Protein kinase CK2 is altered in insulin-resistant genetically obese (*falfa*) rats

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Abstract Hepatic insulin receptor levels in 6-week-old obese (*falfa*) rats were about 2-fold lower than those from lean (*Fal*−) rats, which agrees with their insulin-resistant state. Nuclear protein kinase CK2 activity and protein content in livers from obese (*falfa*) rats were similar to those of lean (*Fal*−) animals but the cytosolic levels were reduced to half, due to a decrease in the 39-kDa catalytic subunit. Marked increases in activity, due to rises in the 44-kDa and 39-kDa catalytic subunits, were seen in the 16 000 × *g* sediments (M1) from insulin-resistant rats, with moderate changes in the 100 000 × *g* sediments (M2). The increase in CK2 binding to M1 did not require increases in the molecular chaperone grp94, which was unaltered in insulin-resistant rats.

Key words: Protein kinase CK2; Grp94; Insulin receptor; Insulin resistance; Obese (*falfa*) rat; Rat liver

1. Introduction

Genetically obese Zucker rats develop severe insulin resistance and show marked hyperinsulinemia as early as 3–4 weeks of age [1] but they maintain normoglycemia until 8–10 weeks of age [2,3]. Zucker rats show marked alterations in liver metabolism, which includes resistance to control of hepatic glycogen synthase by insulin [2,4] and impairment of the glycosyl-phosphatidylinositol signalling pathway [4]. Protein kinase CK2 is one of the possible targets for glycosyl-phosphatidylinositol, whose production is stimulated by insulin [5]. This protein kinase may act on glycogen synthase by direct phosphorylation of the enzyme and through phosphorylation of the inhibitor 2 of protein phosphatase 1, which is a prerequisite for or potentiates the subsequent action of glycogen synthase kinase 3 (GSK3) on these substrates, the latter leading to marked changes in their activity [6–8]. Several groups have indicated that protein kinase CK2 is controlled by insulin in human skeletal muscle, rat liver and in culture cells but controversy exists concerning the type of effect detected [9].

Previous reports on grp94 purification [10,11] have clarified its persistent association with protein kinase CK2, which suggested that it could serve to locate the kinase in membranous fractions. Grp94 is a member of the stress proteins with chaperone activity [12,13] whose synthesis in culture cells is influenced by different metabolic stressors, in particular glucose levels and insulin [14,15]. However, its possible changes under diabetic conditions seem to be largely unexplored. In the present work, the possible alterations in the content and subcellular distribution of hepatic grp94 and protein kinase CK2 have been studied in young obese Zucker rats.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies against a peptide corresponding to an internal region (residues 70–91) of human CK2α subunit were obtained from Upstate Biotechnology Inc. This region is also present in human CK2α′ subunit (residues 71–92) except for the presence of Val-83 in CK2α′ instead of Ile in the corresponding position in CK2α [9] and thus the antibody recognizes both CK2α and CK2α′ subunits. Immobilon P was from Millipore. Mouse monoclonal anti-grp94 from chicken oviduct was from StressGen Biotechnologies Corp. Rabbit polyclonal antibody (α-CT) against a peptide corresponding to the C-terminal region of the rat insulin receptor, purified rat liver protein kinase CK2 and other reagents were obtained as indicated previously [10].

2.2. Subcellular fractionation

Genetically obese female Zucker (*falfa*) rats and lean (*Fal*−) littermates were purchased from IFA-Credo (France) and fed ad libitum with ordinary rat chow. The animals used were 6 weeks of age and they were killed by decapitation after mild ether anesthesia. The body weight of the (*falfa*) Zucker rats (196.3 ± 5.2 g) was about 40% higher than that of the (*Fal*−) littermates (140.8 ± 15.1 g) and a similar increase was observed in the liver weight of the obese rats (8.9 ± 0.5 g) compared to that of the lean rats (6.1 ± 0.7).

