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Glucokinase regulatory protein is associated with mitochondria in hepatocytes

Catherine Arden[1], Simone Baltrusch[2], and Loranne Agius[1#]

[1]School of Clinical Medical Sciences-Diabetes, University of Newcastle upon Tyne, Newcastle upon Tyne, NE2 4HH, UK

[2]Institute of Clinical Biochemistry, Hannover Medical School, 30623 Hannover, Germany.

ABSTRACT: The association of glucokinase with liver mitochondria has been reported (Danial et al., (2003). *Nature.* 424, 952-956). We confirmed association of glucokinase immunoreactivity with rat liver mitochondria using Percoll gradient centrifugation and demonstrated its association with the 68kDa regulatory protein (GKRP) but not with the binding protein phosphofructokinase-2/fructose bisphosphatase-2. Substrates and glucagon induced adaptive changes in the mitochondrial glucokinase / GKRP ratio suggesting a regulatory role for GKRP. Combined with previous observations that GKRP overexpression partially inhibits glycolysis (de la Iglesia et al., (2000) J Biol Chem. 275,10597-603) these findings suggest that there may be distinct glycolytic pools of glucokinase.

Keywords
Glucokinase, Glucokinase regulatory protein, mitochondria, liver

Abbreviations
GK, glucokinase; GKRP, Glucokinase regulatory protein; N/C nuclear / cytoplasmic ratio; PFK-2/FDP-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; fructose 1-P, fructose 1-phosphate; fructose 6-P, fructose 6-phosphate; USF-1, upstream stimulatory factor-1.

(#) Correspondence address:
School of Clinical Medical Sciences-Diabetes, The Medical School,,
Newcastle upon Tyne, NE2 4HH, UK
Tel. 044-191-2227033
Fax. 044-191-2220723
E-mail Loranne.Agius@ncl.ac.uk

1. INTRODUCTION

Hepatic glucokinase (GK) is regulated by a 68 kDa regulatory protein (GKRP), which inhibits GK competitively with glucose by a mechanism potentiated by fructose 6-P and reversed by fructose 1-P [1]. Immunocytochemical studies have shown that GKRP is present predominantly in the nucleus of hepatocytes whereas GK translocates between the nucleus and cytoplasm [2-6]. Conditions that favour binding of GK to GKRP are associated with sequestration of GK in the nucleus, whereas precursors of fructose 1-P and also high glucose concentration cause translocation of GK to the cytoplasm [6,7].

The exclusive cytoplasmic location of GK in GKRP -/- mice is consistent with a role for GKRP as a nuclear receptor for GK [8]. However, whether GKRP has additional roles as a cytoplasmic inhibitor of GK activity [9] remains unresolved. Whilst several studies concluded that GKRP is present predominantly or exclusively in the nucleus [2-5], two independent studies have reported translocation of GKRP together with GK during substrate-stimulation [10,11].

In addition to GKRP, other binding proteins of GK have been reported including a dual-specificity phosphatase [12] and the bifunctional enzyme phosphofructokinase-2 / fructose bisphosphatase-2 (PFK-2/FDP-2) [13]. The latter protein regulates GK activity in pancreatic β-cells [14] and GK expression and compartmentation in hepatocytes [15].

A recent study reported the association of GK with liver mitochondria in a multi-protein complex involving the pro-apoptotic protein BAD, the catalytic unit of protein phosphatase-1, protein kinase A and its anchoring protein WAVE-1 [16]. A role for mitochondria-associated GK in regulation of glucose homeostasis was suggested based on the hyperglycaemia of BAD -/- mice, which showed decreased binding of GK to mitochondria. However, a later study did not detect glucokinase immunoreactivity in association with mouse liver mitochondria [17].

In this study we confirmed the association of glucokinase immunoreactivity with rat liver mitochondria and demonstrate immunoreactivity to GKRP as well as adaptive changes in the glucokinase / GKRP ratio supporting a role for GKRP in the cytoplasm.

2. MATERIALS AND METHODS

2.1. Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Wistar rats (B&K Universal Ltd, Hull, UK) by a collagenase perfusion technique [9] and suspended in MEM supplemented with 5 % (v/v) neonatal calf serum. After cell attachment (4 h), the cells were cultured overnight in serum-free MEM containing 5 mM glucose and 10 nM dexamethasone [9].

