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Overexpression of the Inhibitor Protein IF_1 in AS-30D Hepatoma Produces a Higher Association with Mitochondrial F_1F_0 ATP Synthase Compared to Normal Rat Liver: Functional and Cross-Linking Studies

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According to functional studies, the higher IF_1 content reported in mitochondria of cancerous cells is supposed to induce a higher association with the F_1F_0 complex than in normal cells and therefore a better inhibition of its ATPase activity. The first structural evidence supporting this prediction is here presented. Densitometric analyses of Western blotting experiments indicated a 2-fold increase in IF_1 content of AS-30D submitochondrial particles compared to normal rat liver controls. The ratio of IF_1/F_1 α subunit increased similarly as judged by Westernblot analyses. This IF_1 overexpression correlated with a slower rate of IF_1 release (F_1F_0-ATPase activation) from the F_1F_0 complex in AS-30D than in normal rat liver submitochondrial particles. The IF_1-IF_1, γ-IF_1, and α-IF_1 cross-linkages previously formed with dithiobis(succinimidylpropionate) in bovine F_1F_0I and IF_1 complexes (Minauro-Sanmiguel, F., Bravo, C., and García, J. J. (2002). *J. Bioenerg. Biomembr*. **34**, 433–443) were reproduced in the F_1F_0I-ATP synthase of hepatoma AS-30D cells. However, a much lower yield of IF_1 cross-linkages was found in normal rat liver particles which made them almost undetectable in SMP as well as in the immunoprecipitated F_1F_0I complex. Modeling *in vivo* IF_1 overexpression of cancerous cells by *in vitro* reconstitution of excess recombinant IF_1 with rat liver submitochondrial particles devoid of IF_1 reproduced the same IF_1 cross-linkages observed in AS-30D particles.

KEY WORDS: Inhibitor protein; IF_1; F_1F_0-ATP synthase; cancer; cross-linking; AS-30D.

INTRODUCTION

The mitochondrial F_1F_0 complex synthesizes most of the ATP required by the cell by using Mg^{2+} ADP and Pi as substrates. Because it is a reversible rotary motor enzyme, it can synthesize or hydrolyze ATP according to the working conditions. Thermodynamically, the enzyme is biased to ATP synthesis by the proton motive force ($\Delta\mu_{H+}$) generated by the respiratory chain. The F_1F_0-ATP synthase is also driven by the constant supply of ADP and Pi by adenine nucleotide (ANT) and Pi carriers, respectively. Furthermore, a recent report indicates the presence of an ATP synthasome supercomplex containing F_1F_0, ANT, and Pi carriers (Ko *et al.*, 2003) which ensures a more efficient ATP synthesis turnover. As a further mechanism to prevent ATP hydrolysis, the mitochondrial enzyme contains an inhibitor protein (IF_1) which arrests the forward and backward functioning of

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Abbreviations used: CAPS, 3-(Cyclohexylamino)-1-propanesulfonic acid; DSP, dithiobis(succinimidylpropionate); DTT, dithiothreitol; EDTA, ethylenediamine-tetraacetic acid; F_1, the soluble F_1-ATPase; F_1I, the complex of soluble F_1-ATPase containing the inhibitor protein; F_1F_0I, the native ATP synthase containing the inhibitor protein; IF_1, the inhibitor protein of the mitochondrial ATP synthase; mAb, monoclonal antibody; SDS-PAGE, denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis; SMP, submitochondrial particles; WB, Westernblot.

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the enzyme when the $\Delta\mu_{H+}$ drops (Gómez-Puyou *et al.*, 1979; Harris *et al.*, 1979; Pullman and Monroy, 1963; Schwerzmann and Pedersen, 1986). Re-establishment of $\Delta\mu_{H+}$ releases the inhibitory action of IF_1 (Harris *et al.*, 1979; Gómez-Puyou *et al.*, 1979) and favors ATP synthesis. Therefore, *in vivo* IF_1 produces a net inhibition of ATP hydrolysis. Physiologically, IF_1 protects the heart, and presumably other tissues, from ischemia-reperfusion injury by preventing cellular ATP depletion (Ala-Rämi *et al.*, 2003; Rouslin, 1991). IF_1 inhibits more efficiently the F_1F_0-ATPase in submitochondrial particles isolated from ischemic hearts in comparison with nonischemic controls (Ala-Rämi *et al.*, 2003; Rouslin, 1991). As a further tissue specific regulation, the expression of IF_1 relative to ATP synthase is higher in the heart than in other organs (Rouslin and Broge, 1996). This is in accordance with the high energy demand and fast regulation required by the heart. In contrast, it has been shown that in a more glycolytic tissue like rat liver, the IF_1 is subexpressed relative to the ATP synthase (Schwerzmann *et al.*, 1982), and therefore the ATPase activity of the rat liver F_1F_0 is higher.

