The uncoupling protein 2, UCP2, is a member of a family of inner mitochondrial membrane ion carriers involved in a host of metabolic processes. UCP2 protein is encoded by nuclear genome, but the protein is found exclusively in the mitochondria. The heterogeneous nuclear ribonucleoprotein K (hnRNPK) is an RNA-binding protein involved in many processes that compose gene expression, including mRNA processing and translation. The yeast three-hybrid screen revealed K protein bound to ucp2 mRNA through sites located in the 3'-untranslated region of the transcript. ucp2 mRNA-K protein complexes were associated with polysome-coated mitochondria. Expression of exogenous K protein augmented the insulin-induced mitochondrial level of UCP2 protein that was not accompanied by a corresponding increase in ucp2 mRNA. These results suggest the insulin stimulates translation of ucp2 mRNA in a process that involves K protein.

The bulk of cell fuel comes from mitochondria that generate ATP through oxidative phosphorylation (1). Mitochondria are bounded by outer and inner membranes. The outer membrane is fairly leaky, whereas the inner membrane tightly maintains the electrochemical gradient necessary for proton-driven ATP production. The inner membrane is imbedded with carriers that transport ions and other substrates (2).

Mammalian mitochondria are composed of ~1000 different proteins (3), only 13 of which are encoded by the circular mitochondrial 16–17-kilobase genome (4). Thus, the vast majority of proteins that compose the mitochondria proteome are encoded by the nuclear genome. Of these proteins, some are localized exclusively to the mitochondria, whereas others are also found in other subcellular compartments (5).

The UCP2 protein belongs to a superfamily of inner mitochondrial membrane carriers that not only includes other uncoupling proteins (UCP1–5)¹ but also oxoglutarate and ADP/ATP transporters. The designation of these proteins was based on their ability to uncouple oxidative phosphorylation when overexpressed in yeast, a process thought to represent a proton leak across the inner membrane and dissipation of the transmembrane voltage. Like all the other UCP proteins, UCP2 is encoded by a nuclear gene, but all these carriers are expressed exclusively in mitochondria (6). There is a large body of literature that provides evidence for the involvement of UCP2 protein in the regulation of metabolic processes (7). The role of UCP2 in regulating insulin secretion by pancreatic β cells is considered particularly important (8). Interest in this carrier has been further fueled by the discovery of a common polymorphism of the ucp2 gene that has been associated with metabolic disorders (9, 10) and hypertension (11). Although there is mounting evidence that UCP2 protein plays a key role in regulating metabolic processes (12), reactive oxygen species production (13), and response to tissue injury (14, 15), the mechanisms that regulate its expression are not well known.

The heterogeneous nuclear ribonucleoprotein K (hnRNPK) protein is an ancient RNA-binding factor involved in multiple processes that compose gene expression. K protein interacts with both kinases and factors that regulate chromatin remodeling, transcription, mRNA processing, and translation (16). Its mode of action appears to be that of a docking platform at sites of nucleic acid-directed processes, where it may facilitate cross-talk between inducible kinases and factors involved in different processes that compose gene expression (16). K protein is phosphorylated in response to insulin and other factors (17, 18). As such, K protein has the potential to sense and integrate signals from multiple kinase cascades and generate output targeting factors that regulate nucleic acid-dependent processes (16).

K protein contains three K homology domains that mediate RNA binding (19, 20). Each one of the K homology domains is able to bind RNA but the affinity of interaction with RNA is stronger when all three domains participate in the binding. K protein K homology domains bind strongest those RNA sequences that contain three C-rich patches, suggesting a three-prong interaction where each one of the K homology domains binds one of three short C-rich sequences that are closely spaced within the RNA target (21).

K protein interacts directly and indirectly with a large repertoire of RNAs, associations that are responsive to ligands such as insulin (18). To identify transcripts that bind K protein directly we used full-length K protein as a bait in the yeast three-hybrid system to screen RNA libraries generated from K protein-associated mRNAs in untreated and insulin-treated cells. The most frequently isolated clones represented different fragments derived from the 3'-UTR of the ucp2 transcripts. A series of experiments were done to determine the physiologic relevance of this in vivo interaction. Our studies provide evi-

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The abbreviations used are: UCP, uncoupling protein; UTR, untranslated region; HPC-IR, hepatoma cells expressing human insulin receptor; IP, immunoprecipitation; RT, reverse transcriptase; GFP, green fluorescent protein; ER, endoplasmic reticulum; PVDF, polyvinylidene difluoride.
ducence that K protein plays a role in the insulin-induced expression of UCP2 protein in the mitochondria.

