FEBS Letters 494 (2001) 196–200 FEBS 24777

Functional disruption of IEX-1 expression by concatemeric hammerhead ribozymes alters growth properties of 293 cells

Olaf Grobe[a], Alexander Arlt[a], Hendrik Ungefroren[b], Guido Krupp[c], Ulrich R. Fölsch[a], Wolfgang E. Schmidt[d], Heiner Schäfer[a,*]

[a] *Laboratory of Molecular Gastroenterology, First Department of Internal Medicine, University of Kiel, Schittenhelmstr. 12, D-24105 Kiel, Germany*
[b] *Molecular Oncology Group, Department of General Surgery, University of Kiel, Kiel, Germany*
[c] *Department of Haematopathology, University of Kiel, Kiel, Germany*
[d] *Medical Department 1, St. Josef-Hospital, University of Bochum, Bochum, Germany*

Received 15 March 2001; accepted 19 March 2001

First published online 28 March 2001

Edited by Julio Celis

Abstract The early response gene IEX-1 modulates apoptosis and cell growth in a poorly defined fashion. Here, we describe the effect of hammerhead ribozymes specifically disrupting IEX-1 expression in 293 cells. Compared to vector control, 293 cells exhibit a reduced growth rate and a slowed cell cycle progression, when stably transfected with a concatemeric ribozyme construct. In addition, these 293 cells were much less sensitive to apoptosis induced by an activating Fas/CD95 antibody or by the anticancer drugs etoposide and doxorubicin. By modulating the cell cycle, IEX-1 might be part of a growth signal if favourable growth conditions prevail, whereas under unfavourable conditions, i.e. death receptor activation, IEX-1 facilitates apoptosis.

Key words: Cell cycle; Apoptosis; Antisense; Hammerhead ribozyme

1. Introduction

Induced expression of the early response gene $p22^{PRG1/IEX-1}$ has been demonstrated in association with proliferation, adaptation and stress response of various cell types [1–3]. This is related to the actions of growth factors, like human, plasmid-derived and epidermal growth factors [1,4], of cytokines, like interleukin 1β or tumour necrosis factor α [2,4–6], to the influence of cellular stress, like radiation [2,7], or to viral infection [8]. The IEX-1 promoter contains several functional binding sites for transcription factors like AP-1, nuclear factor (NF)-κB and p53 [9,10], thus underscoring the putative role of IEX-1 as a gene contributing to growth control and apoptosis. A recently described splicing variant, designated IEX-1L, has been reported to account for NF-κB-dependent inhibition of apoptosis [11], but it has been shown that IEX-1L is merely a transdominant negative mutant of IEX-1 that does not exist in vivo [12]. Other studies demonstrated growth-promoting activity of IEX-1 in certain cell lines [7], but growth-inhibiting activity in others [13].

To elucidate in what fashion and to what extent IEX-1 contributes to the regulation of cell growth and survival, we made use of the hammerhead ribozyme technique for functional disruption of IEX-1. This technique offers great potential to cleave an individual mRNA with high specificity and efficacy. Using 293 cells, we investigated the effect of abolished IEX-1 expression on cell cycle progression, growth rate and apoptotic sensitivity. It could be shown that ribozyme-mediated disruption of IEX-1 expression reduces growth rate and cell cycle progression as well as the apoptotic sensitivity of 293 cells.

2. Materials and methods

2.1. Chemicals

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt (MTS) proliferation assay CellTiter 96 was from Promega, Mannheim, Germany; Dulbecco's modified essential medium (DMEM), glutamine and foetal calf serum (FCS) were from Biochrom, Berlin, Germany; puromycin, doxorubicin and etoposide were from Sigma, Deisenhofen, Germany; CH-11 anti-Fas antibody was from Coulter Immunotech, Hamburg, Germany; restriction endonucleases were from Life-Technology, Karlsruhe, Germany.

2.2. Cell culture

293 cells (Invitrogen; Schelp, The Netherlands) were cultured in DMEM (10% FCS, 1% glutamine) at 37°C, 85% humidity and a 5% CO_2 atmosphere.