Liver homogenates, obtained as indicated previously [10], were centrifuged at 600 × *g* for 30 min at 4°C and the pellet, which contained the nuclei, was stored. The supernatant was centrifuged again at 16 000 × *g* for 30 min at 4°C and the sediment was stored and designated membranous fraction M1. The supernatant was centrifuged at 100 000 × *g* for 90 min at 4°C and the sediment was designated membranous fraction M2 whereas the supernatant corresponded to the cytosolic fraction. In all cases, the sediments (either nuclei, M1 or M2) were resuspended with 1–5 ml of 50 mM Tris-HCl, pH 7.5, buffer containing 150 mM NaCl, 5 mM EDTA and the same protease inhibitors as in buffer A (buffer B). Membrane proteins were solubilized with the addition of Triton X-100 to 2% (v/v) and gentle stirring for 90 min at 4°C, and then centrifuged at 120 000 × *g* for 90 min at 4°C.

Aliquots of the samples, including the cytosolic fraction, were then subjected to partial purification by chromatography on DEAE-Sepharose. 0.5 ml of the samples was added to conical centrifuge tubes that contained 0.4 ml of settled DEAE-Sepharose previously equilibrated in buffer B supplemented with 0.1% (v/v) Triton X-100. The mixture was gently stirred overnight at 4°C, and then centrifuged at 2000 × *g* for 5 min at 4°C. The resin was washed three times with 1 ml of buffer B and the retained proteins were eluted by washing the resin with 0.5 ml of 0.8 M NaCl in buffer B and stored until use. Preliminary experiments showed that under these conditions all the immunodetectable grp94 and protein kinase CK2 present in the extracts were retained by the column and were quantitatively recovered in the eluates (data not shown). The amount of protein recovered in this step did not vary between lean and obese rats when referred per g of wet tissue (gwt), and it was higher in cytosol (2.86 ± 0.22 mg/gwt) and nuclei (1.88 ± 0.15 mg/gwt) than in M1 (0.14 ± 0.02 mg/gwt) and M2 (0.12 ± 0.02 mg/gwt). Aliquots of the solubilized proteins obtained from the M1 fraction were also subjected to chromatography on a (1 × 3 cm) wheat-germ lectin-Sepharose column and then eluted with

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0.3 M *N*-acetyl-D-glucosamine as indicated previously [10], in order to partially purify insulin receptors.

2.3. Assays

Protein kinase CK2 activity was assayed as described previously [10] using 4 mg/ml β-casein and 125 μM [γ-^{32}P]GTP as substrates. One unit of protein kinase CK2 activity is defined as the amount that catalyzes the transfer of 1 nmol of phosphate from [γ-^{32}P]GTP to β-casein per min at 30°C. Insulin receptor tyrosine kinase activity on poly(Glu:Tyr) (4:1) and receptor autophosphorylation were assayed as described previously [10]. The protein concentration in the samples was determined by the Bradford method [16] using bovine serum albumin as standard.

For immunological assays, samples were subjected to SDS-PAGE and transferred to Immobilon P membranes [10]. Blocking was performed with 5% w/v of powdered skim milk in 25 mM Tris-HCl, pH 8.0, buffer containing 150 mM NaCl, 3 mM KCl and 0.1% v/v Tween 20 (TTBS) for 2 h at 22°C. Thereafter, the membranes were washed three times with TTBS and incubated for 1 h at 22°C with either anti-insulin receptor antibodies (diluted 1/250), anti-CK2α/α′ antibodies (diluted 1/1000) or anti-grp94 antibodies (diluted 1/1000) and then the membranes were rinsed three times with TTBS. The immunocomplexes were visualized by the alkaline phosphatase reaction using BCIP/NBT alkaline phosphatase-conjugated substrates, and the intensity of the bands was quantified by densitometry.

Statistical analysis of the data was performed with Student's *t*-test. The data represent means ± S.E.M.