On the other hand, several reports have shown that IF_1 is overexpressed in mice (Capuano *et al.*, 1997; Chernyak *et al.*, 1991; Luciakova and Kuzela, 1984) and human (Chernyak *et al.*, 1994) cancerous cells. On the basis of F_1F_0-ATPase determinations, it has been proposed that the excess of IF_1 should prevent ATP hydrolysis more efficiently in cancerous cell lines than in normal cells (Chernyak *et al.*, 1991). Since many cancer cell lines exhibit a high glycolytic capacity, it has been concluded that IF_1 inhibits hydrolysis of the glycolytic ATP in these cells (Chernyak *et al.*, 1991). However, there is no structural evidence supporting a better productive interaction of IF_1 with the ATP synthase in cancerous cells. In this work we developed a new method to determine the productive association of the endogenous IF_1 with the ATP synthase in submitochondrial particles (SMP). The AS-30D cell line from hepatocellular carcinoma origin (Chang *et al.*, 1967; Smith *et al.*, 1970) was used in parallel to normal rat liver to isolate mitochondria and SMP. We took advantage of previous cross-linking studies of IF_1 with rotor (γ and ε) and stator (α) subunits of the ATP synthase that we described before with bovine heart SMP (Minauro-Sanmiguel *et al.*, 2002). We found that the higher expression of IF_1 in rat AS-30D hepatoma compared to rat liver produces a better productive association of IF_1 with the F_1F_0 complex. This was demonstrated by greater IF_1-rotor/stator cross-linking yields as well as by ATPase activity assays.

MATERIALS AND METHODS

Rat liver mitochondria and digitonin-extracted mitochondria from AS-30D hepatoma were obtained as described before (Moreno-Sánchez, 1985; Moreadith and Fiskum, 1984). "Mg-ATP" submitochondrial particles (SMP) containing the endogenous IF_1 were prepared as described elsewhere (García *et al.*, 1995). The endogenous IF_1 was released from the F_1F_0-ATPase of SMP by incubation in "Activation Media" containing Sucrose 125 mM, Tris-SO_4 60 mM, EDTA 2 mM, KCl 100 mM, and ATP 10 mM, pH 8.0. SMP were incubated at 40°C and aliquots were withdrawn at indicated times for spectrophotometric measurement of ATPase activity (García *et al.*, 1995). Activity measurements of liver and hepatoma SMP were carried out immediately after SMP preparation avoiding cycles of freezing and thawing. This procedure improved the stability of IF_1 inhibition and the maximal ATPase activity obtained after activation. SMP devoid of IF_1 were obtained by sonication and incubation in Activation Media as described elsewhere (Klein *et al.*, 1982).

Cross-Linking of IF_1 With Subunits of the F_1F_0I Complex in SMP

Cross-linking experiments were carried out with DSP (Pierce) in Sucrose-phosphate buffer as described before (Minauro-Sanmiguel *et al.*, 2002). Bovine SMP were incubated at a concentration 1 mg/mL; liver and hepatoma SMP were cross-linked at a final concentration of 1.8 mg/mL, with the indicated concentrations of DSP. The reaction was carried out for 30 min at room temperature and arrested with 20 mM *L*-lysine.

Immunodetection of Cross-Linked Subunits

SDS-PAGE was carried out under nonreducing (without DTT) and reducing (+20 mM DTT) conditions. Afterwards, proteins were transferred 2 h to transfer membranes at 100 mA in a buffer containing 100 mM CAPS, 10% methanol, pH 11.0. Monoclonal antibodies (mAb) raised against recombinant rat liver IF_1 or bovine α subunit (García *et al.*, 1995) were used. Reactive bands were immunodetected with secondary goat antimouse IgG conjugated to HRP and the chemiluminescent ECL-Plus kit of Amersham-Pharmacia.