2.3. In vitro transcription of ribozymes, generation of concatemeric vector-based ribozymes

Synthetic DNA oligonucleotides (Biometra-Whatman, Göttingen, Germany) were used as template for T7-primed in vitro transcription using the *AmpliScribe T7 High-Yield* transcription kit (Epicentre). Ribozymal RNA was separated by urea–PAGE and purified as described recently [14]. Vector-based concatemeric ribozyme constructs were generated by single or consecutive ligation of annealed DNA templates via terminal 5′-*Nhe*I and 3′-*Xba*I restriction sites into the pCDNA2.1 vector (Invitrogen). Via *Xba*I and *Pme*I restriction sites, the IEX-1 intron flanked by a 5′-*Xba*I and a 3′-*Pme*I site was ligated directly adjacent to the ribozyme insert. In this way, an *antisense* monomer targeted to position 290, or concatemers targeted to positions 114, 287, 290 and 293 of the IEX-1 mRNA (GenBank accession number S81914) were generated. Via flanking *Bam*HI and *Sal*I restriction sites, the entire inserts were subcloned into the retroviral expression vector p*BabePuro* [15].

*Corresponding author. Fax: (49)-431-597 1302.
E-mail: hschaef@1med.uni-kiel.de

Abbreviations: IEX-1, immediate early gene on X-radiation 1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt; PI, propidium iodide; FCS, foetal calf serum; DMEM, Dulbecco's minimal essential medium

0014-5793/01/$20.00
PII: S0014-5793(01)02344-4

2.4. Transient and stable transfection of ribozyme constructs

Transient transfection was carried out as described [12]. For stable transfection, the p*BabePuro* ribozyme vectors were subjected to in vitro packaging in 293T cells as described elsewhere [16]. The virus supernatants were used for transduction of 293 cells, selected for 3 weeks with 1 μg/ml puromycin.

2.5. Reverse transcription and PCR

Total RNA was isolated using the RNeasy kit (Qiagen), treated with DNase I and reverse transcribed into single-stranded cDNA as described [12]. Genomic DNA was isolated using the Blood and Cell culture DNA Kit (Qiagen). The integration of ribozymal cDNA into the genome was verified by PCR (95°C, 5 min/95°C, 30 s; 63°C, 30 s; 72°C, 30 s; for 25 cycles/72°C, 10 min; GeneAmp PCR System 2400, Perkin-Elmer) using 1.5 U Taq polymerase (Life Technologies) and the provirus-specific primers 5′-cggatcccaagctggctagcag-3′ (forward) and 5′-acagtcgacgctgatcagcggg-3′ (reverse). For RT-PCR, 2 μl cDNA was subjected to PCR (95°C, 2 min/95°C, 60 s; 58°C, 30 s; 72°C, 30 s; for 25 cycles/72°C, 10 min) using 1.5 U Taq polymerase (Life Technologies) and forward/reverse primers specific for virus-integrated ribozyme 9 (5′-gcagaactgatgagtccttg-3′/5′-caccatcgtttcgtcccgaa-3′), ribozyme 2/8/9 (5′-aggtgaactgatgagtcctt-3′/5′-caccatcgtttcgtcccgaa-3′) and ribozyme 2/8/9/10 (5′-aggtgaactgatgagtcctt-3′/5′-atcgtcttttcgtcccgaag-3′). All PCR products were separated by PAGE (8% polyacrylamide) and visualised by ethidium bromide staining.

2.6. Northern and Western blotting

IEX-1 Western blotting was performed as described recently [12]. As control, a monoclonal antibody against α-tubulin (Sigma) was used. Northern blots were performed following the procedure essentially described by Sambrook et al. [17] using a PCR-generated IEX-1 probe (position 610–845), ^{32}P-labelled by random priming.

2.7. Measurement of apoptosis

Apoptosis was measured by means of the annexin V binding assay (*ApoAlert*, Clontech). Briefly, 10^5 cells were pelleted, resuspended in 200 μl of HEPES-buffered saline, and 10 μl of FITC-labelled enhanced annexin V and 100 ng of propidium iodide (PI) were added. Upon incubation for 15 min at room temperature in the dark, samples were brought to 0.5 ml with phosphate-buffered saline. Analysis was done by fluorescence flow cytometry (Galaxy Argon Plus, Dako), and the results were analysed with the *FLOMAX* software (Dako). Those cells exhibiting high staining with annexin V were regarded as being apoptotic.

2.8. Cell cycle analysis

Cells were prepared and stained with PI as described recently [12], and analysed by fluorescence flow cytometry. Cell cycle analysis was carried out using the *FLOMAX* software (Dako).