MATERIALS AND METHODS

Cells—Rat hepatoma cells expressing human insulin receptor (HTC-IR) were grown in plastic cell culture flasks in Dulbecco's modified Eagle's media supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (0.01%), and humidified with 6% CO2/94% air gas mixture. Cells were routinely subcultured using trypsin solution as described before (18).

RNA Co-immunoprecipitation—Cells were lysed with immunoprecipitation (IP) buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5) containing RNase inhibitor (1 unit/μl) and the following inhibitors: 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 30 mM p-nitrophenyl phosphate, 10 mM NaF, 0.1 mM Na3VO4, 0.1 mM Na3MoO4, and 10 mM β-mercaptoethanol. The lysates were centrifuged (15,000 × g, 4°C, 30 min), and the supernatant was first pre-cleared with rabbit IgG (Bio-Rad) by bath sonication for 15 min (4°C) followed by binding to protein A beads for 45 min (4°C). The beads were spun down, and the supernatant was bath sonicated with purified anti-K protein antibody 54 for 15 min. The sonicated complexes were sedimented by adding 200 μl of protein A beads (20 μl) and rotating the slurry for 45 min (4°C). Beads were washed four times with 1 ml of IP buffer. RNA was eluted from the beads with 100 mM NaCl, 1% SDS at 65°C for 10 min in an Eppendorf Thermomixer, then phenol purified and ethanol precipitated. RNA pellets were resuspended in water and stored at -70°C.

Yeast Three-hybrid Screens—Hybrid RNA libraries from transcripts co-immunoprecipitated with K protein from untreated and insulin-treated cells were generated as described previously (20). The pellets were resuspended in RSB-100 buffer containing 0.5% Triton X-100 and incubated by adding 100 μl (20 μl) and rotating the slurry for 45 min (4°C). Beads were washed four times with 1 ml of IP buffer. RNA was eluted from the beads with 100 mM NaCl, 1% SDS at 65°C for 10 min in an Eppendorf Thermomixer, then phenol purified and ethanol precipitated. RNA pellets were resuspended in water and stored at -70°C.

Subcellular Fractionation—Fractionation was performed as described by Mill et al. (23) with few modifications. To obtain soluble cytosolic fraction, HTC-IC cells were resuspended in the RSB-100 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl2 containing digitonin (40 μg/ml)) and inhibitors of proteases, phosphatases, and RNases. The lysates were incubated on ice for 5 min and centrifuged at 2000 × g for 8 min. The supernatant was collected, and the pellet was resuspended in RSB-100 buffer containing 0.5% Triton X-100 and incubated on ice for 5 min. The Triton-extracted fraction was separated by centrifugation at 105,000 × g for 5 min. Each fraction collected and the remaining pellet was resuspended in RSB-100 buffer and sonicated twice for 5 s on ice using a Branson sonicator with a microtip. The sonicated material was centrifuged through a 30% sucrose cushion in RSB-100 buffer at 4000 × g for 15 min.

Total RNA was prepared from subcellular fraction using the TRIzol reagent, and RNA bound to K protein was co-purified using anti-K protein antibody as described. Ethanol-precipitated RNA was further purified using RNAeasy mini columns after DNase treatment. Following ethanol precipitation, total RNA and co-immunoprecipitated RNA pellets were dissolved in water and used for reverse transcription (RT) reaction.

Isolation of Mitochondria—The polysome-free and polysome-bound mitochondria were purified from the cells by differential centrifugation through sucrose gradient as previously described for mammalian cells (Fig. 3A) (24). To generate polysome-free mitochondria, cells were lysed by a Dounce homogenizer in breaking buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, 7.5 mM EDTA, 5 mM 2-mercaptoethanol, 200 μl/mg cycloheximide, 500 μg/ml heparin. To obtain mitochondria coated with polysomes, the breaking buffer contained 5 mM MgCl2 and 100 mM KCl instead of EDTA. All buffers contained inhibitors of proteases, phosphatases, and RNases. The entire isolation procedure was done either in the presence of EDTA (poly-some-free) or MgCl2/KCl (polysome-bound) (Fig. 3A) (25). The homogenates were centrifuged at 700 × g for 10 min at 4°C, and the resulting supernatants were re-centrifuged under the same conditions. The pellets contain nuclei and cell debris (Fig. 3A). The post-nuclear supernatants were centrifuged at 10,000 × g for 30 min at 4°C. The supernatant (post-mitochondrial fraction) contains cytoplasm with endoplasmic reticulum and lysosomes (26). The mitochondrial pellets were resuspended carefully in the homogenization buffer and centrifuged again at 105,000 × g for 30 min at 4°C. Then, the crude mitochondrial fraction resuspended in the homogenization buffer was layered on a 1.0–1.5 M discontinuous sucrose gradient and centrifuged at 80,000 × g for 1 h. Mitochondria were collected from the band at the 1.5–1.0 M sucrose interphase and washed several times with the homogenization buffer. To extract proteins mitochondria were lysed with IP buffer. Purity of mitochondria were assessed with antibody markers (Fig. 3B).

