGRP58 increased in both 1DE or 2DE (spots 1-3 and 1-4) (data not shown). These observations indicate that 12-h fasting
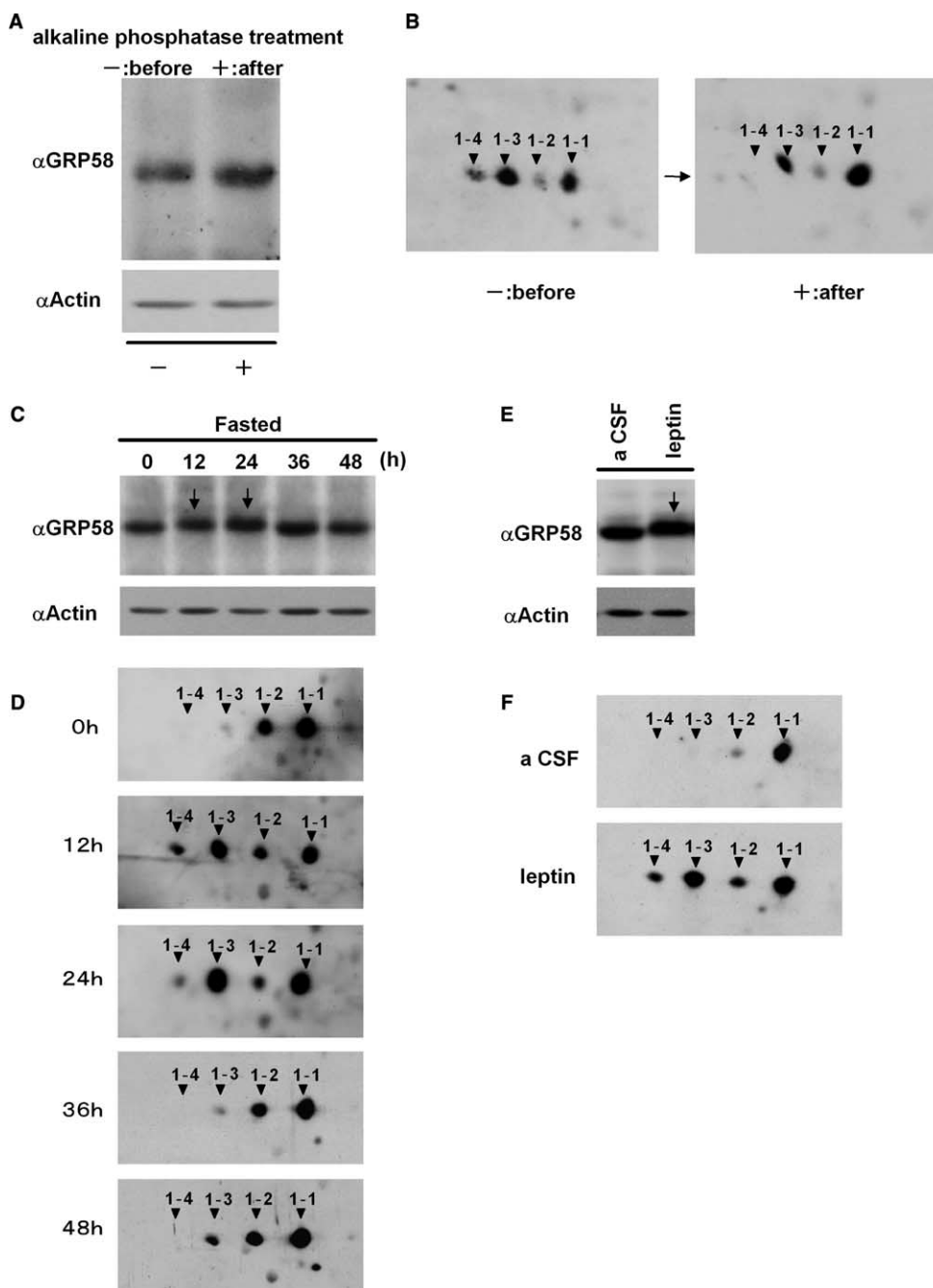
Fig. 2. Mobility changes in 1DE (A) and 2DE (B) after phosphatase treatment (A, B), fasting (C, D), and leptin injection (E, F). (A, B) Liver samples were obtained from rats at ZT11 and immunoblot analyses were carried out using anti-GRP58 antibodies before and after treatment with alkaline phosphatase in 1DE (A) and 2DE (B). (C, D) Effect of fasting on GRPLIS. Livers were obtained from rats at 0, 12, 24, 36 and 48 h after the start of food deprivation, homogenized and loaded onto 1DE and 2DE gels. After electrophoresis, immunoblot analyses were performed using an anti-GRP58 antibodies in 1DE (C) and 2DE (D). (E, F) Effect of leptin on GRP58LIS. Liver samples were obtained 3 h after the administration of leptin or aCSF and immunoblot analyses performed using an anti-GRP58 antibodies in 1DE (E) and 2DE (F).

and intracranial injection of leptin elevate phosphorylation of Ser-150 of GRP58.

