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Fas/APO-1(CD95)-Induced Apoptosis of Primary Hepatocytes Is Inhibited by cAMP

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Fas/APO-1(CD-95) activation induced rapid apoptotic cell death of primary rat hepatocytes in suspension culture. Activators of cAMP-dependent protein kinase (glucagon and N^6-benzoyl-cAMP) protected against apoptosis, whereas the specific cAMP-kinase inhibitor (*Rp*)-8-Br-cAMPS enhanced Fas-induced death. The latter observation indicated that even the basal cAMP level may provide partial protection against Fas-induced hepatocyte apoptosis. Two-dimensional gel electrophoresis revealed decreased phosphorylation of several proteins in Fas-activated cells. Most of these dephosphorylations were attenuated or not observed in cells simultaneously stimulated by anti-Fas and cAMP, indicating a tight correlation between the dephosphorylations and death. Elevation of cAMP rescued the cells not only from the Fas-induced morphological changes and dephosphorylation, but also from functional deterioration. Whereas cells treated with anti-Fas alone quickly lost plating efficiency, hepatocytes co-treated with glucagon retained their ability to adhere and spread on a collagen substratum. © 1997 Academic Press

It has recently been demonstrated that a targeted mutation in the Fas gene causes liver hyperplasia (1), suggesting that the Fas/APO-1 (CD-95) system may be important in the normal physiological turnover of hepatocytes. Fas is a member of the tumor necrosis factor (TNF) / nerve growth factor receptor family (2), and is highly expressed in normal liver (3). Activating antibody against Fas (anti-Fas) induces massive hepatocyte apoptosis (4). The Fas signaling pathway is a major inducer of apoptotic cell death (5-7). Triggered trimerized Fas recruits specific intracellular proteins that bind and activate a pro-protease (FLICE), which in turn can initiate a proteolytic cascade of cysteine proteases (8-10). So far little is known about how this basic machinery is modulated. Several indices point to modulatory roles of protein phosphorylation in Fas- and TNF-induced apoptosis: the Fas- and TNF-associated serine/threonine kinase RIP can induce apoptosis (11, 12) and Fas-associated proteins (alternatively termed CAP 2,3, MORT1 or FADD) are known to exist in phosphorylated states (13). Additionally, elevation of ceramide has been implicated in Fas- and TNF-signaling (14, 15) and shown to activate a serine/threonine kinase as well as a serine/threonine phosphatase (16, 17). Moreover, protein kinase C has been shown to protect against Fas-induced apoptosis (18).

The cAMP pathway, activated by glucagon or the β-adrenergic component of epinephrine, is the major signal of metabolic stress to the hepatocyte. It was therefore considered important to know if activation of cAK, as can occur through metabolic stress in the intact animal, enhanced or protected against Fas-induced hepatocyte apoptosis. We have previously determined exact intracellular activation of cAK in freshly isolated rat hepatocytes in response to graded increases of glucagon (19) and more recently shown that the basal cAK activity can be decreased by the specific cAK inhibitor (*Rp*)-8-Br-cAMPS (20). In view of the indices that protein phosphorylation may modulate Fas-induced apoptosis we also investigated the protein phosphorylation pattern in hepatocytes exposed to anti-Fas in the absence and presence of elevated cAMP.

We here report that primary rat hepatocytes in suspension culture are highly responsive to anti-Fas, and that inhibition of cAK further enhances anti-Fas-induced apoptosis. Activation of cAK effectively protected primary hepatocytes from apoptosis induced by anti-Fas, both based on morphological criteria and long-term cell survival. We also show that cAMP partly counteracts the altered phosphorylation pattern (hypophosphorylation) in cells treated with anti-Fas. It will be concluded that cAK antagonizes anti-Fas action in mature hepatocytes both on the biochemical level (prevention of protein hypophosphorylation) and with regard to induction of apoptosis.

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MATERIALS AND METHODS

Chemicals. Rabbit polyclonal IgG antibodies (type M-20) against murine Fas (anti-Fas) were from SDS (Santa Cruz, USA). Okadaic acid was from Scientific Marketing Associates (Barnet, UK). N^6-benzoyladenosine 3′:5′-cyclic monophosphate (N^6-benzoyl-cAMP), 3-isobutyl-1-methyl-xanthine (IBMX), and collagenase (C-0130) were from Sigma (St. Louis, USA). (*R*p)-8-Br-cAMPS were from Biolog Life Science Institute (Bremen, Germany). Reagents for isotope labeling of hepatocytes, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and autoradiography were as previously described (20).