ucp2 Gene Expression

RESULTS

K Protein Binds to Sequences within the 3′-UTR of the Rat ucp2 mRNA—K protein is associated with many transcripts, interactions that are responsive to a host of ligands including insulin (18, 31, 32). To identify its target transcripts we used full-length K protein as bait in the yeast three-hybrid system to screen (20) a library constructed from RNAs co-immunoprecipitated with K protein from untreated and insulin-treated HTC-IR cells. From a total of 190 true positive clones, 30 (14 from untreated and 16 from insulin-treated cells) represented seven unique fragments of the 3′-UTR of ucp2 (Fig. 1A). Clone 1 did not overlap with clones 5, 6, and 7, whereas clones 2 and
3 did not overlap with clones 6 and 7. These results indicate that K protein binds more than one region within the 3'-UTR of ucp2 mRNA. Multiple binding sites would increase the affinity of interaction and thus explain the high frequency of ucp2 3'-UTR clones isolated in the screen.

We have previously defined three C-rich patches within a consensus sequence that binds K protein, protein CCAUCN$_{2}$wCCwN$_{7-m}$UCAYC, where W represents adenine or uracil and Y designates for pyrimidine (20, 21). Alignment of mammalian ucp2 3'-UTRs identified several such C-rich clusters that are highly conserved in murine, human, canine, and bovine species (Fig. 1B). Putative K protein binding sites in the 3'-UTR of the ucp2 mRNA are also conserved in marsupials (AY233003), spanning 100–150 million years of evolutionary separation (33), but not in the Xenopus (BC044682) (data not shown). The 3'-UTRs play a critical role in the control of mRNA translation and stability (34). Thus, the evolutionary conservation of these C-rich clusters within ucp2 mRNA 3'-UTR suggests that they play a regulatory role.

In agreement with the results of three-hybrid screens, ucp2 mRNA was also the most frequently encountered tag in serial analysis gene expression-based profiling of mRNAs immunoprecipitated with K protein from the HTC-IR cells (21). ucp2 mRNA was also found in RNA co-immunoprecipitated with K protein from untreated and insulin-treated serum-deprived rat HTC-IR cells was used to generate a RNA library for screen in the yeast three-hybrid system using full-length K protein as a probe (20).
The open reading frame (ORF). The coding regions of the rat and RNA co-immunoprecipitated with K protein (K) was used for RT reactions. Primers to the protein Total RNA (Total) and RNA co-immunoprecipitated with K protein (Co-IP) was used for real-time RT-PCR. Amplification was carried out using pairs of oligonucleotide microarrays.2 These results identified ucp2 mRNA as one of the key cellular targets of K protein in mammalian cells.

Evidence That K Protein-ucp2 mRNA Complexes Are Bound to Mitochondria—The above three-hybrid screen of RNAs co-immunoprecipitated with K protein revealed that K protein binds directly ucp2 mRNA in vivo (Fig. 1). During and after their synthesis, mRNAs exist in dynamic ribonucleoprotein complexes whose composition is continually changing (35). K protein is found in the nucleus, cytoplasm, and mitochondria (20), thus the interaction of K protein with ucp2 mRNA could take place in one or more of these subcellular compartments. Differential fractionation (23) and RNA co-immunoprecipitations (18) were carried out to determine the subcellular compartments where K protein interacts with ucp2 mRNA. β-Actin mRNA was used as a control. HTC-IR cells were first permeabilized with a buffer containing a low concentration of digitonin, which disrupts the plasma membrane but leaves the nuclear and mitochondrial envelopes intact (23). The digitonin-soluble fraction represents cytosol (Fig. 2). The remaining pellet was then solubilized with 0.5% Triton X-100 and the resulting supernatant, the Triton fraction, contains some nuclear and organelle components including those that are derived from mitochondria. The remaining pellet was homogenized by probe sonication. This last soluble fraction is referred to as nucleoplasm and includes sheared chromatin and heterogeneous nuclear ribonucleoprotein complexes (23, 36). Each one of the three fractions was used to co-immunoprecipitate RNA with anti-K protein antibody and protein A beads (18).