Purification of Recombinant Rat Liver IF_1 and Reconstitution Into Liver SMP

Sequence of the rat liver ATPase inhibitor protein was excised form the pMalIF1 fusion vector (kindly provided originally by Prof. Pedersen from Johns Hopkins University School of Medicine, Baltimore, USA)

using the synthetic oligonucleotides N1 (GGAATTC-CATATGGGGTCGGACTCG) to insert a NdeI site at the 5′ region of the sequence and the Cl (CC-CAAGCTTTCAATGCTCACTATT) to insert a HindIII restriction site at the 3′ region of the sequence. The PCR generated fragment was restricted with the NdeI and HindIII restriction enzymes and subcloned into a pIN-III-A3 derivated IPTG inducible plasmid (Inouye and Inouye, 1985). The plasmid generated this way was transformed into the *Escherichia coli* BL21 strain for expression.

Protein Overexpression

The protein was overexpressed according to (Zheng *et al.*, 1993). The cultures were started by the addition of 2 mL of an overnight grown culture to a 500 mL LB medium. After 3 h of incubation (or Abs 600 nm = 0.5) at 37°C, the protein expression was induced by the addition of 0.4 mM IPTG, the incubation was continued overnight. The cells were harvested by centrifugation at 5000 rpm and washed twice with 100 mL saline solution. After overnight IPTG induction, cells were broken by sonication followed by DNAse treatment. Cell debris were collected by 15,000 rpm centrifugation at 4°C and the supernatant was precipitated with 50% ammonium sulfate. After a second centrifugation, the supernatant was precipitated with 80% ammonium sulfate. The pellet was resuspended in 20 mM KH_2PO_4 buffer pH 8.0 and IF_1 was concentrated in Amicon membranes (10 kDa cutoff). Afterwards, pH was readjusted to 6.5 and the concentrated sample was loaded into a 3 mL CM-Sepharose column preequilibrated in the same buffer. IF_1 was eluted with a gradient of 20–600 mM of KH_2PO_4 and simultaneously a pH gradient of 6.5–8.7. The IF_1 sample was concentrated and desalted as described above and stored at −80°C until used. The purity of the sample was higher than 90% as judged by SDS-PAGE, Coomassie staining, and densitometry analyses (not shown). Pure recombinant IF_1 was reconstituted with liver SMP devoid of IF_1 as described by Lebowitz and Pedersen (1993).

Other Methods and Materials

The amount of protein in SMP, F_1F_0I, and F_1I preparations was measured with the method of Lowry *et al.* (1951). Densitometric analyses of Coomassie stained bands in SDS PAGE gels were carried out with the digital camera and imaging software "ALPHADIGIDOC" of Alpha Innotech Corporation according to the manufacturers instructions. In each gel the molecular weights of cross-linking products were calculated from interpolation using the appropriate standards. The intensities of subunits were measured by peak integration after densitometry analyses. Coupling enzymes and substrates for ATPase activity were obtained from Sigma. Reactants for SDS PAGE were obtained from Bio-rad laboratories.

RESULTS

Several reports have shown a higher expression of IF_1 in cancerous cells in comparison with normal cells. In order to compare the amount of IF_1 expressed in normal and cancerous cells, we designed a Western-blot titration approach in which loading of 1–50 ng of pure recombinant IF_1 resulted in a linear increase in band intensity as calculated from densitometry analyses. Higher amounts of IF_1 saturated the chemiluminescent signal of the detection system (not shown). Therefore, we interpolated the intensities of the IF_1 band obtained in normal and hepatoma SMP that were in the linear range of the titration curve. After several preparations and experiments carried out with different amounts of loaded protein, we found that the amount of IF_1 in hepatoma was about twice of that found in normal SMP from rat liver (Fig. 1). The amount of IF_1 estimated in liver and hepatoma SMP were 0.55 ± 0.11 and 0.26 ± 0.04 nmol/mg, respectively. This value is higher than that previously reported by Schwerzmann *et al.* (1982) for whole mitochondria. This might reflect the increase in IF_1 content in SMP relative to mitochondria since according to these authors IF_1 is mostly associated to the inner mitochondrial membrane.