Incubation of primary hepatocytes in short term suspension culture. Hepatocytes were isolated and purified from young rats (male Wistar 80-110 g, fed *ad libitum*), by *in vitro* collagenase perfusion, filtration and low speed centrifugations (21). Cells were resuspended (2×10^6 cells/ml) in pre-gassed (5% CO_2/95% O_2) low-phosphate incubation buffer (120 mM NaCl, 5.3 mM KCl, 0.01 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 10 mM Hepes, (pH 7.4) supplemented with 5 mM lactate and 5 mM pyruvate) and kept in suspension by gyratory shaking (175 rev./min, 37°C). The cells were routinely supplemented with cycloheximide (10 μg/ml) from the time of isolation.

Evaluation of apoptotic morphology. Cell aliquots were fixed in 10 fold excess of 2 % glutaraldehyde buffered with 0.1 M Na-cacodylate (pH 7.4). For routine assesment of apoptosis, cell morphology was evaluated by inverted phase microscopy. Apoptotic cells were easily discriminated from non-apoptotic cells by the appearance of surface protrusions. For each determination (% apoptotic cells) at least 300 cells were evaluated. Differential interference contrast microscopy (DIC) was performed as described by Bøe *et al* (22). For ultrastructural analysis the hepatocytes, fixed in 2% glutaraldehyde, were washed in Na-cacodylate buffer, and post-fixed in the same buffer supplemented with 2% OsO_4. The cells were dehydrated in graded alcohol solutions and embedded in Agar 100 resin essentially as described previously (22). Ultrathin sections were stained in uranyl acetate and lead citrate and viewed in a Phillips 300 electron microscope.

Determination of hepatocyte plating efficiency. One parameter of hepatocyte intactness after various treatments in suspension was the ability to attach to and spread on collagen-coated dishes (23). Cell aliquots, washed twice in 25 volumes of incubation buffer, were resuspended and seeded in a defined (synthetic, serum free) culture medium (24) supplemented with 5 nM dexamethasone and 0.2 nM insulin. The medium efficiently supported attachment, spreading and DNA replication of control hepatocytes. After 48 hr of culture the hepatocytes were fixed in 2% glutaraldehyde. The density of attached cells was determined in defined squares of the dishes using phase contrast microscopy.

Two-dimensional gel electrophoresis of extract from $^{32}P_i$-prelabeled hepatocytes. Hepatocytes were preincubated with $^{32}P_i$ (500 μCi /ml) for 45 min to obtain a steady state specific activity of the γ-phosphate of endogenous ATP (25). Thereafter the cells were exposed to various potentially apoptosis modifying agents for 40 minutes in a volume of 100 μl. The reactions were terminated by addition of 10 vol. ice-cold 8% aqueous trichloroacetic acid. The precipitates formed were spun (15,000 × g for 15 min), resuspended in 1 ml of 5% trichloroacetate and re-spun. The pellets were extracted with 1 ml ice-cold ether, air-dried, and dissolved in 100 μl lysis buffer (9.8 M urea, 100 mM dithioerythritol, 1.5% Pharmalyte pH 3.5-10, 0.5% Pharmalyte pH 5-6, 4% CHAPS, 0.2 % SDS in 50 mM Tris/HCl (pH 8.7). Isoelectric focusing (135 kVh) was performed in linear immobilized pH gradients (IPG strips, pH range 4.0-7.0; Pharmacia Biotechnology, Uppsala, Sweden). The strips were subjected to SDS-gel electrophoresis in the second dimension essentially as described previously (20).

All pictures shown (including light- and electron microscopic work) were made by scanning original negatives or autographic films with an Afga Arcus II flatbed scanner and processed using Adobe Photoshop software.