2.2. Percoll gradient fractionation and enzyme activity determination

Freshly isolated hepatocyte suspensions or cultured hepatocyte monolayers were disrupted in MSHE buffer (220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4), 0.5 mM EGTA) [18] using 27 or 23 gauge needles, respectively. The homogenate was centrifuged at 1000g (10 min) and the supernatant layered onto 27% (w/v) Percoll in MSHE buffer with 0.1% BSA and centrifuged at 35,000g (50 min). One ml fractions were collected and washed (x2) with MSHE buffer at 13,000g (15 min). Pellets were resuspended in extraction buffer (200 μl) for determination of enzyme activity and immunoreactivity. For determination of enzyme activity mitochondrial fractions were diluted with 0.05% Triton X-100 and glutamate dehydrogenase [19], citrate synthase, 5'nucleotidase and NADPH cytochrome C reductase determined [20]. In additional experiments, mitochondria were isolated from fresh liver using a standard centrifugation method [17], which generated mitochondrial and cytoplasmic fractions for determination of immunoreactivity.

2.3. Western Blotting and Immunostaining

Western Blotting was performed using the NuPAGE MOPS system (Invitrogen Life Technologies, Paisley, UK). After transfer onto nitrocellulose membranes, the membranes were incubated for 3 h with primary antibody (rabbit derived anti-human-GK (sc-7908, Lot No DO51), USF-1 (sc-229) (Santa Cruz, Autogen Bioclear, UK), sheep derived anti-glutathione-S-transferase-GK fusion protein (a gift from Dr. M.A. Magnuson) [21], BAD (610391, BD Biosciences, Oxford, UK), PFK-2/FDP-2 bisphosphatase domain [13] and GKRP (generously donated by E. Van Schaftingen)). After washing, membranes were incubated with the appropriate peroxidase-conjugated secondary antibody (Jackson Immunoresearch Laboratories, PA, USA), visualized by Enhanced Chemiluminescence and

band intensity determined by densitometry. Immunofluorescent staining of cultured hepatocytes was performed as described in [11] using antibodies raised against GK (sc-7908) and GKRP (sc-6340) (Santa Cruz).

2.4. GK activity determination in liver mitochondria

ATP production and utilisation were determined on mitochondria isolated from rat liver by a two-step centrifugation method [22] as in [23]. Briefly, mitochondria were incubated with 50 mM Hepes (pH 7.8), 100 mM KCl, 0.1 mM EDTA, 0.24 mM ADP, 0.63 mM NADP, 2 mM $MgCl_2$, 5 mM KPi, 5 mM pyruvate, 2.5 mM malate and 2.1U/ml glucose 6-P dehydrogenase without or with 5 mM KCN. ATP production was determined in the presence of 3U/ml yeast hexokinase (Boehringer Mannheim, Lewes, UK) and ATP utilisation in the absence of added hexokinase at 0.5 mM and 50 mM glucose.

3. RESULTS

3.1. Mitochondrial GK is associated with GKRP but not with PFK-2/FDP-2

To test for association of GK with liver mitochondria, homogenates of fresh hepatocyte suspensions were fractionated on a Percoll gradient enabling separation of a mitochondrial peak, based on glutamate dehydrogenase activity, from both plasma membrane and microsomal membranes, based on 5'-nucleotidase and NAD(P)H-cytochrome-C reductase activity, respectively (Fig. 1A). Citrate synthase, another mitochondrial marker, showed a similar distribution and recovery as glutamate dehydrogenase (results not shown). Immunoblotting of every third fraction showed co-localisation of GK (rabbit derived GK-antibody) and GKRP with glutamate dehydrogenase (Fig. 1A). The association of GK with mitochondria was confirmed using a sheep-derived antibody against glutathione-S-transferase-GK fusion protein [21], which showed a similar distribution of immunoreactivity at 50 kDa. To determine whether the association of GK and GKRP with mitochondria is an artefact of percoll density centrifugation, mitochondria were isolated from fresh liver using a standard centrifugation method [17]. Western blotting of these fractions demonstrated association of GK and GKRP immunoreactivity with the mitochondrial fraction, representing 6 % and 7 % respectively, of cytosolic immunoreactivity when equal amounts of cytosolic and mitochondrial protein were loaded (results not shown).