To purify mitochondria we used a sucrose gradient centrifugation-based method previously described for mammalian cells that allows separation of mitochondria from most other organelles (25, 26, 39) (Fig. 3A). Heat shock protein-60, Hsp60 (40, 41), and cytochrome c (39) are exclusively localized to the mitochondria. The glucose-regulated protein, GRP94, is an abundant endoplasmic reticulum protein (42). These proteins were used as organelle markers (39, 40, 42).

HTC-IR cells were Dounce homogenized in the presence of either MgCl2/KCl or EDTA to yield mitochondria associated with or without polysomes, respectively (25). Equal amounts of

![Fig. 2. K protein-ucp2 mRNA complexes exist in multiple subcellular fractions. HTR-IC cells grown in serum were homogenized by probe sonication (23) and RNA co-immunoprecipitations (18) were carried out to determine the subcellular compartments where K protein interacts with ucp2 mRNA.](http://www.jbc.org/)

**Subcellular Fractions**

**Fig. 2.** K protein-ucp2 mRNA complexes exist in multiple subcellular fractions. HTR-IC cells grown in serum were homogenized by probe sonication (23) and RNA co-immunoprecipitations (18) were carried out to determine the subcellular compartments where K protein interacts with ucp2 mRNA. The above three-hybrid screen of RNAs co-immunoprecipitated with K protein revealed that K protein binds directly ucp2 mRNA in vivo (Fig. 1). During and after their synthesis, mRNAs exist in dynamic ribonucleoprotein complexes whose composition is continually changing (35). K protein is found in the nucleus, cytoplasm, and mitochondria (20), thus the interaction of K protein with ucp2 mRNA could take place in one or more of these subcellular compartments. Differential fractionation (23) and RNA co-immunoprecipitations (18) were carried out to determine the subcellular compartments where K protein interacts with ucp2 mRNA. β-Actin mRNA was used as a control. HTC-IR cells were first permeabilized with a buffer containing a low concentration of digitonin, which disrupts the plasma membrane but leaves the nuclear and mitochondrial envelopes intact (23). The digitonin-soluble fraction represents cytosol (Fig. 2). The remaining pellet was then solubilized with 0.5% Triton X-100 and the resulting supernatant, the Triton fraction, contains some nuclear and organelle components including those that are derived from mitochondria. The remaining pellet was homogenized by probe sonication. This last soluble fraction is referred to as nucleoplasm and includes sheared chromatin and heterogeneous nuclear ribonucleoprotein complexes (23, 36). Each one of the three fractions was used to co-immunoprecipitate RNA with anti-K protein antibody and protein A beads (18). Total RNA from each fraction and RNA eluted from the immune complexes was reverse transcribed and then used in real-time PCR. Amplification was carried out using pairs of oligonucleotide primers to either the ucp2 or β-actin gene. As shown in
ucp2 Gene Expression

**Fig. 3.** ucp2 mRNA is associated with polysome-coated mitochondria. A, outline for the procedure used to isolate mammalian polysome-free (buffers with EDTA, in blue) and polysome-bound mitochondria (buffers with MgCl2/KCl, in red) (“Materials and Methods”) (25, 26). Two centrifugations at 700 × g of the cell homogenate separated the nuclei and cell debris (Pellet I) from the cytosol containing organelles (Supernatant I). 10,000 × g centrifugation separated crude cytoplasmic post-mitochondrial fraction (Supernatant II) from the crude mitochondrial preparation (Pellet II). The post-mitochondrial fraction contains cytoplasmic, ER, and lysosomal proteins (26). The washed mitochondrial pellet (Pellet III) resuspended in buffer was applied to the sucrose gradient. After 80,000 × g centrifugation the clear bands at the 1–1.5 M interphase containing purified mitochondria were collected and washed several times. B, mitochondria and cytosolic post-mitochondrial fractions were prepared from HTC-IR cells grown in serum as outlined in A. Organelle markers were used to verify the mitochondria isolation procedure. Equal amounts of protein from the cytosolic post-mitochondrial fraction (Supernatant II) and from the purified mitochondria were resolved by SDS-PAGE and after electrotransfer the PVDF membrane was stained with antibody to the mitochondrial proteins, HSP60 (71), and cytochrome c (39), and to the endoplasmic protein GRP 94 (42). C, total RNA purified from mitochondrial and from cytosolic post-mitochondrial fractions was used in RT. Real-time PCR were carried out using primers to the rat ucp2 (NM_019354) and ribosomal protein L39, RpL39 (NM_012875.1) cDNAs. The results are expressed as levels relative to the amount of RNA in whole cell lysates. Polysome-bound, +, lanes 1 and 3; and polysome-free, −, lanes 2 and 4, fractions. Results represent mean ± S.D. (n = 2).