In order to assay the functional association of IF_1 with the ATP synthase in SMP from AS-30D hepatoma and liver, F_1F_0-ATPase activation assays were carried out. SMP were incubated in conditions that induce a progressive release of IF_1 to the media, i.e., pH 8.0, KCl 100 mM, and 40°C. At $t = 0$ before activation, the F_1F_0-ATPase of AS-30D SMP showed about half of the basal F_1F_0-ATPase activity of the normal rat liver enzyme (Fig. 2(A), $t = 0$). Time courses revealed that the F_1F_0-ATPase from AS-30D SMP was activated with a slower rate than the control enzyme of SMP (Fig. 2(A)). However, both SMP reached similar maximal F_1F_0-ATPase activities, although the enzyme from AS-30D hepatoma had a lower maximal ATPase rate. These results strongly suggest that in comparison with normal liver mitochondria, the higher expression of IF_1 in AS-30D hepatoma results in a better yield of productive association of IF_1 with the F_1F_0 complex. Furthermore, the total amount of F_1F_0-ATPase seems similar in AS-30D and normal liver mitochondria. According to maximal ATPase rates, if there is a difference in the F_1F_0-ATPase content in both systems, that should consist of a slightly lower amount of

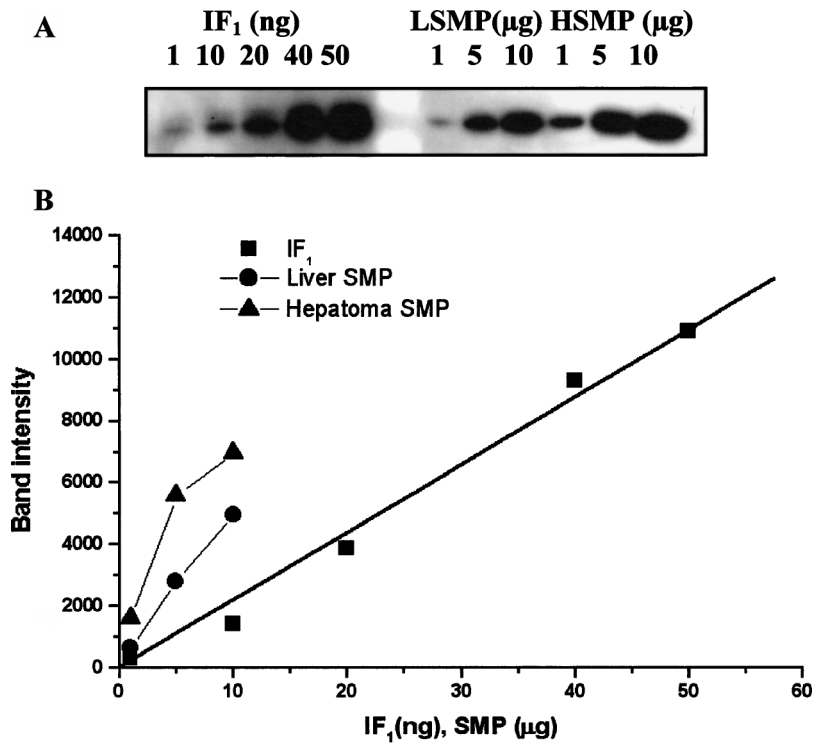

Fig. 1. Amount of IF_1 in liver and hepatoma SMP with Westernblot and densitometry analyses. (A) The shown amounts of recombinant rat liver IF_1, liver (LSMP), and hepatoma (HSMP) submitochondrial particles were loaded on SDS-PAGE. Denaturing electrophoresis and WB were carried out as indicated in Materials and Methods with a mAb anti-IF_1. The two clear spots in the center of the immunoblot membrane correspond to 7 and 14 kDa molecular weight standards which are negatively developed with the chemiluminescent kit, therefore estimated the size of the IF_1 band is 10 kDa, the expected size of IF_1. (B) Densitometry analyses of each band was carried out as described in Materials and Methods. In the range of 1–50 ng of pure IF_1, the band intensity increased linearly. With higher IF_1 amounts the band intensity diminished due to signal saturation (not shown). The content of IF_1 in SMP was estimated with interpolation of the observed band intensities obtained with known amounts of SMP protein in this linear range of IF_1 titration.

F_1F_0-ATPase in AS-30D SMP. To confirm this, we carried out a simultaneous immunodetection of IF_1 and the α subunit of F_1. Densitometry analyses (Fig. 2(B)) showed that the relative amounts of α subunit was slightly lower in AS-30D SMP than in control rat liver particles (α AS-30D/α liver $= 0.9$). However, the ratio of IF_1/α intensities increased in AS-30D hepatoma ($IF_1/\alpha = 1.2$) relative to normal rat liver SMP ($IF_1/\alpha = 0.8$). These observations are in concordance with previous reports where the F_1F_0-ATPase was found reduced in rat hepatoma cells (Kuzela *et al.*, 1977). Furthermore, the β subunit was also found diminished in mitochondria derived from human cancer cells (Capuano *et al.*, 1996, 1997). Taken together, the functional and WB analyses show that IF_1 is indeed overexpressed in AS-30D cells in relation to ATP synthase expression leading to a better inhibiton of the F_1F_0-ATPase in cancer cells.

