Up-regulation of the ATPase Inhibitory Factor 1 (IF1) of the Mitochondrial H⁺-ATP Synthase in Human Tumors Mediates the Metabolic Shift of Cancer Cells to a Warburg Phenotype

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The H⁺-ATP synthase is a reversible engine of mitochondria that synthesizes or hydrolyzes ATP upon changes in cell physiology. ATP synthase dysfunction is involved in the onset and progression of diverse human pathologies. During ischemia, the ATP hydrolytic activity of the enzyme is inhibited by the ATPase inhibitory factor 1 (IF1). The expression of IF1 in human tissues and its participation in the development of human pathology are unknown. Here, we have developed monoclonal antibodies against human IF1 and determined its expression in paired normal and tumor biopsies of human carcinomas. We show that the relative mitochondrial content of IF1 increases significantly in carcinomas, suggesting the participation of IF1 in oncogenesis. The expression of IF1 varies significantly in cancer cell lines. To investigate the functional activity of IF1 in cancer, we have manipulated its cellular content. Overexpression of IF1 or of its pH-insensitive H49K mutant in cells that express low levels of IF1 triggers the up-regulation of aerobic glycolysis and the inhibition of oxidative phosphorylation with concurrent mitochondrial hyperpolarization. Treatment of the cells with the H⁺-ATP synthase inhibitor oligomycin mimicked the effects of IF1 overexpression. Conversely, small interfering RNA-mediated silencing of IF1 in cells that express high levels of IF1 promotes the down-regulation of aerobic glycolysis and the increase in oxidative phosphorylation. Overall, these findings support that the mitochondrial content of IF1 controls the activity of oxidative phosphorylation mediating the shift of cancer cells to an enhanced aerobic glycolysis, thus supporting an oncogenic role for the de-regulated expression of IF1 in cancer.

In oxidative phosphorylation, ATP is synthesized by the mitochondrial ATP synthase, a H⁺-driven rotatory engine of the inner membrane that utilizes as driving force for ATP synthesis the H⁺ electrochemical gradient generated by the respiratory chain (1–4). The cellular expression level of β-F1-ATPase,² which is the catalytic subunit of the H⁺-ATP synthase, is diminished in diverse human pathologies (5), which include cancer (6–9), affording a relevant marker of disease progression (6, 7, 10–12) and of the response to chemotherapy (7, 13–15). Moreover, the down-regulation of β-F1-ATPase in lung carcinomas (12) and colon cancer cells (15) also provides a mechanistic explanation to the increased glucose avidity of carcinomas, i.e. to the enhanced aerobic glycolysis of cancer cells (16, 17). Interestingly, the quantitative determination of β-F1-ATPase relative to the content of glyceraldehyde-3-phosphate dehydrogenase in human tumors has revealed that cancer abolishes the tissue-specific differences in the cellular complement of the bioenergetic β-F1-ATPase protein (18).

It is well established that when mitochondrial respiration is impaired, the H⁺-ATP synthase can function in reverse acting as an ATP hydrolase for maintaining the proton motive force (1, 19). This process is regulated by an inhibitor peptide called ATPase inhibitory factor 1 or IF1 (19–21), a highly conserved inhibitor of the hydrolase activity of the H⁺-ATP synthase (H-F1) ATPase protein (18). The structure and function of IF1 have been studied in detail, and its role as an ATPase inhibitor has been well documented (19, 20, 23). However, the information on IF1 expression in human tissues and its putative contribution to the development of human pathology are unknown. In this study, we demonstrate that IF1 is overexpressed in human carcinomas. Moreover, we document that IF1 plays a regulatory role in controlling cellular energetic metabolism, strongly supporting its participation as an additional molecular switch used by cancer cells to trigger the induction of aerobic glycolysis, i.e. their Warburg phenotype.