Subsequent studies were performed on mitochondria isolated from cultured hepatocytes to enable the investigation of adaptive changes. GK and GKRP immunoreactivity were present in mitochondrial fractions 27 to 29 (Fig. 1.B-C). However, there was no immunoreactivity to the glucokinase binding protein PFK-2/FDP-2 in these fractions (results not shown). We therefore compared the sensitivity of the GK, GKRP and PFK-2/FDP-2 antibodies by immunoblotting against varying protein loadings of hepatocyte extract (Fig. 2). All three antibodies demonstrated appropriate linearity with greatest sensitivity for GKRP and similar sensitivity for glucokinase and PFK-2/FDP-2. The latter suggests that if PFK-2/FDP-2 and GK were similarly distributed, immunoreactivity to PFK-2/FDP-2 would have been detectable in the mitochondrial fractions. Contamination of the mitochondrial fractions with nuclear components was excluded by the lack of immunoreactivity to the transcription factor USF-1 (Fig. 1B-C). Immunoreactivity to the pro-apoptotic protein BAD was detected in the mitochondrial fractions as a single band in the 40kDa region rather than the expected 23kDa as in the homogenate. Immunoreactive GK and GKRP determined in the mitochondrial fractions represented ~2 % of total immunoreactive protein loaded on the gradient (Fig. 1.C). However, this estimate does not take into account the low recovery of glutamate dehydrogenase activity in this fraction (<10%) that is in part due to loss of mitochondria in the bottom fractions as well as loss of mitochondria during washing of the fractions to remove the Percoll.

3.2. Lack of elution of GK or GKRP with fructose 1-P from isolated mitochondria

We tested whether GK or GKRP immunoreactivity could be eluted from isolated mitochondria with glucose 6-P, ligands of GKRP (fructose 6-P and fructose 1-P), or elevated KCl concentrations. There was no detectable release of GK and negligible release of GKRP with any of the treatment conditions tested (Fig. 3).

3.3. Substrate and hormone-induced translocation of mitochondrial GKRP

To test for adaptive changes in nuclear and mitochondria-associated GK and GKRP, hepatocytes were incubated for 3 h either with 5mM glucose or with 25mM glucose plus 250μM sorbitol without or with 100 nM glucagon (Fig. 4). These conditions have no apparent effect on total cellular GK content [15]. Nuclear compartmentation was determined by immunofluorescence staining and mitochondrial compartmentation by immunoblotting of

Percoll fractions. Stimulation of hepatocytes with 25 mM glucose / 250 μM sorbitol decreased the nuclear to cytoplasmic (N/C) ratio for GK from 3.1 ± 0.3 to 1.8 ± 0.1, whilst addition of glucagon reversed this effect (Fig. 4.A-B). The N/C ratio for GKRP was marginally reduced by substrate stimulation, which was counteracted by glucagon, in agreement with previous findings [11,15]. Association of GK with mitochondrial fractions was increased by 16% after substrate stimulation, whereas GKRP immunoreactivity was decreased by 50%, resulting in a 2.8-fold increase in the mitochondrial GK / GKRP ratio (Fig. 4.C-D). This substrate effect was counteracted by glucagon.

3.4. Coupling of hexokinase activity to mitochondrial metabolism

As mitochondrial-bound low-K_m hexokinase activity is coupled to mitochondrial metabolism in brain through an ADP-recycling mechanism [23], we tested whether the mitochondrial GK or hexokinase activity (assayed at 50 mM and 0.5 mM glucose, respectively) is coupled to ATP generated by oxidative phosphorylation. Utilisation of endogenously formed ATP by mitochondria-associated hexokinase and GK was 29% at 0.5 mM glucose and 36% at 50 mM glucose (0.5 mM glucose: 3.9 ± 0.3; 50 mM glucose: 4.8 ± 0.3 nmol/min/mg, means ± SEM, n=10) of total KCN-sensitive ATP production determined with exogenous yeast hexokinase (13.4 ± 1.8 nmol/min/mg). This confirms that hexokinase and / or GK associated with liver mitochondria use ATP generated endogenously by oxidative phosphorylation as shown for low-K_m hexokinase activity in brain [23].

4. DISCUSSION

Danial and colleagues [16] reported the presence of GK in a multi-protein complex containing the pro-apoptotic protein BAD in association with mitochondria isolated from mouse liver. Mitochondrial GK immunoreactivity was diminished in BAD-/- mice which were mildly hyperglycaemic, implicating a role for BAD in anchoring GK to mitochondria and possibly also a role for mitochondrial GK in regulating glucose homeostasis. Hepatocytes from the BAD-/- mice showed diminished glucose-induced oxygen consumption consistent with a putative role for GK in priming mitochondria to generate ATP through pyruvate oxidation as had been reported previously for low K_m hexokinases in mitochondria from brain and other tissues including liver [23,24].