RESULTS

Induction of Apoptosis by Anti-Fas in Primary Rat Hepatocytes

Apoptotic effect of anti-Fas on primary cultured hepatocytes has been reported only in mouse (18) and human (26) cells. In the present study freshly isolated hepatocytes from rat were used as target for anti-Fas-action. Cells were kept in rotatory suspension culture up to three hours and were scored for apoptosis by morphology. The highest sensitivity to anti-Fas was noted for hepatocytes isolated from young (less than 120 g) rats. Hepatocytes pretreated with cycloheximide had a slight, but significantly enhanced sensitivity to anti-Fas-induced apoptosis. For these reasons hepatocytes were isolated from 80 -110 g rats and incubated with 10 μg/ml of cycloheximide throughout the study. The morphological effects induced by anti-Fas, observed after 1.5-2 hr of incubation, were characteristically apoptotic (Fig.1B,F): initial shrinkage of cell volume, loss of microvilli, vacuolization, surface budding, and condensation of nuclear chromatin. The mitochondria and the rough endoplasmic reticulum moved closer to the nucleus whereas numerous vacuoles were found close to the plasma membrane (Fig.1F). Less than 3% of the control (vehicle supplemented) cells underwent spontaneous apoptotic cell death during the experimental period. Non-apoptotic cells (Fig.1A,C) tended to adhere to each other forming increasing number of doublets and small aggregates as a function of incubation time, whereas apoptotic cells remained single (Fig.1B,D).

Anti-Fas-Induced Apoptotic Death Was Blocked by Activators of cAK and Enhanced by cAK Inhibitor

The hydrolysis-resistant cAMP agonists N^6-benzoyl-cAMP (Fig.1C,G,H) and (*S*p)-cAMPS (data not shown) protected hepatocytes from anti-Fas-induced apoptosis, the percentage of apoptotic cells decreasing from 45 ± 4 (mean ± s.e.m.) to 7 ± 1 with 100 μM N^6-benzoyl-cAMP (Fig.1H). Both substances are known to mimick the action of microinjected pure catalytic subunit of cAK in rat hepatocytes (20). Since these activators of cAK protected against anti-Fas, it was tested whether inhibition of the kinase could enhance anti-Fas-induced apoptosis. (*R*p)-8-Bromo-cAMPS, known to antagonize activation of cAK in hepatocytes and other cells (20), did in fact enhance apoptosis induction by anti-Fas (Fig.1D,H). This means that even the modest basal cAK activity of about 18% in isolated hepatocytes (19) must be enough to inhibit somewhat the anti-Fas-