EXPERIMENTAL PROCEDURES

Protein Extraction—Frozen tissue sections obtained from surgical specimens of untreated cancer patients with primary

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² The abbreviations used are: β-F1-ATPase, β catalytic subunit of the H⁺-ATP synthase; IF1, ATPase inhibitory factor 1; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; siRNA, small interfering RNA; NRK, normal rat kidney.
breast (ductal invasive), lung, and colorectal adenocarcinomas as well as squamous lung carcinomas were obtained from the Banco de Tejidos y Tumores, Instituto de Investigaciones Biomédicas Pi y Suner, Hospital Clinic, Barcelona, Spain. Routine histopathological study of all cases had been previously performed by an experienced pathologist, and the histological type, grade, and size of the tumor as well as regional lymph node involvement were recorded (24). Coded samples were received to protect patient confidentiality after approval of the project by the Institutional Review Board. Tissue sections of paired normal and tumor tissue derived from each patient were processed (25). Details of the clinicopathological features of the patients have been recently provided (see Table 1 in Ref. 24). Protein concentration in the extracts was determined with the Bradford reagent (Bio-Rad) using bovine serum albumin as standard.

Monoclonal Antibody Production—To produce antibodies against human IF1, we proceed as described recently (18). In brief, BALB/c mice were immunized with various doses of the antigen against human IF1, which add Sacl and NotI restriction sites, respectively. The resulting product was purified and first cloned into pGEM-Teasy vector (Promega) and after into pQE-Trisystem (18). The resulting plasmid, pQE-IF1 that encodes IF1 with C-terminal His6 and streptavidin tags, was used to transform Escherichia coli BL21 cells. Protein expression was induced by addition of 1 mM isopropyl-1-thio-D-galactopyranoside. After overnight induction, the cells were collected, and the expressed protein was purified using nickel-nitritriacetic acid superflow resin (Qiagen) (18).

Plasmid Constructs—The CDNA (BC009677) encoding human IF1 (AAH96777) was amplified by PCR using the IMAGE 3877506 clone obtained from the ATCC collection (Manassas, VA) and primers 5′-gga-gactctgtagcatcga-gc-3′ and 5′-atagtttagcggccgcatcatcctgg-3′, which add SacI and NotI restriction sites, respectively. The resulting product was purified and first cloned into pGEM-Teasy vector (Promega) and after into pQE-Trisystem (18). The resulting plasmid, pQE-IF1 that encodes IF1 with C-terminal His6 and streptavidin tags, was used to transform Escherichia coli BL21 cells. Protein expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. After overnight induction, the cells were collected, and the expressed protein was purified using nickel-nitritriacetic acid superflow resin (Qiagen) (18).

Cloning, Expression, and Purification of Recombinant IF1—The CDNA (BC009677) encoding human IF1 (AAH96777) was amplified by PCR using the IMAGE 3877506 clone obtained from the ATCC collection (Manassas, VA) and primers 5′-gga-gactctgtagcatcga-gc-3′ and 5′-atagtttagcggccgcatcatcctgg-3′, which add SacI and NotI restriction sites, respectively. The resulting product was purified and first cloned into pGEM-Teasy vector (Promega) and after into pQE-Trisystem (18). The resulting plasmid, pQE-IF1 that encodes IF1 with C-terminal His6 and streptavidin tags, was used to transform Escherichia coli BL21 cells. Protein expression was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. After overnight induction, the cells were collected, and the expressed protein was purified using nickel-nitritriacetic acid superflow resin (Qiagen) (18).

Monoclonal Antibody Production—To produce antibodies against human IF1, we proceed as described recently (18). In brief, BALB/c mice were immunized with various doses of the purified protein (20 μg) and the hybridomas produced by fusing spleen cells with the SP2 myelomas (18). Supernatants of the hybridomas were screened by indirect enzyme-linked immunosorbent assay on IF1-coated polystyrene plates. Bound antibodies were detected using horseradish peroxidase-labeled goat anti-mouse antibodies (1:1,000 DAKO, Carpinteria, CA) (18). The positive colonies were cloned by limiting dilution. Mouse monoclonal antibodies were purified with Montage antibody purification kit (Millipore, Billerica; MA).