In contrast with the findings of Danial et al [16], Bustamante and colleagues [17] reported lack of detection of GK immunoreactivity in isolated mouse liver mitochondria and suggested that the previously reported association of GK immunoreactivity with mitochondria may be an artefact. They failed to detect hexokinase activity associated with mouse liver mitochondria and argued against a role for glucose-induced mitochondrial oxygen consumption in liver. However, association of hexokinase activity with isolated rat liver mitochondria has been reported by others [24-26]. Low K_m hexokinases account for a variable proportion of total hexokinase activity (GK plus low-K_m hexokinases) in rat hepatocytes ranging from 5 to 27 % [27,28]. Although hexokinase isoenzymes I and II are much less abundant than GK in hepatocytes they are associated predominantly with the mitochondria [26] and utilise mitochondrial ATP in preference over exogenous [24].

The current contentious issues [16,17] are whether GK associates specifically with liver mitochondria and whether it has an analogous role in regulating mitochondrial ATP production similar to low-K_m hexokinases [23,24]. In this study we tested for association of GK and its two major binding proteins (GKRP and PFK2/FDP2) with rat hepatocyte mitochondria. We used a Percoll gradient procedure to fractionate mitochondria from other subcellular organelles / membrane vesicles [19,29]. Since GK shuttles between the nucleus and the cytoplasm of hepatocytes [2], association with cytoskeletal filaments [30] and other subcellular organelles is not unexpected. We therefore tested for contamination of the mitochondrial fractions with protein markers of other organelles. We demonstrated a peak of GK immunoreactivity that coincides with the mitochondrial markers glutamate dehydrogenase and citrate synthase. There was a similar peak of GKRP immunoreactivity that was coincident with the GK immunoreactivity, but no detectable PFK2/FDP2 immunoreactivity. We can exclude the possibility that the GK and GKRP immunoreactivity represent contamination by the nuclear fraction based on the lack of colocalisation of the nuclear marker USF-1. In addition, the association of GK and GKRP with mitochondria does not appear to be an artefact of hepatocyte culture since similar co-localisation is observed in freshly isolated hepatocytes. We cannot however exclude the possibility that the GK and GKRP immunoreactivity in the mitochondrial fractions are bound to cytoskeletal proteins in association with the mitochondria. It is unlikely that association of GK and GKRP with the mitochondria represent non-specific association of these proteins during the homogenisation procedure since neither protein dissociated during incubation with conditions expected to

dissociate GK from GKRP. This suggests the involvement of additional proteins in the interaction with mitochondria. Previous work has also shown that purified GK does not bind to isolated mitochondria [31]. This is consistent with the lack of association of GK with mitochondria in Bad-/- mice [16]. We can exclude the possibility that the GK and GKRP immunoreactivity in the mitochondrial fractions represent membrane vesicles of entrapped cytosolic proteins generated during the homogenisation procedure based on the lack of detection of PFK-2/FDP-2 which was present exclusively in the cytosolic fraction. The lack of association of GK with PFK-2/FDP-2 contrasts with the situation in pancreatic β-cells [19]. Since β-cells do not express GKRP [2], the interaction with PFK-2/FDP-2 may represent a tissue-specific difference in interaction of GK with binding partners. Although mitochondria-associated GK and GKRP immunoreactivity represented 6-7% of cytsolic immunoreactivity in the experiments on isolated mitochondria and 2% of that in the homogenate in the Percoll gradient experiments, the latter may be an underestimate because of loss of organelles in the bottom fraction of the gradient and also loss during the washing of the Percoll fractions. It is possible that binding of a multiprotein complex containing GK and GKRP to mitochondria is dependent of divalent ions that are absent from the homogenisation medium. Differences in concentrations of chelating agents in the homogenisation media in the previous studies [16,17] is a possible explanation for the discrepancy between these studies.

We confirmed that low-K_m hexokinases and/or GK associated with liver mitochondria can use ATP generated intramitochondrially by oxidative phosphorylation as suggested previously [23], but we could not ascertain the contribution of GK as opposed to low-K_m hexokinases to the measured activity. We cannot therefore firmly exclude the possibility that the immunoreactive GK associated with mitochondria is a catalytically inactive splice variant [32]. However an alternative explanation is that since GK is less stable than low-K_m hexokinases in media of low ionic strength [32], its activity may be in part lost during the fractionation procedure.