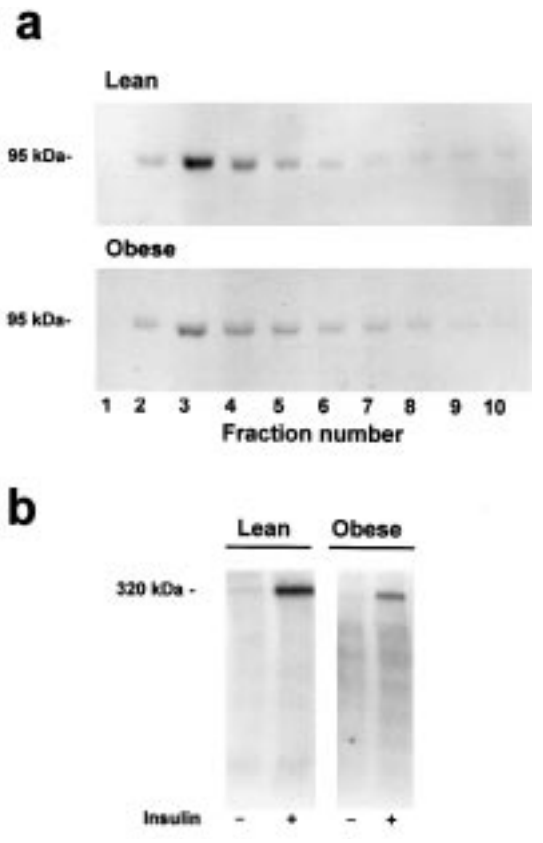

Fig. 1. Wheat-germ lectin-Sepharose chromatography of the solubilized membrane proteins from lean and obese rats. Rat liver membrane proteins solubilized with 2% (v/v) Triton X-100 were applied to a wheat-germ lectin-Sepharose that was eluted with 0.3 M *N*-acetyl-D-glucosamine as indicated in Section 2. Fractions were analyzed for the presence of insulin receptor. a: The indicated fractions were subjected to SDS-PAGE under reducing conditions using 7.5% (w/v) polyacrylamide gel, transferred onto a membrane and checked for reaction with antibodies against insulin receptor β-subunit. b: Fractions 2–5 were pooled and subjected to autophosphorylation with [γ-^{32}P]ATP in the presence or absence of insulin. The reaction was stopped with the addition of sample electrophoresis buffer and the mixtures were subjected to SDS-PAGE under non-reducing conditions using a 7.5% (w/v) polyacrylamide gel and autoradiographed. Values on the side of the plots indicate the apparent M_r of the bands.