Plasmid Constructs—The pCMV-SPORT6-IF1 plasmid containing human IF1 CDNA was used to generate the IF1-H49K mutant by standard techniques. In brief, two overlapping fragments, including the mutation, were amplified using pCMV-SPORT6-IF1 as template and the following primers: SP6 (atttagtgacatacatag) and H49K reverse (gaacagttcttctctgttgg), T7 (taatacgaacctctca) and H49K forward (aaccagaagagaatcctg). The final product was amplified, digested with EcoRI and NotI, and re-cloned into the pCMV-SPORT6 vector. The pTRE2hyg-IF1 and pTRE2hyg-H49K plasmids were generated from pCMV-SPORT6 constructs using BamHI and NotI restriction sites and pTRE2hyg (Clontech). The plasmid pTRE2hyg-Luc containing a luciferase gene (luc) was used as a control (Clontech). The pBI-L-IF1 and pBII-L-H49K plasmids were generated following standard cloning techniques from pCMV-SPORT6-IF1 and pCMV-SPORT6-H49K and the bidirectional plasmid pBII-L (Clontech). The pBII-GL plasmid contains the lacZ gene, which was used as a control (Clontech). All the constructs were checked by sequencing.

Cell Cultures, Transfections, and siRNA Silencing—Mouse hepatoma (Hepa 1–6), normal rat kidney (NRK), human embryonic kidney (HEK293T), human hepatocarcinoma (HepG2), breast (HS578T and T47D Tet-Off), colon (HCT116), lung (A549), and cervix (HeLa Tet-Off) carcinoma cells were cultured following the suppliers’ indications (ATCC and Clontech) up to 70–80% confluence. Transfection of NRK, T47D, HeLa, and Hepa 1–6 cells was performed using Lipofectamine and Plus Reagent (Invitrogen). Transfections of HepG2 cells were carried out with JetPEI (Polyplus transfections). Optimal transfection conditions were previously determined for each cell line. A plasmid encoding a mitochondrial version of green fluorescent protein (26) was co-transfected with the plasmid of interest at a 1:10 ratio to assess transfection efficiencies and for selection of cells in flow cytometry experiments. Harvested cells were lysed and the lysates cleared by centrifugation (26). Luciferase activity was determined in protein extracts with the luciferase assay system kit (Promega). Luminescence was registered using a FLUOstar OPTIMA (BMG Labtech) plate luminometer. siRNA (Qiagen S10098075) was used to suppress the expression of IF1 (23). An inefficient siRNA sequence, Silencer® Select Negative Control number 1 plasmid (Ambion/Applied Biosystems), was used as a control.

Determination of the Rates of Glycolysis and of Oxygen Consumption—Twenty four h after seeding the cells, the culture medium was replaced with fresh medium supplemented with 0.5% fetal bovine serum and with or without 6 μM oligomycin. Aliquots of the medium were collected at various times (up to 2 h), and the lactate content was determined enzymatically to verify a linear production rate of the metabolite (12). Cellular oxygen consumption rates were determined in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells were seeded in XF24-well cell culture microplates (Seahorse Bioscience) at ~70% confluence and incubated at 37°C and 7% CO2 for 24 h. The final concentration and order of injected substances was 6 μM oligomycin, 1 μM FCCP, 1 μM rotenone, and 1 μM antimycin.

Determination of the Mitochondrial Membrane Potential (ΔΨm)—Transfected cells were treated with 0.5 μM tetramethylrhodamine methyl ester (Molecular Probes) and processed for flow cytometry (26). In some experiments, cells were treated with or without 6 μM oligomycin (2 h) or 5 μM FCCP (30 min) before assessing tetramethylrhodamine methyl ester retention. The fluorescence intensity of at least 10,000 events was determined in a FACScan cytometer (BD Biosciences) using CellQuest (BD Biosciences) acquisition software. Data of the green population of transfected cells was analyzed with FlowJo software (TreeStar). To estimate ΔΨm, the fluorescent signal of the cells obtained with FCCP treatment was subtracted from the signals obtained without FCCP treatment.