Protein extracted from purified mitochondria and from the post-mitochondrial fraction were analyzed by Western blotting using antibodies to the above organelle markers (Fig. 3B). Immunoblots showed that Hsp60 and cytochrome c were found in the mitochondrial but not in the post-mitochondrial fractions, indicating that the final pellet contained mitochondria. Although the GRP94 ER marker was detected in the final mitochondria pellet, the amount present was much lower than that found in the cytosolic post-mitochondrial fraction that included ER (Fig. 3B, compare lower panels in lanes 1 and 2 to 3 and 4). As a fraction of ER is closely associated with mitochondria within the cell (43), complete dissociation of ER from mitochondria is very difficult to achieve in vitro (43). Predictably, the levels of these proteins in the mitochondrial and the post-mitochondrial fractions were the same whether they were purified with or without polysomes.

Next we tested the partition of ucp2 mRNA between the mitochondrial and cytosolic post-mitochondrial fractions (Fig. 3C). When harvested with attached polysomes in the presence of MgCl2/KCl, the level of ucp2 mRNA co-purified with the mitochondria was higher than that found in the post-mitochondrial fraction (Fig. 3C, compare upper graph, bars 1 and 3). When purified in the presence of EDTA, which strips the ribosomes from the mitochondria (25), little of ucp2 mRNA was recovered in the mitochondrial pellet and instead a much greater fraction was found in the cytosolic post-mitochondrial supernatant (Fig. 3C, compare upper graph, bars 2 and 4). These results suggest that a major fraction of ucp2 mRNA is associated with polysomes that are bound to mitochondria. Like ucp2 mRNA, the ribosomal protein L39 mRNA strongly binds K protein (21). We wondered how the RpL39 transcript partitions between these fractions. The real-time RT-PCR results showed that more RpL39 mRNA was recovered in the post-mitochondrial supernatant than in the mitochondria and the levels were the same regardless whether or not the harvesting of mitochondria was done with or without attached polysomes (compare bars 1 and 2 to 3 and 4). These experiments shows that although both ucp2 and RpL39 mRNAs bind K protein strongly, only the ucp2 transcript is preferentially associated with the mitochondria decorated with polysomes.

To test if ucp2 mRNA-K protein complexes are associated with mitochondria that are coated with ribosomes we prepared lysates from mitochondria for RNA co-immunoprecipitations with K protein. As before, the levels of total RNA and RNA co-immunoprecipitated with K protein were assessed by real-time PCR (Fig. 4). The RT-PCR revealed that a subset of the ucp2 transcript was bound to K protein when mitochondria were purified with attached polysomes. Much lower levels of
ucp2 mRNA-K protein complexes were found in mitochondria stripped of ribosomes. In agreement with previous studies, oxa1 mRNA was associated with the mitochondria-bound poly-somes but unlike ucp2, oxa1 transcript did not bind K protein. Cytochrome c oxidase 2 (Cox2) is encoded by the mitochondrial genome and is translated within the organelle (4). Real-time PCR showed that the number of Cox2 mRNA copies found in mitochondria decorated with or without polysomes was the same. Predictably, this result indicates that Cox2 mRNA is restricted to the organelle. These results show that ucp2 mRNA behaves similar to the mitochondria-bound oxa1 transcript (25), suggesting that these two transcripts might be similarly translated in the vicinity of mitochondria.

Mitochondria and ER are attached in vivo and cannot be completely separated during their purification (Fig. 3B, GRP 94) (43). Thus, it is formally possible that the ucp2 mRNA is associated with ribosomes bound to the fraction of ER co-purified with mitochondria. Sec61α is highly conserved exclusively in the ER membrane protein that is a part of a complex that anchors ribosomes to this organelle (44). Most of sec61α mRNA is associated with ER (45) serving as another example of localized mRNAs that provide templates for protein synthesis directly at sites where these factors act (46). Real-time RT-PCR revealed that the sec61α transcript was found in the mitochondrial fraction, but unlike ucp2 and oxa1 mRNA, sec61α mRNA was not removed from this fraction when mitochondria were harvested without attached polysomes (Fig. 4) (25). The association of ribosome with ER is known to be strong (45, 47).

