Inducible cAMP early repressor (ICER) suppresses gastrin-mediated activation of cyclin D1 and c-fos gene expression

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Running head: ICER suppresses gastrin-induced cyclin D1 and c-fos

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ABSTRACT

The gastric hormone gastrin and its precursors promote proliferation in several gastrointestinal cell types. Here we show that gastrin induces transcription of cell cycle gene cyclin D1 and proto-oncogene c-fos in the neuroendocrine pancreatic cell line AR42J and that this gastrin response is inhibited by endogenous Inducible cAMP Early Repressor (ICER). The transcriptional repressor ICER is known to down-regulate both its own expression and the expression of other genes containing cAMP-responsive elements (CRE). Using siRNA we show that CRE promoter elements are the targets of endogenous ICER also in AR42J cells as well as in the neuroendocrine cell line RIN5F. Our results suggest that ICER plays an important role in molecular mechanisms governing gastrin-mediated growth by modulating gastrin’s transcriptional activation of growth-related genes. Our finding that ICER modulates Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)-activated gene expression, indicates a regulatory effect of ICER in the responses of neuroendocrine cells also to other peptides than gastrin.

Keywords: Neuroendocrine gene regulation, Gastrointestinal proliferation, Gastrin
INTRODUCTION

THE PEPTIDE HORMONE GASTRIN is well characterized as a stimulant of gastric acid secretion by stimulating ECL-cells to produce histamine, which in turn stimulates the parietal cells in the oxyntic mucosa to release HCl (36, 48). Gastrin is an important growth factor for the fetal pancreas (50) and a potent stimulant for the growth of gastric mucosa (4, 5, 46). Transgenic mice overexpressing gastrin exhibit increased proliferation of the oxyntic mucosa (51), while gastrin deficient mice develop abnormal gastrointestinal (GI) mucosa with immature cells (7, 12). In addition, there is abundant evidence to suggest that gastrin may play an important role in tumor biology, as gastrin is shown to regulate tumor cell growth (4, 5), and stimulate tumor cell invasion (4, 5, 13). Hypergastrinemia is associated with the occurrence of gastric enterochromaffin-like (ECL) cell carcinoid tumors (4, 5, 28, 46) and with an increased risk of gastric and colorectal carcinoma (5), indicating a role in carcinogenesis. The biological actions of gastrin are exerted through binding to the gastrin/cholecystokinin (CCK2) receptor. This receptor has been shown to be coexpressed with gastrin in several GI tumor cell lines (53) and in human gastric carcinoids (38), indicating the existence of an autocrine growth-stimulatory loop. The malignant potential of carcinoids is associated with hypergastrinemia (24). These studies suggest that gastrin plays an important role in neuroendocrine tumor biology.

Inducible cAMP Early Repressor (ICER) is a transcriptional repressor encoded by the CREM gene, and a member of the CREB/CREM/ATF-1 family of transcription factors which bind to cAMP responsive promoter elements (CREs) (14, 32, 41). The activity of ICER is mainly influenced by its protein levels which are controlled by the CRE-regulated P2 promoter of the CREM gene (18). Once increased, ICER down-regulates its own expression, as well as the expression of other CRE-regulated genes (6, 18). Interestingly, increased ICER expression has been linked to both proliferative and anti-apoptotic responses. Thus, a robust induction of ICER expression occurred during regeneration of rat liver after partial hepatectomy (34), and proliferation of pancreatic β-cells.
also seems to involve ICER (11). In the myeloid leukemia cell line IPC-81, on the other hand, high levels of ICER proteins protected cells from cAMP-induced cell-death, indicating that ICER proteins block transcriptional activation of genes encoding proteins necessary for apoptosis (31). Also, over-expression of ICER reverses the transformed phenotype of prostate tumor cells indicating a role in cancer prevention (54). Taken together, these studies suggest a role for ICER in growth regulation.