2.9. Proliferation assay

For the determination of cellular growth rate, stably transfected 293 cells (5000 per well) were seeded onto 96-well plates and cultured for 24 h. Upon overnight serum starvation, cells were cultured in the absence or presence of FCS for 30 h. Then, 25 μl of the MTS reagent (Promega) was added to each well (200 μl medium) and further incubated. After periods of 30, 60, 120 and 240 min, plates were analysed on an ELISA reader at 490 nm.

3. Results

Using synthetic DNA oligonucleotides as template that are composed of the ribozymal backbone sequence, the catalytic region and the bipartite substrate binding region, 12 hammerhead ribozyme RNAs were generated by means of T7-primed in vitro transcription (Table 1). These antisense ribozymes were targeted to various potential cleavage sites (GUC/A) of IEX-1 mRNA since the trinucleotide GUC/A should be an efficient target [18] and were used for cleavage of in vitro transcribed substrate RNA consisting of exon 1 or exon 2 of IEX-1. As shown by PAGE and subsequent ethidium bromide staining, 11 of the 12 ribozymes specifically cleaved the RNA templates at the expected positions (Fig. 1A), whereas no cleavage occurred on any of the other exon-derived IEX-1 RNAs (data not shown). As demonstrated for one of these ribozymes, cleavage was rapid and time-dependent, producing an increasing signal of the cleavage product, whilst the amount of intact RNA decreased (Fig. 1B).

Ribozymes were further tested on an in vitro transcribed RNA substrate consisting of the entire coding region of IEX-1 and a small part of the 3′-untranslated region. As shown in Fig. 1C, the RNA substrate was again cleaved by eight different ribozymes, yielding fragments of the expected sizes. In another experiment, the efficacy of the ribozymes was tested on total RNA isolated from 293 cells. As shown by Northern blot analysis, significant amounts of the 1.3-kb IEX-1 mRNA were detectable (Fig. 1D). Compared to untreated RNA, ribozyme treatment reduced the amount of IEX-1 mRNA, whereas 18S or 28S rRNAs remained unaffected (not shown). Obviously, ribozymes 8 and 9, which cleave IEX-1 mRNA at adjacent positions, were most efficient since they not only reduced the amount of the 1.3-kb RNA molecule, but also produced significant amounts of a smaller cleavage product.

In order to provide a ribozyme construct with higher transcription efficacy and enhanced cleavage activity, ribozymes 2, 8 and 9 or 2, 8, 9 and 10 were fused to each other, 3′-end-

Table 1
Nucleotide sequences of the DNA templates for anti-IEX-1 hammerhead ribozymes

Ribozyme number/cleavage position	Sequence
Exon 1:	
Ri1/36	**accatgtg**tttcgtcccgaaggactcatcag**actctcg**cctatagtgagtcgtatta
Ri2/134*	**tcctgaga**tttcgtcccgaaggactcatcag**ttcacct**cctatagtgagtcgtatta
Ri3/143	**cttcacct**tttcgtcccgaaggactcatcag**gaccctc**cctatagtgagtcgtatta
Ri4/40	**tgtgtcac**tttcgtcccgaaggactcatcag**tcgcagc**cctatagtgagtcgtatta
Ri5/150	**ttcgaccct**tttcgtcccgaaggactcatcag**tcccgga**cctatagtgagtcgtatta
Ri6/193	**ccagcgcc**tttcgtcccgaaggactcatcag**tcgcggg**cctatagtgagtcgtatta
Exon 2:	
Ri7/49	**aaaaggct**tttcgtcccgaaggactcatcag**tctttct**cctatagtgagtcgtatta
Ri8/69*	**gctcacca**tttcgtcccgaaggactcatcag**gtcttct**cctatagtgagtcgtatta
Ri9/72*	**caccatcg**tttcgtcccgaaggactcatcag**ttctgcc**cctatagtgagtcgtatta
Ri10/75*	**catcgtct**tttcgtcccgaaggactcatcag**tgccaga**cctatagtgagtcgtatta
Ri11/197	**atctgact**tttcgtcccgaaggactcatcag**ggagccc**cctatagtgagtcgtatta
Ri12/258	**ggccgcct**tttcgtcccgaaggactcatcag**taactgg**cctatagtgagtcgtatta

Twelve oligonucleotides were used as templates for in vitro transcription of hammerhead ribozymes targeted to various positions of exons 1 and 2 of IEX-1. Nucleotides in bold indicate IEX-1-specific regions. *The corresponding positions of Ri2, 8, 9 and 10 in the IEX-1 mRNA (GenBank accession number S81914) are 114, 287, 290 and 293, respectively.