To obtain structural evidence for the association of IF_1 with the ATP synthase, we carried out DSP cross-linking of IF_1 with rotor (γ, ε) and stator (α) subunits of the F_1F_0 complex as we described before with the bovine heart enzyme (Minauro-Sanmiguel *et al.*, 2002, and Fig. 3). SMP from liver and AS-30D hepatoma were incubated with DSP as described in Materials and Methods and cross-linking was arrested with L-Lysine. Afterwards, aliquots containing 30 μg of protein were loaded into SDS-PAGE with or without reduction by DTT. Figure 3(B) shows that in the SMP from normal liver, essentially no cross-linked products were obtained. However, in the SMP from hepatoma, three cross-linking products were clearly defined of about 43, 46, and 67 kDa, according to computer calculations carried out as described in Materials and Methods. The products of lower molecular weight above IF_1 correspond to ε-IF_1 and IF_1-IF_1 cross-linkages

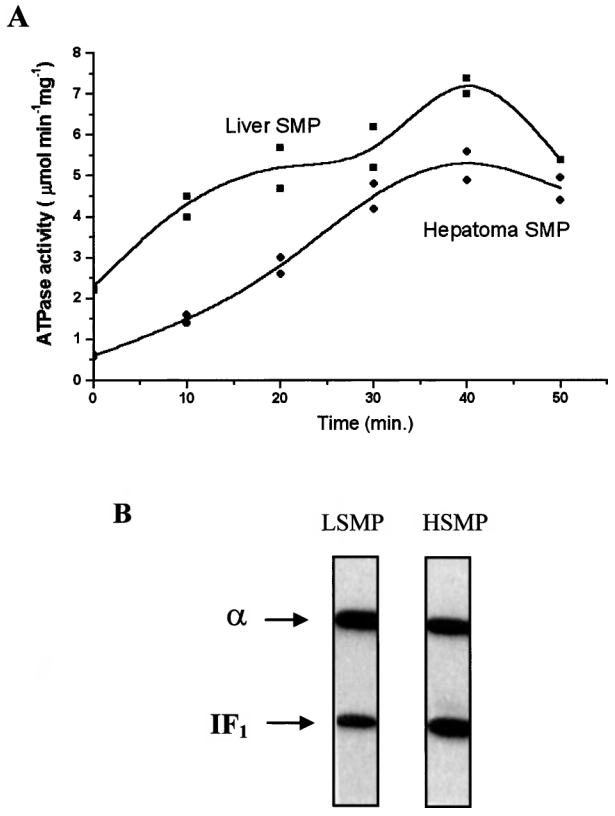

Fig. 2. Time course of F_1F_0-ATPase activation of liver and hepatoma SMP under conditions of IF_1 release. (A) Freshly prepared liver and hepatoma SMP were incubated at $t = 0$ in activation media as explained in Materials and Methods. At the times shown, 5 μL aliquots carrying 5 μg of protein were withdrawn for spectrophotometric measurement of ATPase activity. Basal activity at $t = 0$ was not modified by incubation of SMP in dilution media (see Materials and Methods) by 50 min. (B) Five micrograms of liver (LSMP) and hepatoma (HSMP) submitochondrial particles were loaded into SDS-PAGE and afterwards, Western blot was carried out as indicated in Materials and Methods with a mixture of anti-α and anti-IF_1 mAbs. Densitometry analyses showed the following band intensities in LSMP ($IF_1 = 1724$, $\alpha = 2440$) and hepatoma ($IF_1 = 2604$, $\alpha = 2223$) therefore, the IF_1/α ratios were 0.8 and 1.2 for liver and hepatoma SMP, respectively.