Gastrin regulates CRE-driven gene expression via \( G_{q}/G_{11} \)-coupled signaling pathways involving a variety of protein kinases (42) and induces expression of all four ICER splice variants, ICER I, ICER I\( \gamma \), ICER II and ICER II\( \gamma \) (43). The aim of the present study was to characterize ICER functions in gastrin-mediated responses. Our focus here is the role of ICER in modulating CRE-driven gene expression involved in gastrin-induced responses coupled to proliferation in neuroendocrine cells. It was therefore of interest to investigate a possible involvement of ICER in gastrin-mediated regulation of expression of the proto-oncogene c-fos (17) and of cyclin D1. Cyclin D1 controls G1/S transition by regulating the activity of the cyclin-dependent kinases (CDK4 and CDK6) (22). Gastrin is known to activate expression of both c-fos (44) and cyclin D1 (40, 56). In order to enable focus on endogenous gene regulation, we measured cyclin D1 and c-fos mRNA, and used RNA interference to knock down ICER. Our results show that gastrin-mediated activation of cyclin D1 and c-fos gene expression is inhibited by endogenous ICER in AR42J neuroendocrine cells. Using reporter-gene studies we confirm that in AR42J endogenous ICER targets CRE promoter elements known to be important for both cyclin D1 (52) and c-fos (41) expression. These findings suggest that ICER modulates gastrin-mediated CRE-driven gene expression involved in cell proliferation.

**EXPERIMENTAL PROCEDURES**

*Cells and Reagents.* AR42J (rat pancreatic acinar cell derived, ATCC, Rockville, MD) were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose (Invitrogen, Carlsbad, CA), 15% fetal calf serum (FCS) (Euroclone Ltd, Devon, UK), 1 mM sodium pyruvate, 0.1 mg/ml L-
glutamine (Invitrogen), 10 U/ml penicillin/streptomycin (Invitrogen), and 1 μg/ml fungizone (Invitrogen). RIN5F cells (rat insulinoma, ATCC) were grown in RPMI 1640 with 2 g/liter glucose (Invitrogen) supplemented with 10% FCS (Euroclone Ltd), 0.1 mg/ml L-glutamine (Invitrogen) and 10 U/ml penicillin/streptomycin (Invitrogen). All cells were grown at 37°C in a humidified atmosphere at 5% CO₂. Gastrin-17 and forskolin was purchased from Sigma Chemical (St. Louis, MO) and PACAP-38 was obtained from Bachem (Bobendorf, Switzerland). The peptides were stored dried and frozen (-20°C).

_Growth factor treatment of cells and isolation of RNA and protein._ AR42J cells were subcultured in 75 cm² tissue culture flasks with 2×10^6 cells/10 ml DMEM containing 15% FCS. After 72 h (post-confluence), growth medium was replenished with 10 ml serum-free DMEM and the cells were serum-starved for 24 h before medium was removed and replaced with 5 ml serum-free DMEM containing 10 nM gastrin (G-17). The quiescent cells were treated with gastrin (G-17) for intervals between 15 min to 24 h. Treated and untreated cells were grown in parallel and harvested at the same time points (15 min – 24 h) after onset of gastrin stimulation. Total RNA was isolated using RNeasy® Midi kit (Qiagen, Germantown, MD) according to the manufacturer’s instruction. The samples were kept frozen at -80°C until further processing. The flow through from RNA isolation on RNeasy RNA absorption chromatography columns was collected for protein analysis. Protein concentration was determined, by an automated color-binding method using a Pentra 400 machine (Horiba ABX Diagnostics, Montpellier, France), and found to be in the range of 500-800 ng/μl for all samples. Prior to western blot analysis flow through samples were mixed with glycerol to 17% and bromphenolblue to 0.01%.

_Real Time RT-PCR._ cDNA synthesis was performed with 500 ng total RNA in a 10 μl reaction of Euroscript Reverse Transcription Core kit according to the manufacturer’s instruction (Eurogentec, Seraing, Belgium). After synthesis, the cDNA was diluted 1:2 with RNase free water. **TaqMan Real Time PCR** was performed with 1x Quantitect Probe PCR Master Mix (Qiagen), 400 nM forward
primer, 400 nM reverse primer, 200 nM TaqMan Probe (Eurogentec) and cDNA equivalent to 62.5 ng total RNA in a total reaction volume of 25 µl. SYBR Green Real Time PCR were run in 1x Quantitect SYBR Green PCR kit (Qiagen) at the same conditions as TaqMan Real Time PCR but without the TaqMan probe. Both Real-Time PCRs were performed in Stratagene’s Mx3000P Real Time PCR system; 10 min at 95°C, 35-45 thermal cycles of 30 s at 95°C, 1 min at 56°C and 30 s at 76°C(TaqMan)/72°C(SYBR Green). PCR products from the SYBR Green analysis were verified by a dissociation curve analysis in order to confirm primer specificity. All PCR products were analyzed by agarose gels.