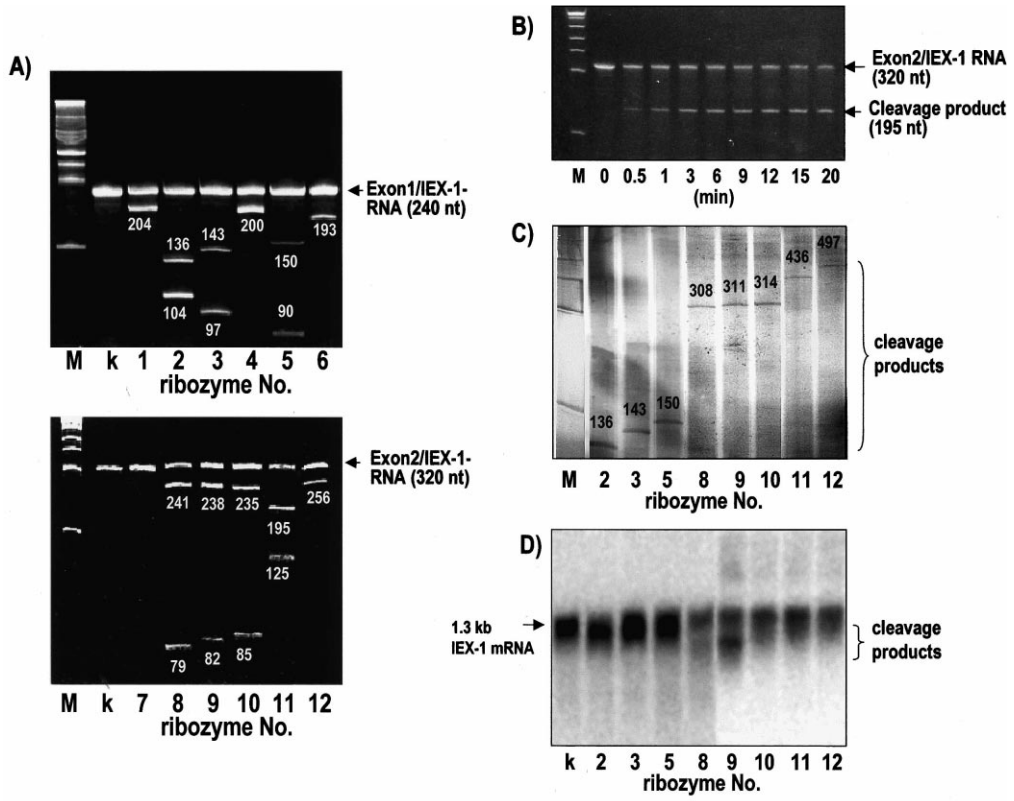

Fig. 1. Testing of in vitro transcribed hammerhead ribozymes on in vitro transcribed IEX-1 RNA or on total RNA from 293 cells. A: Upon in vitro transcription, 12 different ribozymes (20 pmol) were used for cleavage (20 min, 37°C) of 10 pmol in vitro transcribed IEX-1/exon 1 RNA (ribozymes 1–6; upper panel) or with IEX-1/exon 2 RNA (ribozymes 7–12; lower panel). The reaction (20 μl total volume, 40 mM Tris–HCl, pH 7.5) was started after 5 min pretreatment at 65°C by addition of 1 μl $MgCl_2$ (500 mM) and terminated by addition of 2 μl 300 mM EDTA. B: Time-dependent cleavage of 10 pmol IEX-1/exon 2 RNA by 20 pmol of ribozyme 11 (conditions as described above). Products of cleavage reactions were separated by PAGE and visualised by ethidium bromide staining. A representative result of three independent experiments is shown. Sizes (bp) of cleavage products are indicated. C,D: In vitro transcribed ribozymes 2, 3, 5, 8, 9, 10, 11 and 12 (20 pmol) were used for cleavage of (C) 10 pmol in vitro transcribed IEX-1 mRNA (position 1–568), or (D) 20 μg total RNA from 293 cells containing native IEX-1 mRNA (1.3 kb). Upon cleavage reaction (conditions as described above), products were analysed by (C) PAGE and silver staining (sizes (bp) of cleavage products are indicated), or (D) Northern blotting. A representative result of three independent experiments is shown.