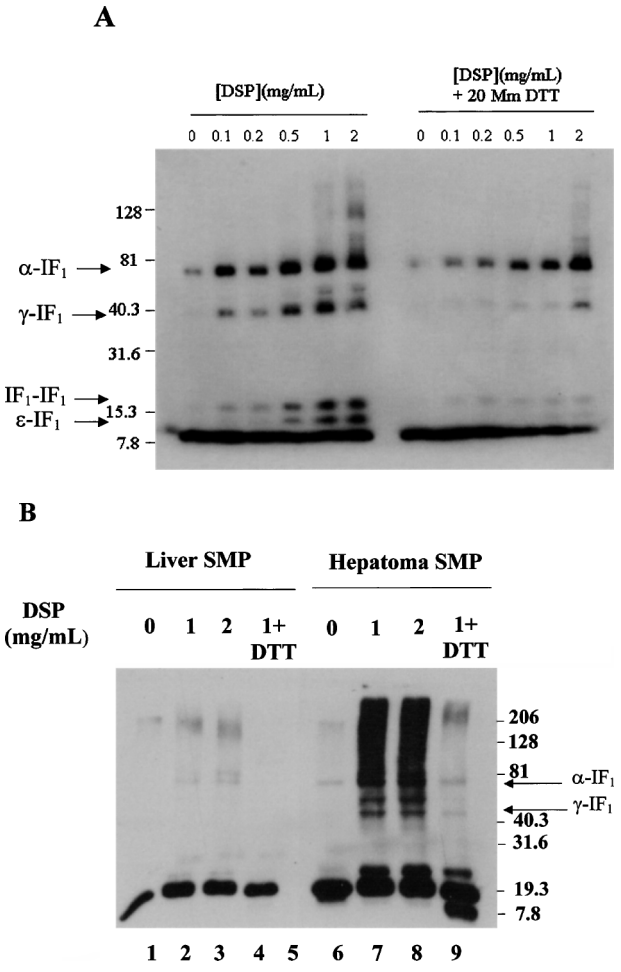

Fig. 3. IF_1 DSP cross-linking with ATP synthase subunits in bovine heart, rat liver, and rat hepatoma SMP. (A) Bovine heart SMP (1 mg/mL) were incubated by 30 min with the indicated amounts of DSP and cross-linking was arrested with 10 mM L-lysine. Afterwards, aliquots were withdrawn to load 20 μg of SMP protein in each lane of SDS-Page and anti-IF_1 Westernblot was carried out as described before. The previously identified α-IF_1 and γ-IF_1 products were observed besides a third 46 kDa product observed between them with 1 and 2 mg/mL of DSP. The presence of DTT reduced most of the γ-IF_1 and 46 kDa products as well as two smaller products that correspond to ε-IF_1 and IF_1-IF_1 cross-linkages (see Minauro-Sanmiguel *et al.*, 2002). (B) The same cross-linking, SDS-PAGE and WB procedures were followed with Liver and hepatoma SMP with the exception that the initial SMP protein concentration was 1.8 mg/mL and 40 μg of SMP protein were loaded per lane. The same α-IF_1, γ-IF_1, and 46 kDa products observed in bovine heart SMP (A) were also obtained in liver and hepatoma SMP. DTT (20 mM) reduced most of these products.

previously described (Minauro-Sanmiguel *et al.*, 2002), whereas the products of about 43 and 67 kDa correspond to γ-IF_1 and α-IF_1 cross-linkages that we found in the bovine heart enzyme. The identity of these products was confirmed with α and γ immunoblots, respectively (Minauro-Sanmiguel *et al.*, 2002). The product of about 46 kDa was not identified. The noncross-linked control of AS-30D SMP showed consistently about twice the intensity of the IF_1 monomer than in the normal liver SMP (Fig. 3(B), lanes 1 and 6). Formation of these cross-linked products was reversed by DTT reduction, except for the α-IF_1 product which, as in the bovine heart enzyme, was only partially reduced (Fig. 3(A)). Assuming that these products could be formed with very low yields in SMP from normal liver, further gels were overloaded and the same products showed as faint bands (not shown).

In order to discard the possibility that the IF_1 cross-linkages observed in AS-30D hepatoma SMP could result from nonspecific interactions, we carried out an *in vitro*