Primers and probes. Using computer software [Oligo v5 (National Bioscience) and Primer Designer (Sci Ed Central)], primers and probes were designed to recognize rat cyclin D1 (GenBank Accession number NM_171992), c-fos (GenBank Accession number X06769), ICER I (GenBank Accession number Z15158), ICER II (GenBank Accession number Z15158), and β-Actin (GenBank Accession number BC063166) mRNA sequences. The different ICER splice variants and the location of the ICER primers are shown in Fig. 1. The sequences of primers used for PCR analysis are as follows:
cyclin D1: 5’- TGGAACTGCTTCTGGTGGAAC-3’ (sense) and 5’- TTCACATCTGTGGCCACAGAG-3’ (antisense), c-fos: 5’- GTAGAGCAGCTATCTCCTGA-3’(sense) and 5’- AACGCAGACTTCTCGTCTTC-3’ (antisense) and 6-FAM-5’-TCTGTCTCCGTGGAGCTGA-3’-Dark Quencher (probe), ICER I: 5’- AGATGAAACTGATGAGGAGACT-3’(sense) and 5’- CAGTCAACCAGGTCCAAGTCAA-3’ (antisense) and 6-FAM-5’- TCTGTCTCCGTGGAGCTGA-3’-Dark Quencher (probe), ICER II: 5’- AGATGAAACTGATGAGGAGACT-3’(sense) and 5’- AGCAACTGTACATGCTGTAATC-3’ (antisense) and 6-FAM-5’-TCTGTCTCCGTGGAGCTGA-3’-Dark Quencher (probe), β-Actin: 5’- CTGGCTCCTAGCACCATGA-3’(sense) and 5’- AGCCACCAATCCACACAGA-3’ (antisense) and 6-FAM-5’-CAAGATCATTGCTCCTCCTG -3’-Dark Quencher (probe).
RNA interference experiments. Two different siRNA duplexes were designed to knock down all ICER splice variants and the sequences are as following: ICER-1: 5’-GACCACUCUGUAGCAAAA-3’, ICER-2: 5’-CUGUAACUGGAGAUGAAACU-3’. The sequences for the control siRNAs were (20): CAT: 5’-GAGUGAAUACCACGACGAUU-3’, EGFP: 5’-GCAAGCUGACCCUGAAGUUC-3’. Cells (1 x 10^6/well) were seeded out in 6-well plates and transfected after 24 h of cultivation. The cells were transfected with 0.8 μg siRNA (47 nM) and 4 μl XtremeGENE transfection reagent (Roche, Indianapolis, IN) pr well. 24 h after transfection the cells were treated with 10 nM gastrin for 1 to 24 h before RNA and protein was isolated. RNA was isolated by use of the RNeasy® Mini kit (Qiagen) according to manufacturer’s instruction. Protein was harvested and detected as described under western blot analysis.

Reporter gene assay. The plasmid pCRELuc containing 4xCRE somatostatin consensus promoter elements (TGACGTCA) was obtained from Stratagene (La Jolla, CA). Cells (5 x 10^4/well) were seeded out in 96-well plates and transfected after 24 h. The cells were transfected with 0.15 μg luciferase reporter plasmid DNA, 0.08 μg siRNA (44 nM) and 0.8 μl XtremeGENE transfection reagent per well. After cultivation for 1 day in the presence of plasmid, siRNA and transfection agent, cells were treated with different stimuli followed by medium removal and lysis in 20 μl of Promega lysis buffer. Luciferase activity was measured by Wallac 1420 Victor3 plate reader (Perkin Elmer, Boston, MA) with the Luciferase Reporter Assay System (Promega, Madison, WI), as recommended by the manufacturer.