To examine the possible role of GRP58 phosphorylation at aa 150 in intracellular signal transduction, an immunoprecipitation study was carried out using mouse anti-STAT3 monoclonal antibody. In an control experiment, it was observed that GRP58 was not precipitated with an anti-BIT (brain immunoglobulin-like molecule with tyrosine-based activation motifs) monoclonal antibody; 1D4, which is kindly given by

Dr. Shin-ichiro Sano (Mitsubishi Kasei Industitute of Life Sciences, Japan) [12]. Therefore, above observation seems to be specific.

In this experiment, liver samples were obtained 3 h after the intracranial injection of leptin or aCSF and immunoreactivities to anti-GRP58 and anti-phosphoGRP58 antibodies were examined. As seen in Fig. 3C, immunoprecipitation using anti-STAT3 antibody precipitated GRP58LIS in the livers of rats injected with either leptin or aCSF. However, the immu-
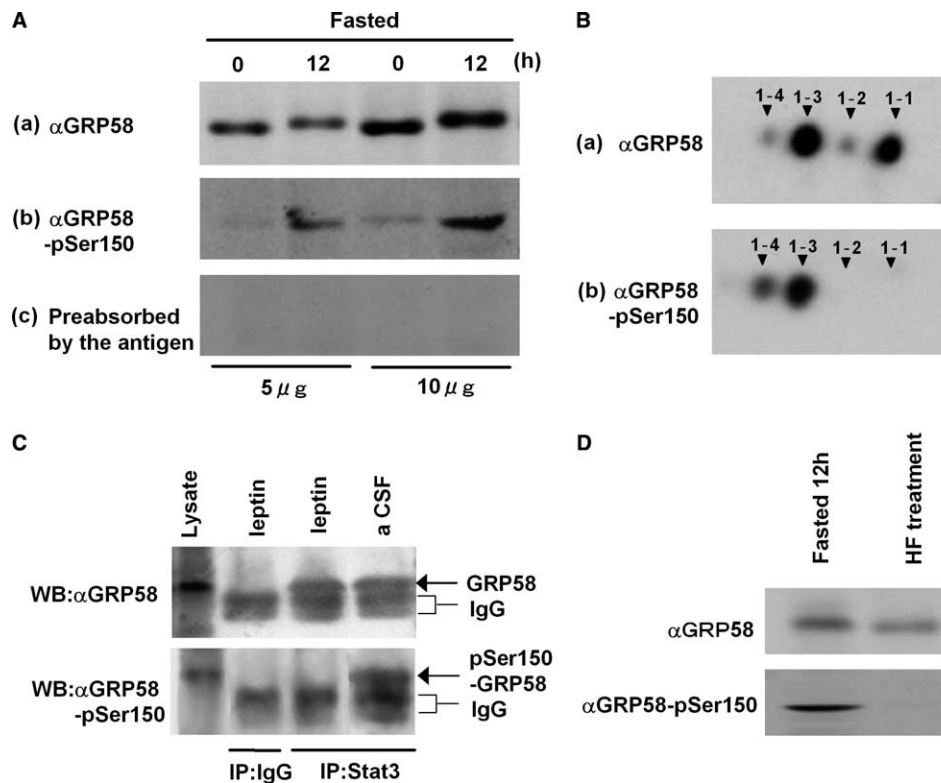
Fig. 3. Changes in immunoreactivity to anti-phosphoGRP58 antibody due to fasting and intracranial injection of leptin. (A, B) Immunoblot analyses of liver samples obtained at 0 and 12 h fasting using an anti-GRP58 and anti-phosphoGRP58 antibodies in 1DE (A) and 2DE (B). Specificity of the anti-phosphoGRP58 antibody was confirmed by preabsorbing it with the antigen. (C) Immunoprecipitation studies using anti-STAT3 antibody and IgG. Liver samples were obtained from rats 3 h after injections of leptin and aCSF and analyzed via immunoblotting. (D) Effect of dephosphorylation of GRP58 using hydrogen fluoride–pyridine on anti-GRP58 and anti-phosphoGRP58 immunoreactivity in livers obtained after 12-h fasting.

noreactivity to anti-phopshoGRP58 was immunoprecipitated by the anti-STAT3 antibody in the liver of aCSF-injected rats but not in that of leptin-injected rats, which indicates that the GRP58 phosphorylated at serine 150 is not able to bind STAT3.

To confirm the phosphorylation of GRP58 after fasting for 12 h, the effect of a dephosphorylation treatment using 70% hydrogen fluoride–pyridine on the immunoreactivity to anti-phosphoGRP58 antibodies in rats was examined. As seen in Fig. 3D, the immunoreactivity to the anti-phosphoGRP58 observed in rats fated for 12 h, disappeared following treatment with hydrogen fluoride–pyridine. This observation also confirms that GRP58 is phosphorylated at aa 150 after fasting.