**FIG. 4.** K protein-ucp2 mRNA complexes are bound to mitochondria. HTC-IR cells grown in serum were Dounce homogenized in buffer containing either EDTA (polysome-free mitochondria) or MgCl2/KCl (polysome-bound mitochondria). After serial centrifugations through sucrose gradient, mitochondrial pellets were resuspended in homogenization buffer and mitochondrial lysates were prepared (Fig. 3A). Mitochondrial lysates were divided into two equal aliquots, one used for the isolation of total RNA and the other for co-immunoprecipitation of RNA with K protein. Total RNA (Total) and RNA co-immunoprecipitated with K protein (Co-IP) were used in RT. Real-time PCR were carried out using primers to the protein coding regions of the rat ucp2 (NM_013505), oxa1 (NW_047454), and Sec61α (NW_047694) nuclear genes and to the Cox2 (NC_001665) mitochondrial gene. The bars show RNA copies in the mitochondrial fractions. Results represent mean ± S.D. (n = 2).
Expression of exogenous K protein augments insulin-induced UCP2 protein levels in mitochondria. Serum-deprived HTC-IR cells infected with either GFP (Adenovirus-GFP) or K protein (Adenovirus-K) adenovirus were treated either without (−) or with (+) insulin (10−8 M, 60 min). Cells were harvested and fractionated into cytosolic (lanes 1–4) and mitochondrial (lanes 5–8) fractions. Cytosolic and mitochondria lysates were resolved by SDS-PAGE and electro-transferred to PVDF membrane. Blotted proteins were assessed by immunostaining (IS) with antibodies to actin (α-actin), K (αK), or UCP2 (αUCP2) proteins. Shown is one of three independent experiments.

Additionally, following cell lysis, the high fraction of ER forms inside-out microsomes where ribosome inside these vesicles are protected from dissociation agents (48). The inability of EDTA to remove ER-bound sec61α mRNA from these fractions shows that the ucp2 mRNA and sec61α transcript were localized to different compartments and that the ucp2 transcript is not attached to ER that co-purifies with mitochondria. Taken together, these results (Figs. 3 and 4) suggest that like oxa1 (25), ucp2 mRNA is associated with polyribosomes bound to mitochondria, where a fraction of the ucp2 transcript exists in a complex with K protein.

Expression of K Protein Increases UCP2 Protein Levels in the Mitochondria—UCP2 is thought to be a metabolic regulator and is localized to mitochondria (6, 7). Insulin stimulates a host of mitochondrial processes (49–51), effects that may in part reflect insulin up-regulated expression of ucp2 mRNA (52, 53) and protein (54). K protein regulates translation through its interactions with the 3′-UTRs of target transcripts (16, 55). K protein responds to insulin (18) and forms complexes with ucp2 mRNA (Figs. 2 and 3) by binding 3′-UTR sites (Fig. 1).

Based on these observations we wondered if insulin alters UCP2 protein expression, and if so, whether or not K protein is involved in this process. Serum-deprived HTC-IR cells infected with either adenovirus expressing GFP (control), or with K protein were treated without or with insulin (1 h at 10−7 M). Cells were harvested and cytosolic post-mitochondrial and mitochondrial fractions were prepared as before. Proteins resolved by electrophoresis were transferred to PVDF membrane, and immunostained with either anti-K protein (Fig. 5, middle panels, lanes 1–8) or anti-UCP2 antibody (Fig. 5, lower panels, lanes 1–8). Actin is attached to mitochondria (56). Immunostaining of PVDF membranes with anti-actin antibody was used to ensure equal protein loading (Fig. 5, upper panels, lanes 1–8). Predictably, expression of K protein increased its level in the cytosolic (lanes 1–4) and nuclear fractions (data not shown). Insulin increased K protein levels in mitochondrial fractions of cells expressing either GFP or K protein (middle panel, lanes 1–7) and insulin-induced (lower panel, lanes 6 and 8) levels of UCP2 protein in the mitochondrial fraction. No UCP2 protein was detected in the cytosolic fraction. We used densitometric analysis of bands (OptiQuant Image Analysis Software, Packard) to estimate the percent change in mitochondrial UCP2 protein levels in response to insulin in adenovirus-transfected cells. In GFP-transfected cells, insulin increased UCP2 protein levels by 68 ± 19%, in untreated K protein-transfected cells UCP2 protein levels were 39 ± 12% higher than in GFP-transfected cells, and in K-transfected insulin-treated cells UCP2 protein levels were 298 ± 28% higher than in the untreated GFP-transfected cells (mean ± S.D., n = three separate experiments). Compared with untreated GFP-transfected cells the increase in UCP2 protein levels in K-transfected insulin-treated cells (298%) was greater than the sum of the increases in GFP insulin-treated plus K protein untreated cells (68 + 39 = 107%). This analysis reveals a synergistic effect, suggesting that K protein facilitates insulin-inducible mitochondrial UCP2 protein expression.