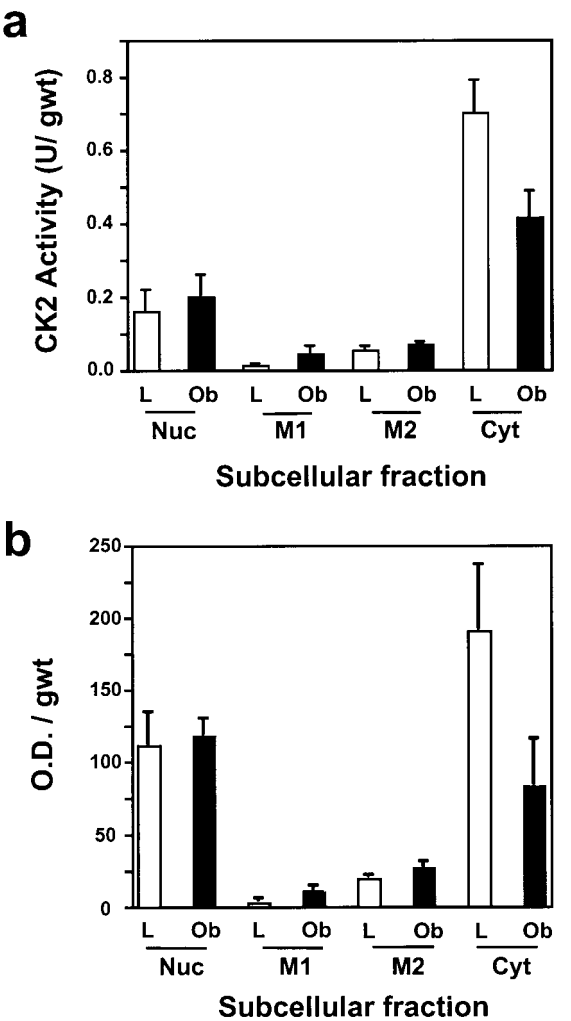

Fig. 2. Total protein kinase CK2 content and catalytic activity in the subcellular fractions from lean and obese rats. a: CK2 activity in the samples from the different subcellular fractions, determined using [γ-^{32}P]GTP and β-casein as substrates, was referred per g of wet tissue. b: Values of total CK2 catalytic subunits ($CK2_T$, or α+α′, see also the legend to Fig. 3) were also expressed per g of liver. Open bars correspond to lean rats and solid bars to obese rats. $^*P \leq 0.05$ compared to lean rats.

3. Results

3.1. Insulin receptor levels in lectin-Sepharose eluates obtained from lean and obese rats

Extracts from rat liver membranes were obtained and subjected to chromatography in wheat-germ lectin-Sepharose as indicated in Section 2. The elution profile of insulin receptor β-subunit protein from genetically obese (*falfa*) rats was similar to that observed with their lean littermates (Fig. 1a), but the amount detected in the obese (*falfa*) rats was about half that present in the lean rats. Fractions 2–5 were pooled and used for insulin binding and tyrosine kinase activity assays. When compared with their lean littermates, the pooled fractions from obese rats also showed a two-fold decrease in in-

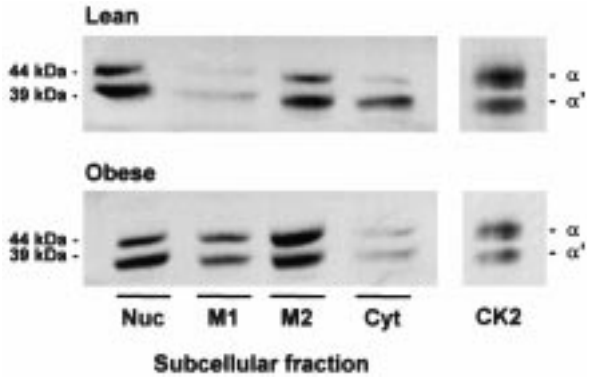

Fig. 3. Immunodetection of CK2 catalytic subunits in the hepatic subcellular fractions from lean and obese rats. Samples of each subcellular fraction (Nuclei, M1, M2 and Cytosol) obtained were subjected to SDS-PAGE under reducing conditions using a 10% (w/v) polyacrylamide gel, transferred onto a membrane and checked for reaction with antibodies against protein kinase CK2α/α′ subunits. Total protein applied to the gel was 40 μg for nuclei, 10 μg for M1, 14 μg for M2, and 7.5 μg for cytosol. Protein kinase CK2 purified from rat liver (0.1 μg upper blot, 0.05 μg lower blot) was analyzed under the same conditions as positive controls. Values on the left denote the apparent M_r of the bands and the position corresponding to the α and α′ subunits of purified rat liver CK2 are indicated on the right.

sulin-stimulated receptor autophosphorylation (Fig. 1b), which was accompanied with a decrease in its tyrosine kinase activity towards poly(Glu:Tyr) (4:1) from 255 ± 19 mU/mg protein in lean rats to 145 ± 28 mU/mg protein in obese animals.

3.2. Protein kinase CK2 activity and immunodetection

Protein kinase CK2 activity, expressed per g of wet liver, was higher in cytosol and nuclei than in the membrane fractions in control (*fa/fa*) rats. Alterations of the enzyme activity on β-casein (Fig. 2a) were detected in obese insulin-resistant (*fa/fa*) rats, with an increase in the membranous fractions and a marked decrease in the cytosol.