Protein Electrophoresis and Western Blot Analysis—Proteins extracts were fractionated on SDS-PAGE, and the fractionated proteins were transferred onto polyvinylidene difluoride membranes. The primary antibodies used were as follows: rabbit
anti-β-F1-ATPase (1:20,000) and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Abcam, 1:20,000), anti-α-tubulin (Sigma, 1:2,500) and anti-IF1 (1:200) antibodies. Peroxidase-conjugated anti-mouse or anti-rabbit IgGs (Nordic Immunology, 1:3,000) were used as secondary antibodies. The mitochondrial expression of IF1 in carcinomas, cell lines, and silencing experiments was assessed relative to the mitochondrial β-F1-ATPase. In overexpression experiments, we used as loading controls the cellular markers glyceraldehyde-3-phosphate dehydrogenase and/or tubulin. The ECL system was used to visualize the bands. The intensity of the bands was quantified using a Kodak DC120 digital camera and the Kodak 1D Analysis software.

Statistical Analysis—Statistical analyses were performed using a two-tailed Student’s t test. Analysis of variance with post hoc Dunnett’s test was used for multiple comparisons to the control, using the SPSS 17.0 software package. The results shown are means ± S.E. The number of experiments (n) is indicated. A p < 0.05 was considered statistically significant.

RESULTS

The recombinant human IF1 protein was expressed in bacteria, purified from inclusion bodies (Fig. 1A), and used to immunize mice for the development of monoclonal antibodies. The expression of IF1 in normal human tissues was assessed relative to the expression of the mitochondrial β-F1-ATPase protein (Fig. 1B). Human tissues derived from the digestive track had the highest relative expression level of IF1 (Fig. 1B), whereas breast and lung expressed nondetectable levels of IF1 (Fig. 1B). The relative expression of IF1 in cell lines indicated a very large variability in the mitochondrial content of the ATPase inhibitor (Fig. 1C). Immunofluorescence microscopy revealed the mitochondrial localization of IF1 (Fig. 1D). Next, we analyzed the relative expression of IF1 (IF1/β-F1-ATPase ratio) in paired normal and tumor biopsies derived from breast, colon, and lung cancer patients (Fig. 1, E–H). Remarkably, in all human tissues analyzed carcinogenesis promoted a significant increase in the relative tumor content of IF1 (Fig. 1, E–H).

The augmented expression of IF1 in tumors (Fig. 1, E–H) suggested that the protein might regulate the energetic metabolism of aerobic cells by modulating the activity of the H⁺-ATP synthase. Therefore, cell lines displaying low basal levels of IF1 (Fig. 1C) were transiently transfected with control, IF1, or the mutant H49K plasmid, and the glycolytic flux was determined (Fig. 2). The NRK (Fig. 2A) and Hepa 1–6 (Fig. 2B) cell lines showed a significant increase in aerobic glycolysis when IF1 or the mutant H49K was overexpressed. Similar findings were

FIGURE 1. Expression of IF1 in human tumors. A, purification of recombinant IF1 (r-IF1). The gel shows protein extracts from noninduced (−) and induced (+) bacterial extracts and the purified recombinant IF1. B and C, IF1 and β-F1-ATPase (βF1) expression in different human tissues (B) and cell lines (C). The migration of the native IF 12-kDa isoform (n-IF1) is indicated. In the right panel, two different exposures of the IF1 film are presented. Cells with high and low IF1 content could be distinguished. D, immunofluorescence microscopy of HeLa cells stained with 200 nm MitoTracker (red) and with the IF1 monoclonal antibody (green) revealing the co-localization (Merge) of IF1 in mitochondria. Images are shown at ×63 magnification. Bar, 20 μm. E–H, Western blots of IF1 and β-F1-ATPase (βF1) in paired normal (N) and tumor (T) biopsies derived from three representative patients are shown. The histograms represent the fold of control of the IF1/βF1 ratio in ductal invasive breast (E, n = 9), colon (F, n = 12), and lung (G, n = 15) adenocarcinomas and squamous lung carcinomas (H, n = 7) relative to paired normal samples. *, p < 0.05 when compared with normal by Student’s t test.
Inhibition of the H⁺-ATP Synthase in Cancer