Expression of exogenous K protein augments insulin-induced UCP2 protein levels in mitochondria. Serum-deprived HTC-IR cells infected with either GFP (Adenovirus-GFP) or K protein (Adenovirus-K) adenovirus were treated either without (−) or with (+) insulin (10−8 M, 60 min). Cells were harvested and fractionated into cytosolic (lanes 1–4) and mitochondrial (lanes 5–8) fractions. Cytosolic and mitochondria lysates were resolved by SDS-PAGE and electro-transferred to PVDF membrane. Blotted proteins were assessed by immunostaining (IS) with antibodies to actin (α-actin), K (αK), or UCP2 (αUCP2) proteins. Shown is one of three independent experiments.

In rat skeletal muscles, insulin (10−8 M) increased ucp2 mRNA levels by more than 3-fold (52). Insulin treatment of serum-deprived HTC-IR increased ucp2 mRNA levels by only 30–40% but its -fold effect on the level of K protein-ucp2 mRNA complexes found in whole cell lysates was greater (Fig. 6). The increased level of UCP2 protein in cells overexpressing K protein, even without insulin treatment (Fig. 5), may reflect a K protein-mediated increase in ucp2 mRNA. To test this possibility, we compared ucp2 mRNA levels in whole cell lysates from HTC-IR cells transfected with adenovirus encoding either GFP or K protein (Fig. 7), and treated with or without insulin. There was constitutive expression of ucp2 mRNA in GFP-transfected cells, and a small increase in response to insulin (Fig. 7, upper panel). Interestingly, in cells expressing K protein the constitutive and insulin-induced levels of ucp2 mRNA were lower than that seen in insulin-treated cells expressing GFP. As a control, we examined levels of β-actin mRNA, and saw no response to insulin (Fig. 7, middle panel). Because K protein regulates c-myc gene expression in a number of systems (57–59), we also compared levels of c-myc mRNA in insulin-treated cells without or with exogenous K protein (Fig. 7, bottom panel). Here the levels of c-myc mRNA were the same in GFP and K protein expressing cells, and in both types of cells insulin elevated the level of c-myc mRNA. The above results showed that the insulin- and K protein-induced increase in UCP2 protein levels was not matched by an increase in ucp2 mRNA and that the levels of the ucp2 mRNA were even lower in cells expressing K protein.

Although the level of ucp2 mRNA in whole lysates of these cells was not much increased by insulin or K protein (Fig. 7), the levels of this transcript bound to mitochondria could be higher in the treated cells. If translation of ucp2 occurs in the vicinity of the mitochondria, higher levels of the transcript attached to the organelle could explain the up-regulation of UCP2 protein in response to insulin and K protein expression (Fig. 5). This possibility was tested next.

Mitochondria were isolated from untreated and insulin-treated HTC-IR cells expressing either GFP or K protein. Total ucp2 mRNA and ucp2 mRNA co-immunoprecipitated with K protein was assessed by RT and real-time PCR (Fig. 8). The total ucp2 mRNA attached to mitochondria reflected levels seen in whole cell lysates (Fig. 7), a small increase in response to insulin, and the levels bound to the organelle were lower in K expressing cells (Fig. 8, Total). The oxa1 mRNA levels did not change at all. Insulin increased K protein-ucp2 mRNA complexes bound to mitochondria in GFP and K protein expressing cells (Fig. 8, Co-IP). There was little or no binding of K protein to oxa1 mRNA. These results show that up-regulation of UCP2 protein in insulin-treated cells and in K protein-expressing cells (Fig. 5) were not accompanied by a matching increase in the level of ucp2 transcript bound to mitochondria.

Treatment with Cycloheximide Blocks the Insulin-induced Expression of Mitochondrial UCP2 Protein—The insulin-increased levels of UCP2 protein could reflect either increased
synthesis or enhanced stability of the protein in the organelle. To differentiate between these two possibilities we examined the effects of insulin on the mitochondrial UCP2 protein levels in cells pretreated with the protein synthesis inhibitor cycloheximide (60). As shown in Fig. 9, cycloheximide blocked the insulin-induced increased UCP2 protein levels in the mitochondrial fraction. These results show that increased protein stability was not altered by insulin. Because there was only a
Fig. 8. The levels of ucp2 mRNA bound to mitochondria in insulin-treated cells. Serum-deprived HTC-IR cells infected with either GFP (Adenovirus-GFP) or K protein (Adenovirus-K) adenovirus were treated with insulin (10^{-8} M, 60 min). Mitochondria were prepared as in Fig. 3. Mitochondrial lysates were divided into two equal aliquots, one was used for the isolation of total RNA and the other for co-immunoprecipitation of RNA with K protein. Total RNA (Total) and RNA co-immunoprecipitated with K protein (Co-IP) were used in the RT reaction. Real-time PCR were carried out using primers to the rat ucp2 as described in the legend Fig. 2. Results represent mean ± S.D. (n = 2).