Immunological detection of protein kinase CK2 catalytic subunits revealed a set of different bands present in all of the samples from either lean or obese rats, with the 39-kDa and 44-kDa ones being the more abundant ones (Fig. 3), whose mobility coincided with that of the CK2α and CK2α′ subunits present in purified CK2 preparations from rat liver cytosol. Since both CK2α and CK2α′ are catalytically active and can substitute for each other in forming CK2 holoenzyme [17], the values on the total CK2α/α′ content were also considered. When total protein kinase CK2 content (39-kDa+44-kDa) subunits was expressed per g of wet tissue it became evident that most of the protein kinase CK2 was present in cytosol and nuclei in both control and obese (*fa/fa*) rats (Fig. 2b). Furthermore, the changes in protein kinase CK2 detected in membrane fractions and in cytosol correlated with alterations in total CK2 content, but the rise in CK2 content observed in the membranous fractions was substantially lower than the decrease observed in cytosol. This indicates that the changes observed in obese (*fa/fa*) rats were not simply due to translocation of the enzyme but to a decrease in total cellular protein kinase CK2.

The results shown in Fig. 3 indicate that the changes in CK2 were not due to similar alterations in both catalytic subunits. In order to quantify these changes, fractions from each experiment were analyzed by Western blot using different amounts of each sample and then the intensity detected in the 44-kDa and 39-kDa bands was normalized to the protein applied to the gel. The data obtained for each band in three different experiments performed in different weeks were pooled and their means ± S.E.M. are shown in Fig. 4. As can be observed, the obese rats showed marked increases in the 44-kDa band in M1 (4.48 ± 1.38-fold), and more moderate

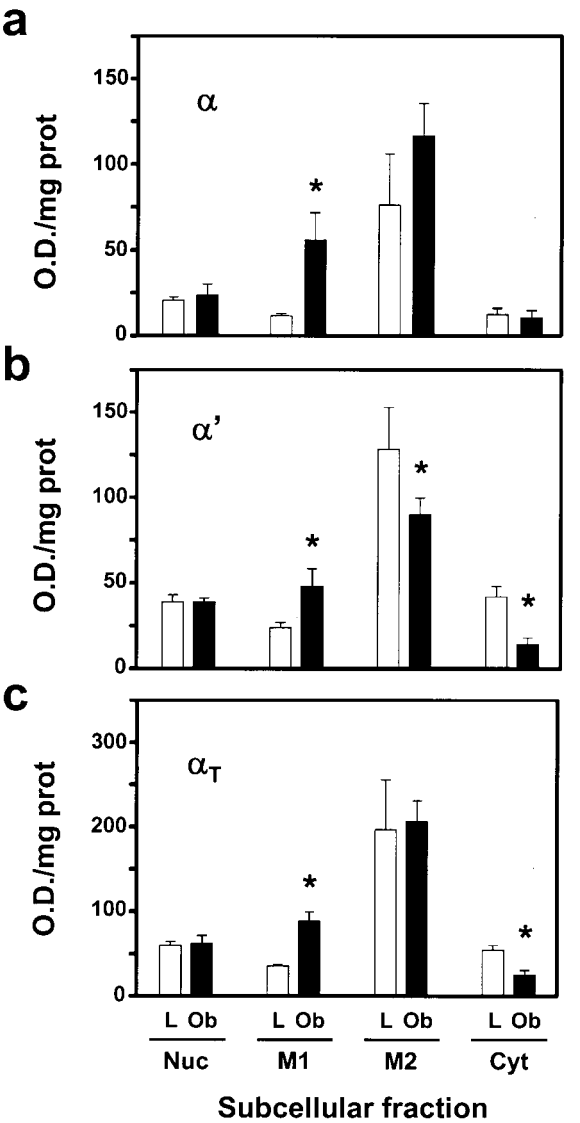

Fig. 4. Subcellular distribution of protein kinase CK2 catalytic subunits in lean and obese rats. Three different preparations of each subcellular fraction from lean and obese rats were analyzed as indicated in the legend to Fig. 2, using at least two different amounts of total protein in the range of 19–45 μg for nuclei, 3–22 μg for M1, 4–14 μg for M2, and 4–9 μg for cytosol. The intensity of the bands detected with antibodies against protein kinase CK2α/α′ subunits was quantified by densitometry and their values divided by the amount of protein applied to the gel. The data obtained were pooled and the means ± S.E.M. are shown. a: Quantification of CK2α (44-kDa). b: Quantification of CK2α′ (39-kDa). c: Quantification of total CK2 catalytic subunits ($CK2_T$, or α+α′). Open bars correspond to lean rats and solid bars to obese rats. *$P \leq 0.05$ compared to lean rats.