The finding of adaptive changes in the GK/GKRP ratio in association with mitochondria is suggestive of a physiological role. The role of GKRP as a nuclear receptor [8] and inhibitor [1] of GK are established. However, whether GKRP has a role in the cytoplasm has remained elusive. Whilst immunocytochemical studies on liver sections or isolated hepatocytes [2-5] have shown predominant localisation of GKRP in the nucleus, this

technique does not allow accurate determination of the cytoplasmic distribution because of non-specific staining and hepatocyte autofluorescence. Nuclear / cytoplasmic ratios for GKRP (on a unit area basis, rather than whole cell basis) ranging from 2 to 14 have been reported [3-5, 11]. However, since nuclear volume is about 10% of cell volume, nuclear / cytoplasmic ratios on a whole cell basis are much smaller than on an area basis. Binding of GK to GKRP has been determined by a digitonin release assay [6] based on the fractional release of GK during permeabilisation of hepatocytes in the presence of Mg^{2+}. The validity of this method was supported by the correlation between rates of glucose phosphorylation and free GK activity in cells with varying GKRP overexpression [9]. Whilst changes in free (unbound) GK correlate with translocation of GK to the cytoplasm, the possibility remains that the bound GK fraction comprises both the GK in the nuclear compartment and cytoplasmic GK bound to GKRP.

The mitochondrial ratio of GK / GKRP was affected by the pre-incubation conditions in a manner not readily explained from changes in the nuclear / cytoplasmic distribution of GK and GKRP. The decrease in the mitochondrial GKRP / GK ratio after incubation of hepatocytes with glucose plus sorbitol, which causes translocation of GK to the cytoplasm [11], could be explained by fructose 1-P induced dissociation or translocation of GKRP from bound GK. Likewise, the reversal by glucagon could be due to the lowering of fructose 1-P by the hormone [7]. Failure to elute GKRP or glucokinase by addition of fructose 1-P to the isolated mitochondria may be due to involvement of additional proteins in the interaction of both GK and GKRP with mitochondria. Binding of both GK [30] and GKRP [33] to actin microfilaments is consistent with such an explanation.

The decrease in the mitochondrial GKRP / GK ratio after glucose and sorbitol treatment and its reversal by glucagon are suggestive of a role for GKRP in association with hepatocyte mitochondria. A previous study that determined the effect of titrated overexpression of GKRP in hepatocytes demonstrated progressive inhibition of glycogen synthesis but only partial inhibition of glycolysis [9]. These findings could tentatively be explained by the presence of two distinct cytoplasmic GK glycolytic pools that are either sensitive or insensitive to GKRP. The latter may represent association of GK with PFK-2/FDP-2 [13-15] and may be involved in lactate formation, whereas the former may represent the mitochondrial pool in conjunction with GKRP [9]. Such a model would predict that

GKRP may have a role in regulating either oxidative metabolism of glucose or lipogenesis rather than lactate formation.

5. ACKNOWLEDGEMENTS We thank Diabetes UK for equipment and project grant support and MRC/JREI for equipment grant support.

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7. FIGURE LEGENDS

Figure 1. Distribution of GK and GKRP in Percoll gradient fractions from hepatocytes. **A.** Percoll fractionation on freshly isolated hepatocytes. Glutamate dehydrogenase, 5'nucleotidase and NADPH-cytochrome C reductase were determined enzymatically and GK and GKRP immunoreactivity by Western blotting. Recovery of activity or immunoreactivity is expressed as % total loaded on the gradient. **B, C.** Percoll fractionation on hepatocytes cultured overnight at 5mM glucose. Immunoreactivity to GK, GKRP, USF-1 and BAD and glutamate dehydrogenase activity are expressed as % total loaded. **C.** Representative immunoblots. Results are representative of 3 (**A**) or 6 (**B**, **C**) experiments, except for BAD, which is representative of 2 experiments.

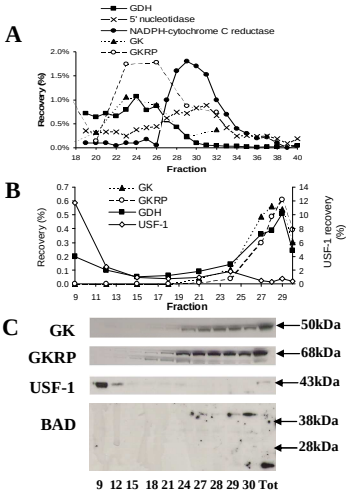
Figure 2. Sensitivity of GK, GKRP and PFK-2 antibodies
Hepatocyte homogenate in MSHE (1.25-20μg per loading) buffer was fractionated by SDS-PAGE and immunoblotted with antibodies to GK, GKRP and PFK-2/FDP-2. **A**. Representative immunoblots. **B**. Immunoreactivity expressed as % of 20μg total protein. Results are representative of 2 experiments.