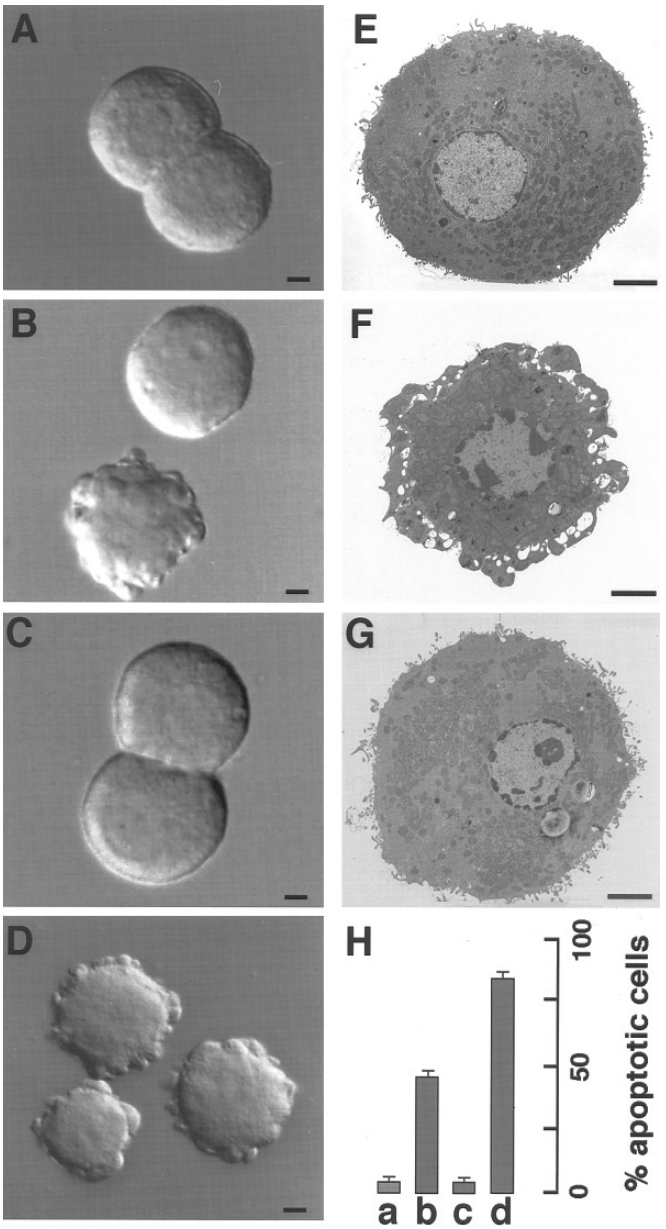

FIG. 1. Anti-Fas-induced hepatocyte apoptosis is oppositely modulated by agonistic (N^6-benzoyl-cAMP) and antagonistic ((*R*p)-8-Br-cAMPS) analogs of cAMP. Panel A shows a differential interference contrast micrograph and panel E an electron micrograph of normal (control) hepatocytes in suspension culture. As shown in column a of panel H less than 3% of control cells had apoptotic morphology. Panels B,F show cells treated for 2 hr with 15 μg/ml anti-Fas, which led to about 50% apoptosis (column b of panel H). Panels C,G show cells treated with 15 μg/ml anti-Fas + 100 μM N^6-benzoyl-cAMP. The addition of N^6-benzoyl-cAMP nearly abolished the apoptogenic effect of anti-Fas (column c of panel H). Panel D shows cells treated with 15 μg/ml anti-Fas + 300 μM (*R*p)-8-Br-cAMPS, which increased the number of apoptosis to about 80% (column d of panel H). Bar, 3 μm.

induced apoptosis. In order to prove that physiologically relevant changes in cAMP level could regulate anti-Fas-induced hepatocyte death, the cells were exposed to concentrations of glucagon which only moderately elevated endogenous cAMP in hepatocytes (19). Glucagon protected hepatocytes against anti-Fas-induced apoptosis with half-maximal effect observed at 1.3 nM glucagon (Fig.2). These data strongly suggest a role for cAMP in modulating anti-Fas-mediated hepatocyte death.

It was tested whether cAMP could protect the hepatocytes against other indices of death than loss of morphological integrity. The ability of hepatocytes to attach to and spread on collagen coated dishes was lost after a short exposure to anti-Fas (Fig.3A,C). Only 10 min of treatment with anti-Fas was enough to decrease the ability to attach by 50%. This means that irreversible loss of plating efficiency occurs prior to the onset of morphological effects typical of anti-Fas-induced apoptosis. Glucagon (and N^6-benzoyl-cAMP) protected cells from anti-Fas-induced loss of plating efficiency (Fig.3A,D). The cells treated with anti-Fas and glucagon that were able to attach normally also had normal morphology after more than 2 days in monolayer culture (Fig.3D).

Cyclic-AMP Antagonizes Anti-Fas-Induced Dephosphorylation of Hepatocyte Proteins

Anti-Fas specifically affected the phosphorylation of several hepatocyte proteins. Effects on protein phosphorylation were observed after exposure to anti-Fas for 15 min, when several proteins were moderately hypophosphorylated and others hyperphosphorylated (data not shown). After 40 minutes of incubation the most prominent feature was selective protein dephosphorylation, only a few proteins remaining hyperphosphorylated (Fig.4B). The modulations of protein phosphorylation by anti-Fas were found prior to the alter-

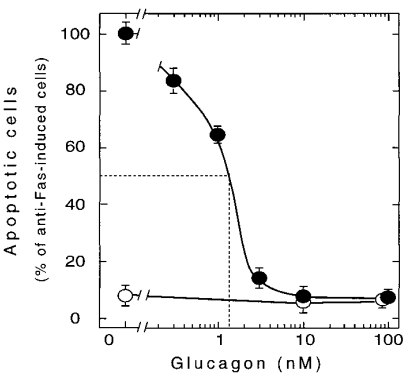

FIG. 2. Anti-Fas-induced apoptosis is inhibited by low nM concentrations of glucagon. Hepatocytes were incubated for 2 hr in modified Krebs-Ringer solution with 50 μM isobutylmethylxanthine (IBMX) and various concentrations of glucagon in the absence (○) or presence (●) of 15 μg/ml anti-Fas. IBMX by itself did not affect anti-Fas-induced cell death which was 45± 4% in the absence of IBMX and 44± 5% in its presence. Symbols represent the mean ± s.e.m. of three separate experiments.