DISCUSSION

The present study provides compelling evidence that that ucp2 mRNA is complexed with K protein in mammalian cells. This association likely reflects direct binding of K protein to sites within the 3′-UTR. Expression of K protein increased insulin-induced mitochondrial UCP2 protein levels without a matching increase in mRNA. These results suggest that K protein facilitates insulin-induced translation of UCP2 protein.

The vast majority of mitochondrial proteins are encoded by the nuclear genome (61). Whereas some of these proteins are localized exclusively within the organelle, others are found in multiple subcellular compartments (5). There are several avenues by which nuclear-encoded mitochondrial proteins are delivered to the organelle (62, 63). The best studied pathway is one where the mitochondrial proteins are synthesized by cytoplasmic ribosomes and the amino-terminal peptides serve as signals to recognize the translocase in the outer mitochondrial membrane (TOM) (64). The originally described amino-terminal amphipathic α-helix is one of several known mitochondrial-targeted sorting signals (64). This class of polypeptides is delivered either post- or co-translationally to the mitochondria. In the former group, synthesis is carried out entirely by cytoplasmic ribosomes. In the latter case, translation is initiated by cytoplasmic ribosomes and then the amino-terminal peptide sorts the transcript to the mitochondria where the translation is completed in the vicinity of the organelle (65). It has recently been recognized that there is yet another class of nuclear-encoded mitochondrial proteins where the mRNAs are bound to mitochondria before the initiation of translation. Synthesis of these mitochondrial proteins also takes place in the vicinity of the organelle (24, 25, 66).

UCP2 protein is not detectable in the cytoplasm (Fig. 5) and it does not have an obvious mitochondrial localization signal. A substantial fraction of ucp2 mRNA appears to be associated with polysomes bound to mitochondria (Fig. 3). These observations are consistent with a model where mitochondrial import of UCP2 protein occurs co-translationally. For example, translation of ucp2 mRNA could begin in the cytoplasm and be completed in the vicinity of the organelle. Alternatively, as in the case of oxa1 (25), translation of ucp2 mRNA could be exclusively carried out by the mitochondria-bound ribosomes. Regardless of the specific site(s) of translation, the current data provides evidence that synthesis of UCP2 protein is regulated by insulin, a process that involves K protein.

Expression of K protein increased the level of K protein-ucp2 mRNA complexes (Figs. 6 and 8). This effect suggests, but does not prove, that the increased complex formation between K protein and ucp2 mRNA is causally related to the augmented mitochondrial UCP2 protein expression in insulin-treated cells.

The involvement of K protein in the insulin-induced synthesis of UCP2 protein likely reflects its ability to bind ucp2 mRNA and receive insulin signals (18). Ribonucleoprotein complexes are dynamic in that their protein composition changes not only during transit of the mRNA from the nucleus to the
site of translation (35, 67) but also in response to extracellular signals (16). Adenovirus-mediated overexpression of K protein in HTC-IR cells increased levels of mitochondrial UCP2 protein (Fig. 5) without a corresponding increase in ucp2 mRNA (Fig. 7). This experiment uncovered the role of K protein in insulin-induced translation of the ucp2 mRNA. K protein is known to either activate or repress translation (16), effects that are modulated by extracellular signals (31). The general function of K protein appears to be that of a docking platform (16). According to this model K protein could assemble insulin-responsive kinases and translation factors at the 3′ end of ucp2 mRNA. The ability of K protein to facilitate the interaction of insulin-responsive kinases with translation factors at the 3′-UTR may explain the up-regulation of UCP2 protein expression seen in these experiments.

UCP2 protein levels have been shown to be regulated by a host of ligands and changes in the extracellular environment influencing transcription (68), translation (69), and post-translational processes (70). Because the number of processes correlated with UCP2 protein levels is so broad (6, 54), it is likely that the list of agents regulating ucp2 expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes. Aberrant expression is far from complete. K protein could be a major portal for regulation of the expression of these two genomes.
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