linked to the IEX-1 intron and cloned into a retroviral expression vector (p*BabePuro*), as well as ribozyme 9 alone. Upon selection with puromycin for 3 weeks, stably transfected 293 cells were tested for the integration of the expression construct into the genome. As shown in Fig. 2A, PCR on genomic DNA detected the ribozyme templates in ribozyme-transfected, but not in mock-transfected cells. The expression of concatemeric ribozymal RNAs in ribozyme-transfected cells was analysed by RT-PCR. As shown in Fig. 2B, specific PCR products were produced from DNase-treated total RNA prior to reverse transcription. In mock-transfected cells, no PCR product could be detected. Northern and Western blotting revealed significantly reduced levels of the 1.3-kb IEX-1 mRNA and the 22-kDa IEX-1 protein, respectively, in ribozyme-expressing 293 cells (Fig. 2C,D) that were otherwise abundantly present in serum-treated 293 cells transfected with the empty vector. Compared to the single ribozyme Ri9, the concatemeric ribozymes Ri2/8/9 and Ri2/8/9/10 were slightly more and much more efficient, respectively.

To test the effect of ribozyme expression on the cell cycle, 293 cells were treated with serum (5% FCS). As shown by cell cycle analysis (Fig. 3), mock-transfected 293 cells did not exhibit an alteration in the cell cycle profile compared to untransfected cells. In contrast, the concatemeric ribozymes Ri2/8/9/10 and Ri2/8/9 and to a lesser extent Ri9 caused a shift in the cell cycle profile: the fraction of 293 cells residing in the G1 phase increased slightly from 60–64% of mock- or untransfected cells to 67–70% if the anti-IEX-1 ribozymes were expressed. Concomitantly, the fraction of G2/M cells was reduced from 20% to 10–14% if 293 cells expressed the ribozymes 9, 2/8/9 or 2/8/9/10. Under these conditions, the number of cells residing in the S phase (19–21%) did not change significantly.

In order to elucidate whether disruption of IEX-1 expression affects cell growth, ribozyme- or mock-transfected as well as untransfected 293 cells were assayed with the colorimetric MTS test. As shown in Fig. 4, the number of cells upon treatment with increasing amounts of serum was significantly lower compared to mock- or untransfected 293 cells, if anti-IEX-1 ribozymes were expressed. Ri2/8/9/10 exhibited the strongest effect: in the presence of 3% FCS for 48 h, the intensity of MTS staining was reduced by almost 50%, and in cells treated with lower amounts of FCS (1.5% or 0.5%), MTS staining was decreased by 37% and 20%, respectively. Ri2/8/9 affected the growth rate of 293 cells at 3% and 1.5% FCS to a similar extent (45% and 30% reduction, respectively) whereas Ri9 was less efficient (20% and 12% reduction, respectively).