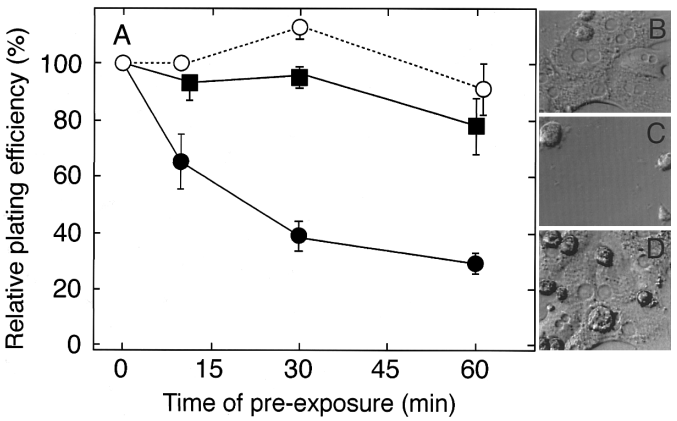

FIG. 3. Glucagon protects against anti-Fas-induced loss of hepatocyte plating ability. Hepatocytes were preincubated in modified Krebs-Ringer solution without anti-Fas (○), in the presence of 15 μg/ml anti-Fas (●), or with 15 μg/ml anti-Fas + 100 nM glucagon / 50 μM IBMX (■). After various periods of time (abscissa) cell aliquots were removed, extensively washed in Krebs-Ringer solution, resuspended in culture medium, and seeded (30, 000 cells / cm^2) on collagen-coated culture dishes. The number of attached cells were scored after cultivation for 48 hr, and compared to the numbers for cells plated directly without preincubation. After 90 min of preincubation with anti-Fas and 48 hr of cultivation the culture dishes contained only few attached hepatocytes (panel C), whilst dishes with cells treated with 15 μg/ml anti-Fas + 100 nM glucagon / 50 μM IBMX appeared confluent (panel D) like control dishes (panel B). Symbols represent the mean ± s.e.m. of 3-4 separate experiments.

ations of cell morphology, which occurred after 1.5-2 hr of incubation (Fig.1). Thus, modulation of protein phosphorylation by anti-Fas correlated more closely with loss of cell viability, as determined by loss of cell attachment (see previous paragraph and Fig.3), than with appearance of apoptotic morphology. The dephosphorylation induced by anti-Fas was anatagonized by the cAK activators glucagon or N^6-benzoyl-cAMP (Fig.4D). This effect of cAMP could hypothetically be due to counteraction of putative anti-Fas-induced proteolysis of phosphoproteins, but since the silver two-dimensional protein staining pattern was the same in anti-Fas- treated cells with or without cAMP stimulation (not shown) this appeared unlikely. Interestingly, the protein hyperphosphorylation noted in response to anti-Fas (Fig.4B) was not prevented by cAK activation (Fig.4D), suggesting that it 1) occurred upstream of the point(s) where cAK modulated anti-Fas signaling and 2) was not sufficient to induce death.

DISCUSSION

The present study demonstrates rapid induction of apoptosis upon triggering of Fas/APO-1(CD95) in freshly isolated rat hepatocytes suspended in simple, defined medium. The first functional deficiency detected in anti-Fas-treated hepatocytes was impaired plating ability (Fig.3), which preceded the development of typical apoptosis, characterized by loss of microvilli, evagination of the plasma membrane, and condensation of nuclear chromatin. Similar morphological features have been reported in the liver of animals treated with anti-Fas (4), and in monolayer cultures of mouse (18) and human (26) hepatocytes exposed to anti-Fas. The induction of apoptosis in the isolated rat hepatocytes was more rapid and synchronous than in primary