4. Discussion

In this study, proteins showing daily changes in their rat liver expression patterns were identified via proteome analysis using 2DE and mass spectrometry. As a result, time-dependent changes in protein spots were detected with GRP58 being identified as one of the protein spots showing daily changes (Fig. 1; Table 1). GRP58 is a stress protein that is localized in the lumen of the endoplasmic reticulum because the C-terminus has an ER-retention signal. It is thought to be important for disulfide bond formation of proteins in the endoplasmic reticulum [13–15]. Recent data confirms the association between GRP58 and STAT3 in cytosolic statsome I complexes and

indicates that both GRP58 and STAT family members co-associate in the plasma membrane compartment [4]. Thus, it is speculated that GRP58 might regulate signal transduction by sequestering active and inactive STAT3.

Therefore, it was of interest to characterize the daily changes in GRP58 expression in the rat liver. Although no daily change in the amount of GRP58LIS was observed, a shift in its mobility was observed at ZT11 in 1DE (Fig. 1B). Four GRP58LIS spots with similar molecular sizes but with different isoelectric points were observed in a time-dependent manner by immuno-blotting in 2DE (Fig. 1C). Since horizontal scattering of spots in 2DE derived from the same protein with similar molecular weight is often the result of protein phosphorylation, it was hypothesized that GRP58 in the rat liver might be time-dependently phosphorylated in a time-dependent manner. This hypothesis was tested by examining the effect of an alkaline phosphatase on the migration of GRP58LIS obtained from liver samples at ZT11 in 1DE and 2DE. The phosphatase treatment slightly increased the mobility of GRP58LIS in 1DE (Fig. 2A) and reduced the immunoreactivities of the two GRP58LIS spots on the more acidic side (1-3 and 1-4) while enhancing the immunoreactivity of the spot on the most alkaline side (1-1) in 2DE gel (Fig. 2B). Altogether, these findings suggest that GRP58 is phosphorylated at ZT11.

Since ZT11 is almost at the end of the light period, it was possible that the mobility changes of GRP58 in 1DE and 2DE might be caused by food deprivation. This idea is supported by a recent report showing changes in the mobility of

ERp57 (GRP58) during recovery from ATP depletion using a cell culture based reversible ATP depletion model [16]. Therefore, the effect of food deprivation on the mobility of GRP58 was examined. As seen in Fig. 2C and D, food deprivation for 12 and 24 h caused a shift in the mobility of GRP58LIS in 1DE and increased the immunoreactivity of GRP58LIS on the acidic side (1-3 and 1-4 in Fig. 2D) in 2DE. These results indicate that GRP58 is phosphorylated after 12- and 24-h fasting. Glycogenolysis and gluconeogenesis in the liver are induced at ZT11 and after certain periods of fasting. Thus, the phosphorylation of GRP58 might be related to the mechanism of these processes.

As mentioned above, GRP58 is known to interact with STAT3 [4] and is thought to be involved in the intracellular signal transduction of leptin. As leptin might induce phosphorylation of GRP58, the effect of intracranial injection of leptin on the mobility of GRP58LIS in 1DE and 2DE was examined. Leptin induced a mobility shift of GRP58LIS in 1DE (Fig. 2E) and increased the immunoreactivities of GRP58LIS on the more acidic side in 2DE (1-3 and 1-4 in Fig. 2F), suggesting that GRP58 phosphorylation is induced by leptin.

Phosphorylation of GRP58 was found to occur at Ser150 after 12-h fasting and leptin injection. Moreover, it was found that dephosphorylation of GRP58 using hydrogen fluoride–pyridine eliminated immunoreactivity to anti-phosphoGRP58 antibody in liver samples obtained from rats fasted for 12 h (Fig. 3D). This observation confirmed that fasting induces phosphorylation of GRP58.

Immunoprecipitation using anti-STAT3 antibody (Fig. 3C) indicated that phosphorylation of GRP58 at Ser150 renders in unable to bind to STAT3. Since it has been suggested that GRP58 and STAT3 co-associate in the plasma membrane compartment [4], it is possible that unphosphorylated, but not phosphorylated GRP58 binds to STAT3. Thus, phosphorylation of GRP58 at Ser150 may constitute the signal that frees STAT3 from the plasma membrane compartment, resulting in the activation of the downstream signal transduction pathway. In this respect, it was found that GRP58 is present in STAT3-DNA complexes within the nucleus and is a necessary component them [17], suggesting that GRP58 is related to the nuclear entry and transcriptional regulation of STAT3.

In the current report, it is known that GRP58 of rat spleen is phosphorylated in three tyrosines (Y 444, Y 453 and Y466) by the Src-like tyrosine kinase Lyn [18]. Therefore, it is possible that spots of 1-1 and 1-2 may represent different phosphorylation states of these tyrosine residues.

These possibilities will be examined in future studies together with analyses of the constituents of spots 1-1 and 1-2, and of kinase responsible for the phosphorylation of aa 150 of GRP58.

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