In addition to the effect on the cell cycle, the presence of

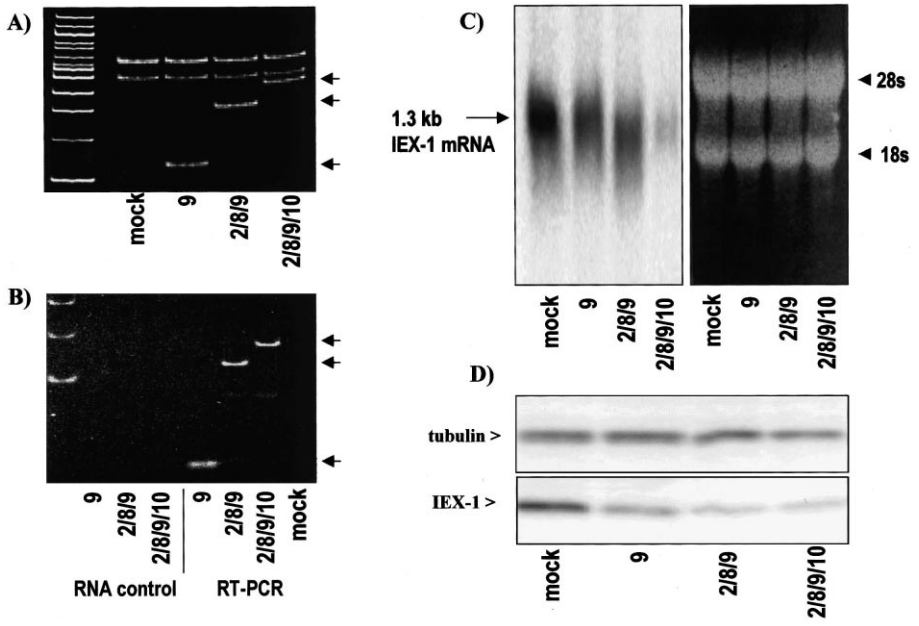

Fig. 2. Ribozyme expression in stably transfected 293 cells. 293 cells were transduced with a retroviral expression vector (p*BabePuro*) encoding the single ribozyme 9, the concatemeric ribozymes 2/8/9 and 2/8/9/10, or the empty vector (mock). A: The integration of ribozymal cDNA into the genome of 293 cells was checked by PCR; arrows indicate specific PCR products. B: RT-PCR analysis on total RNA samples pretreated with DNase I was performed for the detection of ribozymal RNA; arrows indicate specific PCR products. C,D: The effect of ribozyme expression on IEX-1 expression was checked by (C) Northern blotting including an rRNA control and (D) Western blotting using α-tubulin as control. A representative result from three independent experiments is shown.

Ri2/8/9/10 reduced the cytotoxic effect of various apoptotic triggers with the highest efficacy. As shown in Fig. 5, the increase of apoptotic 293 cells (basal, 7 ± 2%) in response to an agonistic Fas antibody (CH-11) amounted to 400%, if these cells were transfected with the empty vector (30 ± 4% apoptotic cells). In contrast, Ri2/8/9/10 transfectants did not exhibit this gain in apoptotic cell number (13 ± 3%) upon CH-11 treatment. If induced by the anti-cancer drugs etoposide (20 μM) and doxorubicin (0.5 μM), apoptosis in vector or control transfectants amounted to 35 ± 6% and 43 ± 11, respectively, whereas Ri2/8/9/10-transfected cells became significantly less apoptotic (11.3 ± 2% and 18 ± 7%, respectively). A decrease of apoptosis was also noted in cells treated with the proteasomal inhibitor MG132 that induced apoptosis in 25 ± 3% of mock- or untransfected cells, but only in 10 ± 2% of cells expressing Ri2/8/9/10. In a similar fashion, Ri2/8/9, and to a lesser extent *Ri9*, also diminished the apoptotic response of 293 cells to the different stimuli.

4. Discussion

In the present study we elucidated to what extent disruption of IEX-1 expression affects cell growth and cell viability of 293 cells. In order to abolish IEX-1 expression, antisense hammerhead ribozymes were generated. Maximal efficacy in cleaving IEX-1 mRNA was achieved by generating a concatemer of four different ribozymes joined to an intron sequence de-

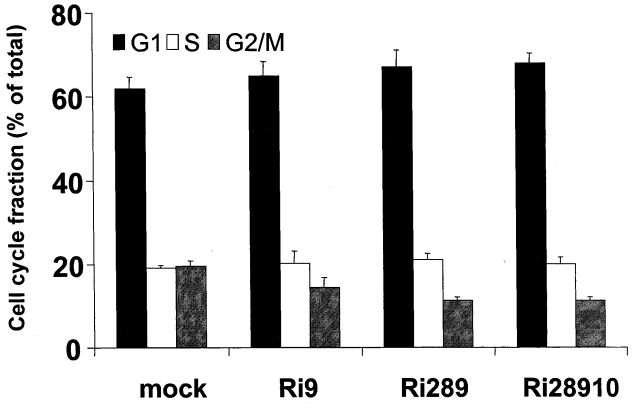

Fig. 3. Cell cycle characteristics in stably transfected 293 cells. Transduced 293 cells (mock, and ribozymes 9, 2/8/9 or 2/8/9/10) were serum-starved for 16 h, followed by stimulation with 5% FCS for 24 h. Cell cycle analysis was performed using the PI staining procedure. Data represent the mean ± S.D. of four independent experiments.

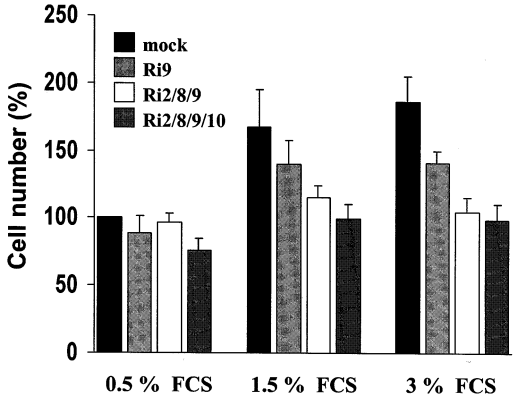

Fig. 4. Growth properties of stably transfected 293 cells. 293 cells were grown for 24–36 h, serum-starved for 12–26 h and stimulated with increasing amounts of FCS (0.5%, 1.5% or 3%). Then, after an additional 48 h, cell number was determined by the colorimetric MTS test. Data represent the mean ± S.D. of four independent experiments performed in quadruplicate.