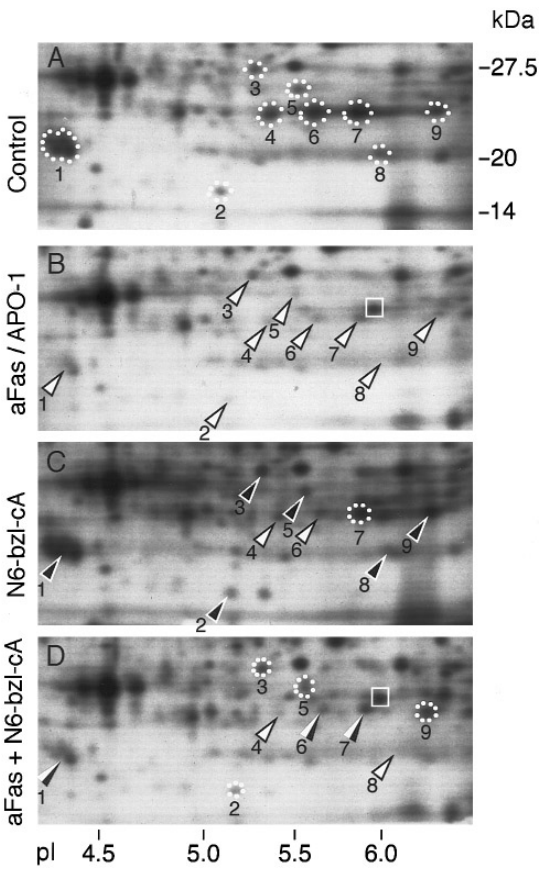

FIG. 4. Opposing effects of anti-Fas and cAMP analog on endogenous hepatocyte protein phosphorylation. Hepatocytes were prelabeled with $^{32}P_i$ and treated for 40 min without added agents (A), with 15 μg/ml anti-Fas (B), 100 μM N^6-benzoyl-cAMP (C), or with 15 μg/ml anti-Fas + 100 μM N^6-benzoyl-cAMP (D). The panels show a region (P_i 4.0 - 6.6; MW 12 - 30 kDa) of autoradiographs of 2D-PAGE of extracts from the hepatocytes. The phosphoprotein spots shown in panel A (spot #1-9) had decreased intensity after anti-Fas treatment (white arrows; panel B). In response to N^6-benzoyl-cAMP (panel C) six of them showed increased intensity (#1,2,3,5,8,9; black arrows), one spot (#7) showed no change and two showed decreased intensity (#4,6). In response to the combinatory treatment (panel D) four spots (#2,3,5,9) returned to about normal intensity, three (#1,6,7; white/black arrows) increased in intensity compared to treatment with anti-Fas alone, and only two spots (#4,8) remained at the low intensity. The square box shows a spot selectively increased in intensity in response to anti-Fas (panel B), which was not downregulated by cAMP analog (panel D). The figure shown is from one representative set of experiments out of three separate series of experiments.

monolayer cultures of rat (unpublished observations), mouse (18) or human (26) hepatocytes, and similar to that noted in Jurkat cells (27), which has been a favoured system for study of Fas-mediated death in malignant cells.

Two-dimensional electrophoresis of extracts from hepatocytes treated with anti-Fas revealed considerable dephosphorylation of proteins (Fig.4). This occurred before the hepatocytes became morphologically apoptotic, indicating, but not proving that dephosphorylation could have a causal role in apoptosis development. The marked protein dephosphorylation in anti-Fas-treated hepatocytes supports a role of phosphatase activation in signaling through Fas. Phosphatase activation has not yet been stressed in Fas-signaling, but has received attention as a downstream event following increased cellular ceramide (16). Both anti-Fas (15, 28) and TNF (14) can stimulate ceramide production. Protein dephosphorylation has been noted in UV-radiated and heat-shocked cells (29).

The main finding of the present study was that increase of cAMP, through activation of the cAMP-dependent protein kinase (cAK), protected the hepatocytes against anti-Fas-induced apoptosis as well as loss of plating ability. The effects did not require supraphysiological increases of cAMP and extreme activation of cAK as modest concentrations of glucagon were sufficient (Fig.2). In fact, even the basal cAK activity level, previously calculated to be 18% of maximal cAK activity (19), was sufficient to provide some protection against anti-Fas-induced apoptosis. This is based on the observation that anti-Fas-induced hepatocyte death was enhanced (Fig.1H) when the cAK activity was brought to a level below basal using the inhibitor (*R*p)-8-Br-cAMPS (20).

The fact that cAK activation restored concomitantly viability and phosphorylation of several proteins in anti-Fas-treated cells (Fig.4D) supports the contention that the hypo-phosphorylation induced by anti-Fas may be important for induction of apoptosis. A recent study of Fas-mediated apoptosis in Jurkat cells pointed to activation of the c-jun N-terminal kinase as pivotal in induction of apoptosis and showed that cAMP counteracted jun kinase activation (30). Another, even more recent study did not support a positive role of the jun kinase in apoptosis (31), and we believe that to search for the molecular mechanism whereby cAK interferes with protein hypophosphorylation may be an alternative approach to find how cAMP abrogates apoptosis.

The biological significance of cAMP protection against Fas-mediated hepatocyte death may be to prevent hepatocyte death under periods of metabolic stress when increased liver capacity is required. This may be particularly important during liver regeneration, when the cAK activity is consistently increased (32).

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