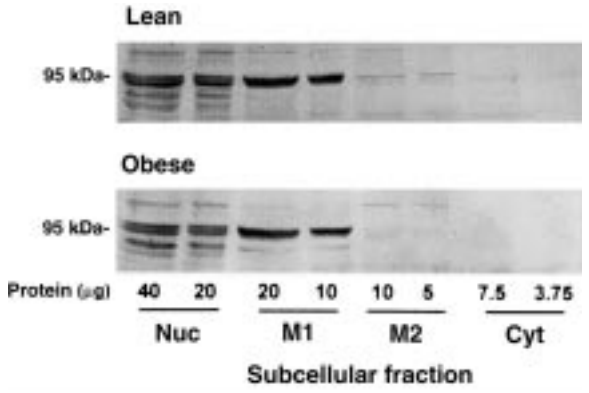

Fig. 5. Immunodetection of grp94 in hepatic subcellular fractions from lean and obese rats. Samples of each subcellular fraction (Nuclei, M1, M2 and Cytosol) obtained from lean or obese rats after DEAE-chromatography were subjected to SDS-PAGE under reducing conditions using a 10% (w/v) polyacrylamide gel, transferred onto a membrane and checked for reaction with antibodies against grp94. Each individual fraction was assayed using two different amounts of total protein applied to the gel. These values were set the same for lean and obese rats within each fraction and are indicated in the figure. Values on the left denote the apparent M_r of the bands.

in M2 (1.88 ± 0.51-fold) with slight decreases (0.84 ± 0.10-fold) in the cytosolic fraction (Fig. 2a). On the other hand, the 39-kDa band increased (2.07 ± 0.42-fold) in M1, did not vary or decreased slightly (0.73 ± 0.21-fold) in M2 and was markedly reduced in the cytosolic fraction (0.34 ± 0.04-fold) (Fig. 3b). This analysis showed that the total amount of protein kinase CK2 catalytic subunits increased (Fig. 3c) in M1 (2.49 ± 0.76-fold), did not essentially vary in M2 (1.07 ± 0.30-fold) and in the nuclear fraction (1.11 ± 0.24-fold) and decreased in the cytosolic fraction (0.47 ± 0.06-fold) from obese (*falfa*) rats compared to their lean littermates.

3.3. Subcellular distribution of grp94 in lean and obese rat livers

Immunodetection of grp94 showed that it was much more abundant in the M1 fraction than in the M2 fraction (Fig. 5). No differences were observed between the lean and obese rats concerning the presence of grp94 in the M1 fraction. Similar results were obtained in three different experiments (data not shown). Multiple bands were detected with the anti-grp94 antibody in the samples from nuclei. Whether they correspond to different proteins homologous to grp94 or degradation products derived from it is unknown. Slight differences in the distribution of the three major bands were detected between the lean and obese rats, with an increase in the lower-M_r form associated with a decrease of the intermediate-M_r band. However, when the three bands were considered together no differences were observed between the lean and obese rats. No bands were detected in cytosol from either lean or obese rats.

4. Discussion

Down-regulation of hepatic insulin receptors has been observed previously in obese rats, but a discrepancy exists concerning the effects on insulin receptor tyrosine kinase activity since both decreases and increases have been reported [6–9]. The data obtained in our present study would agree with the idea that the insulin resistance shown by the obese (*falfa*) rats is due mainly to a decrease in the number of insulin receptors. In turn, these receptors would not present marked differences in their tyrosine kinase autophosphorylation activity when compared with those from lean controls.