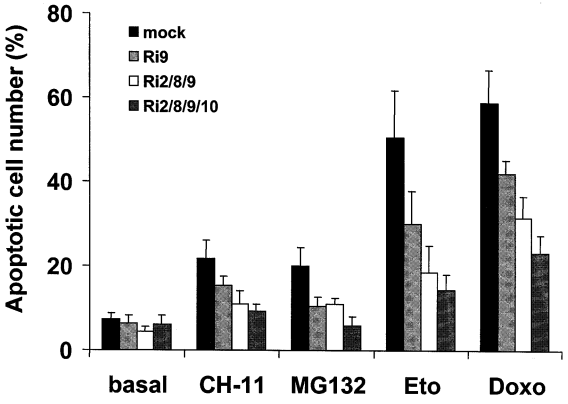

Fig. 5. Fas- and anti-cancer drug-induced apoptosis in stably transfected 293 cells. 293 cells were grown for 24–36 h. Then, cells were treated with an agonistic anti-Fas antibody (clone CH-11, 150 ng/ml) for 16 h, or with doxorubicin (0.5 μM), etoposide (20 μM) or MG132 (50 ng/ml) for 24 h. Apoptosis was quantified by the FITC–annexin V staining procedure (ApoAlert). Data represent the mean ± S.D. of four independent experiments.

rived from the IEX-1 gene. Upon retroviral transfection, mixed clones of 293 cells were established stably expressing this concatemeric ribozyme RNA and exhibiting reduced expression of IEX-1 compared with mock transfectants. These cells displayed a significant alteration in cell cycle progression in that the fraction of cells residing in G2/M was lower compared to mock-transfected cells, whereas the G1 fraction increased. In accordance with this inhibition of cell cycle progression, the growth rate of 293 cells decreased if IEX-1 expression was abolished by the concatemeric ribozyme. Interestingly, the efficacy in reducing IEX-1 expression (in the rank order Ri9 < Ri2/8/9 < Ri2/8/9/10) was exactly paralleled by the efficacy in reducing the cell cycle progression as well as the growth rate. Thus, disruption of IEX-1 expression obviously affects signalling pathways during proliferation. These findings support earlier observations indicating a growth-promoting activity of IEX-1 in certain epithelial cell lines [7] and a close association of IEX-1 expression with growth-promoting signals in vitro [1,4] as well as in vivo [3]. Furthermore, an accelerating effect of IEX-1 on cell cycle progression was observed in HeLa cells [19]. During growth stimulation, IEX-1 protein enters the nucleus [12] and accumulates in distinct subnuclear structures (H. Schäfer, unpublished result) that might be related to the so-called nuclear dots [20] or PML oncogenic domains. Recent studies have revealed that these particular nuclear structures are involved in growth regulation, apoptosis and stress response [21–23]. Still, the exact mechanisms by which IEX-1 exerts its cell cycle-promoting effect are unknown.

Another effect caused by abolished IEX-1 expression was a decrease of the sensitivity to apoptotic triggers, like Fas activation or anti-cancer drug treatment. This could be explained by the fact that particularly those cells are prone to apoptosis that proceeded in the cell cycle, whereas those cells residing in G1 arrest are more refractory. A line of evidence supports this link of apoptotic sensitivity and cell cycle (reviewed in [24–26]). It has been demonstrated that the exaggerated activity of c-myc and E2F accelerates cell cycle progression but also triggers apoptosis induced by various apoptotic stimuli. As IEX-1 contributes to cell cycle progression, it might similarly increase the sensitivity to apoptosis. Under conditions that favour cell growth, IEX-1 might be part of a growth signal, as demonstrated by previous work. However, under unfavourable conditions, i.e. death receptor activation, IEX-1 facilitates apoptosis. This may be due to a signal conflict that emerges if inappropriate cell cycle progression occurs [27], as already described for other cell cycle-promoting mediators [28].