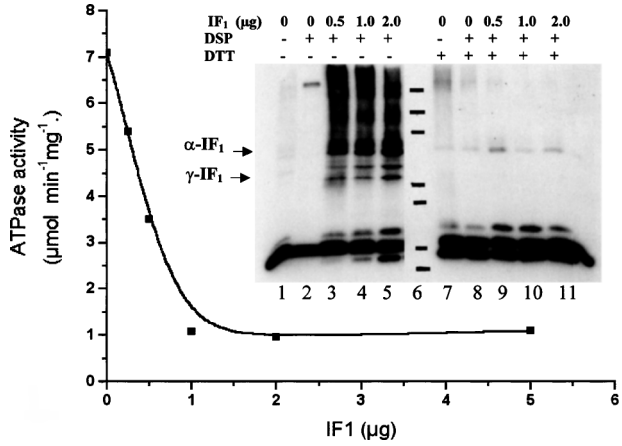

Fig. 4. Reconstitution of recombinant IF_1 into F_1F_0-ATPase of rat liver SMP. The shown amounts of IF_1 were reconstituted into liver SMP as indicated in Materials and Methods and 10 μl aliquots containing 4 μg of protein were withdrawn for measurement of ATPase activity. Inset, the remaining of SMP samples were centrifuged once at 45,000 rpm in order to remove excess nonreconstituted IF_1 and the SMP pellets were dissolved directly in SDS-PAGE sample buffer to load 60 μg of SMP. Where indicated, 20 mM DTT was added 10 min before gel loading. Anti-IF_1 Westernblot was carried out as described before.

reconstitution experiment as a model for the *in vivo* overexpression of IF_1 in cancer cells. Thus, recombinant rat liver IF_1 was reconstituted with SMP devoid of IF_1 before DSP cross-linking. Reconstitution produced about 90% inhibition of ATPase activity (Fig. 4) with an affinity similar to that previously reported by Lebowitz and Pedersen (1993) with recombinant rat liver IF_1. Western blotting of these reconstituted samples showed exactly the same DTT-sensitive cross-linking products as in the endogenous IF_1 overexpressed in cancer cells (Fig. 4, inset). As an additional control, we measured the IC_{50} of recombinant IF_1 for the rat liver and hepatoma F_1F_0 complex of SMP devoid of IF_1 by titration and reconstitution experiments in order to confirm that the enzyme from hepatoma retained a similar affinity for IF_1. We found the same IC_{50} of about 0.5 μg of IF_1 (Fig. 4) in liver and hepatoma SMP (not shown). Taken together, these experiments demonstrate structurally that the overexpression of IF_1 in AS-30D cells leads to a higher amount of endogenous IF_1 associated with the rotor and stator of the ATP synthase.

As a further effort to confirm that the observed IF_1-cross-linking products were formed within the intact F_1F_0I complex, the latter was immunoprecipitated as we described before for the bovine heart enzyme (Aggeler *et al.*, 2002; Minauro-Sanmiguel *et al.*, 2002). We obtained about half the yield of immunoprecipitated F_1F_0 from hepatoma than from liver SMP, consistent with the lower amount of ATP synthase in the former system. DSP cross-linking decreased even further the yield of F_1F_0I complex. However, after loading about 5 μg of immunoprecipitated enzyme, the γ-IF_1 and α-IF_1 cross-linkages were detected almost exclusively in the enzyme immunoprecipitated from hepatoma SMP (Fig. 5). These results confirm that IF_1 is associated with higher yield in the F_1F_0I complex of hepatoma than in that of normal rat liver mitochondria.

DISCUSSION

This work shows the first structural evidence demonstrating that overexpression of the inhibitor protein (IF_1) in cancer cells produces a higher amount of association with the mitochondrial F_1F_0-ATP synthase than is found in normal cells. We developed a new approach of DSP cross-linking useful to assay the degree of association of IF_1 with the native ATP synthase in SMP. Furthermore, Westernblot titration experiments allowed estimation of a 2-fold relative increase of IF_1 expression in mitochondria and SMP isolated from AS-30D cells relative to normal rat liver cells. Coimmunodetection of α and IF_1 subunits demonstrated that this overexpression is not a parallel increase of the whole F_1F_0 subunits, but that IF_1 is over-expressed in relation to the ATP synthase. Furthermore, our functional (Fig. 2(A)), WB (Fig. 2(B)), and immunoprecipitation studies indicate a tendency of AS-30D cancer cells to subexpress the F_1F_0 ATPase. These results are in concordance with previous reports where a lower amount of F_1F_0-ATPase was observed in other cancer cells (Capuano *et al.*, 1996, 1997; Kuzela *et al.*, 1977). Previous careful estimations have shown that rat liver IF_1 is expressed with a stoichiometry of 0.4 IF_1 molecules per ATP synthase (Schwerzmann *et al.*, 1982). Therefore, our determinations of 2.0-fold increase of IF_1 expression indicate that this ratio in AS-30D cancerous cells could be increased to 0.8, or higher, recalling the apparent lower content of F_1F_0 in comparison with normal cells. Thus, as predicted from previous functional studies (Chernyak *et al.*, 1994), IF_1 associates to F_1F_0 with better efficiency in cancerous cells than in normal cells.