Figure 3. Dissociation of mitochondrial-associated GK and GKRP
Mitochondria isolated by Percoll fractionation from hepatocytes cultured at 5 mM glucose were incubated for 20 min in the absence (C) or presence of 5mM glucose 6-P (G), 1mM fructose 1-P (F1), 5mM fructose 6-P (F6), 25mM KCl (K1), 25mM KCl + 1mM fructose 1-P (KF) or 150mM KCl (K2). Fractions were centrifuged at 13,000g and GK and GKRP immunoreactivity determined in supernatant (SN) and pellet (P) fractions. Results are representative of 3 experiments.

Figure 4. Substrate and hormone-induced changes in GK and GKRP in the nuclear and mitochondrial compartments
Hepatocyte cultures were incubated for 3 h at either 5 mM glucose (Con) or 25 mM glucose + 250 μM sorbitol without (G/S) or with 100 nM glucagon (G/S/G). Cells were fixed and immunostained for GK or GKRP (**A/B**) or mitochondria isolated by Percoll fractionation for determination of GK and GKRP immunoreactivity (**C/D**). **A**. Representative images for GK

and GKRP nuclear staining. **B**. GK and GKRP N/C ratio determined for 15 nuclei per condition. Means ± SEM. n = 12. **C.** Representative immunoblots for mitochondria-associated GK and GKRP. **D**. Mitochondria-associated GK and GKRP immunoreactivity (IR) % total. Means ± SEM. n = 4. * $p < 0.05$ relative to control. # $p < 0.05$ relative to G/S.

Fig.1


A
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Recovery (%)
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GK
GKRP
GDH
USF-1
Recovery (%)
USF-1 recovery (%)
Fraction
C
GK
GKRP
USF-1
BAD
50kDa
68kDa
43kDa
38kDa
28kDa
9 12 15 18 21 24 27 28 29 30 Tot

Fig.2

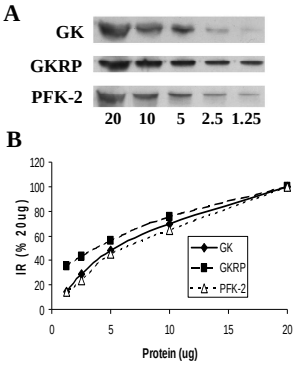

A
GK
GKRP
PFK-2
20 10 5 2.5 1.25
B
IR (% 20ug)
120
100
80
60
40
20
0
0 5 10 15 20
Protein (ug)
GK
GKRP
PFK-2

Fig.3

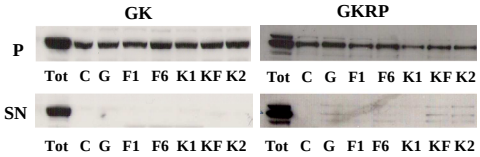
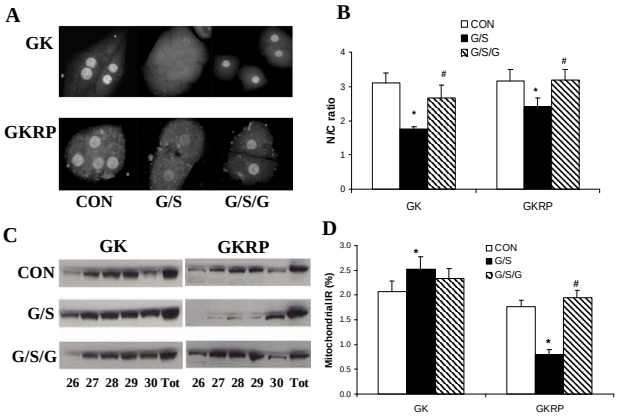

GK
GKRP
P
SN
Tot C G F1 F6 K1 KF K2
Tot C G F1 F6 K1 KF K2
Tot C G F1 F6 K1 KF K2
Tot C G F1 F6 K1 KF K2

Fig. 4


A
GK
GKRP
CON
G/S
G/S/G
B
CON
G/S
G/S/G
N/C ratio
GK
GKRP
C
GK
GKRP
CON
G/S
G/S/G
26 27 28 29 30 Tot 26 27 28 29 30 Tot
D
CON
G/S
G/S/G
MitochondrialIR (%)
GK
GKRP