Determination of oligomycin-sensitive respiration in NRK cells transfected with IF1 showed that the oxygen consumption rates were significantly diminished when compared with controls (Fig. 3A), indicating that IF1 is interfering with the activity of the H⁺-ATP synthase. A more stringent inhibition of oxidative phosphorylation was noted when the cells expressed the pH-insensitive H49K mutant (Fig. 3A), consistent with the nonregulated binding activity of the mutant to β-F1-ATPase (22).

In normal aerobic conditions, the H⁺-ATP synthase utilized the H⁺ gradient for the synthesis of ATP. If the enzyme is inhibited, mitochondrial hyperpolarization ensues due to the interruption of the backflow of H⁺ into the matrix. Consistently, when any of the cell lines under study were treated with oligomycin, the mitochondrial membrane potential (ΔΨ m) increased (Fig. 3, B–D). Similarly, NRK (Fig. 3B) and Hepa 1–6 (Fig. 3C) cells transfected with IF1 or H49K showed significant increases in ΔΨ m when compared with control cells. The same studies in HepG2 cells using a bidirectional plasmid with a doxycycline-regulated promoter that expresses luciferase and any of the two transgenes confirmed that IF1 or H49K promoted an increase in ΔΨ m (Fig. 3D).

The siRNA-mediated silencing of IF1 in HeLa cells that naturally overexpress the inhibitor (Fig. 1C) promoted a large and significant reduction in the rates of aerobic glycolysis (Fig. 4A). Remarkably, oligomycin treatment of IF1-silenced cells restored the original rates of aerobic glycolysis (Fig. 4A), strongly supporting the link between the bioenergetic activity of mitochondria and glucose consumption rates (12, 15). Consistently, the determination of the activity of oxidative phosphorylation indicated that IF1-silenced cells had a higher oligomycin-sensitive respiratory rate than controls (Fig. 4B).

**DISCUSSION**

We show for the first time that human carcinomas have an increased expression of IF1. We support that the expression level of IF1 participates in the regulation of tumor energetic metabolism by controlling the synthase activity of the H⁺-ATP synthase. Indeed, cells that overexpress IF1 or H49K show a remarkable increase in their rates of aerobic glycolysis and a significant decrease in the activity of oxidative phosphorylation concurrent with an increase in ΔΨ m. All these findings are consistent with IF1 interfering with the activity of the H⁺-ATP synthase as illustrated by the results obtained when using the H⁺-ATP synthase inhibitor oligomycin. Moreover, the silencing of IF1 resulted in a sharp reduction in the rates of aerobic glycolysis and the stimulation of oxidative phosphorylation. These findings are of utmost importance for understanding the metabolism of cancer cells (9, 27).
independent mechanisms affect the overall bioenergetic activity of the H+-ATP synthase in cancer. Two of them limit the tumor content of the catalytic β-F1-ATPase (7, 9, 12) either by masking the translation of the transcript (34, 35), as recently shown in breast, colon, and lung tumors (24), or by limiting the amount of β-F1-ATPase mRNA after hypermethylation of the promoter of the ATP5B gene in leukemia (36). The third mechanism limits the activity of the complex and is triggered by increasing the mitochondrial content of IF1 as described in this study.

It is worth mentioning that IF1 not only plays a role in limiting oxidative phosphorylation and thus in promoting glycolysis. Glycolysis is known to provide an advantageous phenotype that favors cellular proliferation and invasion (15, 37). Moreover, limiting the activity of the H+-ATP synthase also contributes to tumor growth because oxidative phosphorylation is required for the efficient execution of cell death (38–40). We suggest that studies aimed at characterizing the mechanisms that regulate IF1 expression in cancer as well as the basic cell biology of the protein will provide promising targets to halt disease progression.

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