In functional studies, the release of IF_1 demonstrated by ATPase activation was slower in AS-30D SMP than in the controls, indicating that the F_1F_0I complex formed in AS-30D mitochondria contains a higher amount of endogenous IF_1 (Fig. 2). Since the IF_1 cross-linkages obtained in both systems were the same, it seems that only the yield of IF_1 cross-linking products was increased in AS-30D SMP. According to our IF_1 saturation curves, this

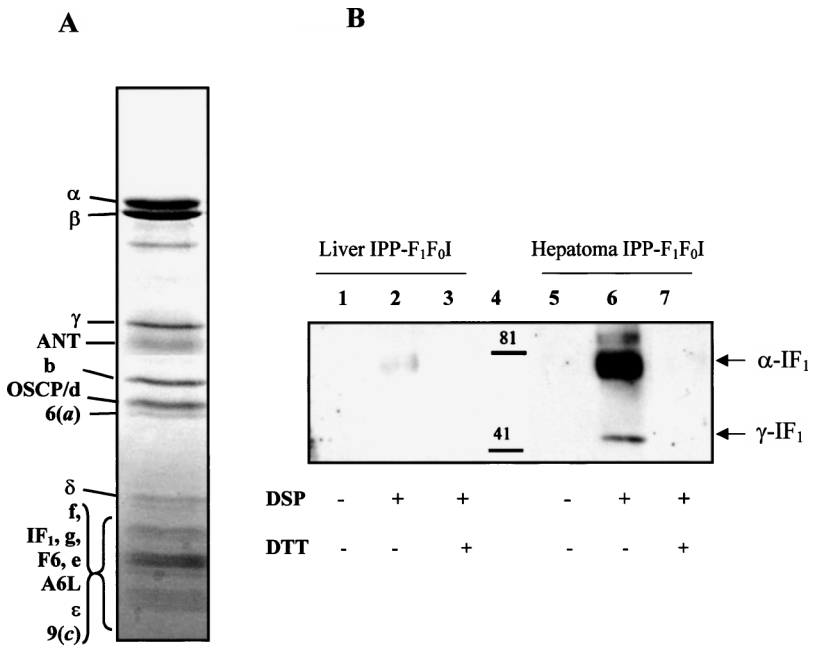

Fig. 5. Cross-linking and immunoprecipitation of the F_1F_0I complex from liver and hepatoma SMP. (A) Rat liver F_1F_0I complex was immunoprecipitated from liver SMP and the purity and subunit composition was analyzed by SDS-PAGE. The identity of subunits was confirmed previously for the bovine heart enzyme (Aggeler *et al.*, 2002) and subunit composition was assumed the same for the rat liver enzyme. (B) Before and after DSP cross-linking, liver and hepatoma F_1F_0I complexes were immunoprecipitated from the corresponding SMP. After determination of protein, 5 μg of immunoprecipitated F_1F_0 complexes were loaded per lane. The gel was over-run to improve resolution of IF_1 products. The main IF_1 band ran off from the gel. The γ-IF_1 and α-IF_1 products were detected in the F_1F_0I complex from hepatoma, but not in the enzyme immunoprecipitated from liver SMP. A faint band of α-IF_1 product was preserved in the F_1F_0I complex from liver SMP. No IF_1 product of 46 kDa was detected, however, the α-IF_1 and γ-IF_1 products were reduced by previous incubation with 10 mM DTT. The center empty lane contains molecular weight standards indicated as lines.

higher association of IF_1 with F_1F_0 in cancer cells is not the result of a shift in the affinity of the enzyme for IF_1. However, these results do not rule out structural differences among the F_1F_0I complexes of normal and AS-30D mitochondria. For example, the lower yield of immunoprecipitation of the F_1F_0I complex in AS-30D cancer cells might indicate structural differences with the normal rat liver F_1F_0I complex in addition to lower expression levels. We are in progress of site directed mutagenesis studies to identify the lysine residues of the rat liver IF_1 that form these cross-linking products by reconstitution of the recombinant IF_1 into rat liver SMP.

Taken together, these results provide structural and kinetic support to the previous proposals suggesting that cancer cells are protected more efficiently to prevent energy dissipation through the F_1F_0-ATPase activity since IF_1 is overexpressed and binds with higher efficiency to the enzyme.

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