The effect of insulin on protein kinase CK2 in whole animals and in cell cultures is a matter of controversy concerning not only the response induced but also its link to changes in CK2 protein content [9,18–20]. Previous studies in streptozotocin-diabetic rats [21,22] have shown that total protein kinase CK2 activity per g of tissue was unaffected in liver and skeletal muscle, although in the latter the specific activity of the enzyme decreased by 30%. On the other hand, protein kinase CK2 activity in human skeletal muscle from insulin-resistant non-diabetic patients was found to be higher than that from normal insulin-sensitive or insulin-resistant diabetic humans but no differences in CK2 protein were detected either between the three groups [23]. More recently, it has been shown that the increase in nuclear protein kinase CK2 activity detected in insulin-stimulated Chinese hamster ovary cells overexpressing human insulin receptors [CHO(Hirc)] was not linked to alterations in either the total nuclear CK2 protein or the subcellular distribution of CK2 subunits [24]. In contrast, changes in CK2 protein have been shown to support both the increase in protein kinase CK2 activity in tumor cells [9,25,26], and its decrease in the brain cortex of schizophrenic and Alzheimer patients [27]. The data obtained in our present work show that both the decrease in hepatic protein kinase CK2 activity detected in cytosol and its increase in the membranous fraction M1 from insulin-resistant obese (*falfa*) rats were in agreement with the changes in the amount of enzyme protein observed in these fractions. This would suggest that under these conditions the control of protein kinase CK2 was exerted through changes in the enzyme protein levels rather that by altering its intrinsic catalytic activity.

The possible physiological implications of the changes in protein kinase CK2 are unknown but would alter the phosphorylation level of at least some of its multiple protein substrates [17,18]. The obese (*falfa*) rats show impaired control of glycogen synthesis and protein kinase CK2 may affect it by phosphorylation of glycogen synthase or through protein phosphatase-1 by phosphorylation of inhibitor-2 [6–8]. Insulin receptor substrate 1 (IRS1) is another substrate for protein kinase CK2 [28] with potential implications in the alterations observed in insulin resistance. Even the insulin receptor itself seems to be a substrate for protein kinase CK2, but controversy exists on this matter due, at least in part, to the presence in receptor preparations of grp94, and other protein substrates for this enzyme [10]. Interestingly, experiments with synthetic peptides corresponding to portions of the sequence of the β-subunit of insulin receptor have demonstrated that Thr-1160 located in the activation loop is indeed a target for protein kinase CK2, and its efficiency is enhanced by prior phosphorylation of Tyr-1163, one of the autophosphorylation sites present in this sequence [29]. However, whether this residue is also phosphorylated in vivo and has any effect on receptor activity is still unknown. The similarity in the changes in hepatic insulin receptor protein content and autophosphorylation activity observed in obese (*falfa*) rats would suggest that the alterations in either cytosolic or membrane-bound protein kinase CK2 did not influence the tyrosine kin-

ase activity of insulin receptor in these insulin-resistant animals.

The reason for the increased association of protein kinase CK2 with the membranous fractions could reside in the fact that the membranes of the endoplasmic reticulum contain grp94 and other integral and peripheral proteins that are substrates for protein kinase CK2 [10,30,31], but which one of these components serves to anchor the enzyme is unknown. In any case, this possible binding would not require an increase in grp94 since its total content did not vary in obese (*fa/fa*) rats. The absence of changes in the levels of total grp94 in the livers of obese (*fa/fa*) rats, which have insulin levels 5–10-fold higher than lean rats is in contrast with the observation that insulin induces grp94 in L929 cells even in the presence of glucose [15] and it could reflect that the obese (*fa/fa*) rats also show insulin resistance to the induction of grp94.

The differences in the pattern of protein kinase CK2 catalytic subunits detected in cytosol and membranous fractions from obese (*fa/fa*) rats are intriguing. Changes in the pattern of catalytic subunits have been described previously in human kidney carcinomas [26] and in regenerating rat liver nuclei [32]. The nature of these bands cannot be fully ascertained with the data at hand but the 44-kDa band would most likely correspond to the CK2α subunit. As regards the 39-kDa band, it could correspond either to the CK2α′ subunit or to some proteolytic fragment derived from CK2α. In any case, our data show that the changes detected in obese (*fa/fa*) rats are not due merely to changes in the cytosolic/membrane distribution of these two forms and suggest that the mechanisms that control the levels of the catalytic subunits of protein kinase CK2 are altered in these insulin-resistant animals.

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