Acknowledgements: The authors wish to thank Maike Breitenbroich for her excellent technical work. This work was supported by a grant (SFB-415/C3) from the German Research Society (DFG).

References

[1] Charles, C.H., Yoon, J.K., Simske, J.S. and Lau, L.F. (1993) Oncogene 8, 797–801.
[2] Kondratyev, A.D., Chung, K.N. and Jung, M.O. (1996) Cancer Res. 56, 1498–1502.
[3] Schäfer, H., Trauzold, A., Siegel, E.G., Fölsch, U.R. and Schmidt, W.E. (1996) Cancer Res. 56, 2641–2648.
[4] Schäfer, H., Lettau, P., Trauzold, A., Banasch, M. and Schmidt, W.E. (1999) Pancreas 18, 378–384.
[5] Pietzsch, A., Büchler, C., Aslanidis, C. and Schmitz, G. (1997) Biochem. Biophys. Res. Commun. 235, 4–9.
[6] Kobayashi, T., Pittelkow, M.R., Warner, G.M., Squillace, K.A. and Kumar, R. (1998) Biochem. Biophys. Res. Commun. 251, 868–873.
[7] Kumar, R., Kobayashi, T., Warner, G.M., Wu, Y., Salisbury, J.L., Lingle, W. and Pittelkow, M.R. (1998) Biochem. Biophys. Res. Commun. 253, 336–341.
[8] Domachowske, J.B., Bonville, C.A., Mortelli, A.J., Colella, C.B., Kim, U. and Rosenberg, H.F. (2000) J. Infect. Dis. 181, 824–830.
[9] Pietzsch, A., Büchler, C. and Schmitz, G. (1998) Biochem. Biophys. Res. Commun. 245, 651–657.
[10] Schäfer, H., Diebel, J., Arlt, A., Trauzold, A. and Schmidt, W.E. (1998) FEBS Lett. 436, 139–143.
[11] Wu, M.X., Ao, Z., Prasad, K.V., Wu, R. and Schlossman, S.F. (1998) Science 281, 998–1001.
[12] Schäfer, H., Arlt, A., Trauzold, A., Hünermann-Jansen, A. and Schmidt, W.E. (1999) Biochem. Biophys. Res. Commun. 262, 139–145.
[13] Segev, D.L., Ha, T.U., Tran, T.T., Kenneally, M., Harkin, P., Jung, M., MacLaughlin, D.T., Donahoe, P.K. and Maheswaran, S. (2000) J. Biol. Chem. 275, 28371–28379.
[14] Gaur, R.K. and Krupp, G. (1997) Methods Mol. Biol. 74, 69–89.
[15] Morgenstern, J.P. and Land, H. (1990) Nucleic Acids Res. 18, 3587–3596.
[16] Hinz, S., Trauzold, A., Boenicke, L., Sandberg, C., Beckmann, S., Bayer, E., Walczak, H., Kalthoff, H. and Ungefroren, H. (2000) Oncogene 19, 5477–5486.
[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Zoumadakis, M. and Tabler, M. (1995) Nucleic Acids Res. 23, 1192–1196.
[19] Arlt, A., Grobe, O., Sieke, A., Kruse, M.-L., Fölsch, U.R., Schmidt, W.E. and Schäfer, H. (2001) Oncogene 20, 69–76.
[20] Brasch, K. and Ochs, R.L. (1992) Exp. Cell Res. 202, 211–223.
[21] Wang, Z.G., Delva, L. and Gaboli, M. et al. (1998) Science 279, 1547–1551.
[22] Everett, R.D., Lomonte, P., Sternsdorf, T., van Driel, R. and Orr, A. (1999) J. Cell Sci. 112, 4581–4588.
[23] Fogal, V., Gostissa, M., Sandy, P., Zacchi, P., Strensdorf, T., Jensen, K., Pandolfi, P.P., Will, H., Schneider, C. and Del Sal, G. (2000) EMBO J. 19, 6185–6195.
[24] Amati, B., Alevizopoulos, K. and Vlach, J. (1998) Front. Biosci. 3, D250–D268.
[25] Guo, M. and Hay, B.A. (1999) Curr. Opin. Cell Biol. 11, 745–752.
[26] Blagosklonny, M.V. (1999) BioEssays 21, 704–709.
[27] Evan, G. and Littlewood, T. (1999) Science 281, 1317–1322.
[28] Sofer-Levi, Y. and Resnitzky, D. (1996) Oncogene 13, 2431–2437.