Western blot analysis. Cells were washed twice in phosphate-buffered saline and harvested directly in 0.5 ml of SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 9 % glycerol, 1% w/v SDS, 5% v/v 2-β-mercaptoethanol, 0.005% w/v bromphenolblue). Viscosity was reduced by drawing the suspension through a 21-gauge needle, cell debris were removed by centrifugation (15,000 x g, 10 min), and the supernatant was stored at -80°C. 35 μl of each extract was boiled and separated on a 10% Bis-Tris
SDS-polyacrylamide gel in 1x MOPS running buffer (Invitrogen) prior to electrotransfer onto Hybond-P membranes (Amersham Pharmacia Biotech, Pittsburgh, PA). The transfer was performed in 1x Transferbuffer (Invitrogen) supplemented with 20% v/v methanol for 1 h at 175 mA. The membranes were dried overnight, moistened quickly in 100% methanol and incubated for 5 min in TBST buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20). Subsequently, the membranes were treated with 5% nonfat dry milk (Nestlé) in TBST for 1 h at room temperature and incubated with primary antibodies diluted in TBST with 1% bovine serum albumin for 2 h at room temperature. The membranes were then incubated with peroxidase-conjugated secondary antibodies diluted in TBST with 1% bovine serum albumin (Sigma chemicals) for 1 h at room temperature. After washing (4x15-min washes in TBST), binding of secondary antibodies was visualized by the ECL detection system (Amersham Pharmacia Biotech) and KODAK Image station 2000R (KODAK, Pittsburgh, PA). The following antibodies were used: rabbit anti-human CREM-1 (X-12) raised against full length polyhistidine tagged CREM-1 fusion protein (1:100-1:50) (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse Akt (1:1000-1:500) (Cell Signaling, Beverly, MA) and horseradish peroxidase conjugated goat anti-rabbit IgG (1:1000) (Cell Signaling).

Statistical analysis. Real Time RT-PCR experiments were repeated three times in three biological replicates with three wells per experiment. Reporter gene experiments were repeated three times with four wells per condition per experiment. Results are shown as the mean values for separate experiments ± SD. Statistically significant differences were determined using non-parametric Mann-Whitney test, and p-values of $P \leq 0.05$ were considered significant.
RESULTS

*Gastrin-mediated expression of cyclin D1 and c-fos in AR42J follows dissimilar kinetics*

We and others have previously shown that gastrin induces proliferation in the neuroendocrine cell line AR42J (10, 33, 35, 44). Due to this fact, AR42J is well suited for studying molecular mechanisms involved in gastrin-regulated proliferation. To investigate gastrin-mediated activation of the endogenous genes cyclin D1 and c-fos in AR42J, we measured fold changes in mRNA levels of these genes in a time course study during a period of 15 min to 24 h. The results showed that cyclin D1 increased gradually during the entire period and reached almost 2-fold induction after 24 h of gastrin stimulation (Fig. 2A). c-fos, on the other hand, as a typical early response gene, showed nearly 20-fold induction after 1 h of gastrin stimulation, followed by a rapid decrease and return to baseline after approximately 6 h of stimulation (Fig. 2B). Our results show that the endogenous genes of both cyclin D1 and c-fos are activated by gastrin in AR42J, and that the kinetics of their mRNA levels differs markedly.

*ICER suppresses gastrin-induced endogenous cyclin D1 expression*

The cyclin D1 promoter contains CRE elements (52) which have been shown to be important for gastrin-mediated activation of cyclin D1 transcription (23). In order to explore whether ICER may be involved in modulation of gastrin-mediated regulation of cyclin D1 gene expression, we measured fold changes in cyclin D1 mRNA levels in AR42J cells treated with ICER specific siRNA. The siRNA was designed to target the common region of all four ICER splice variants. We found that gastrin induced markedly higher amounts of cyclin D1 transcripts in cells treated with siICER compared to cells treated with siRNAs against either the bacterial gene Chloramphenicol Acetyltransferase (siCAT) or against the jellyfish gene Enhanced Green Fluorescent Protein (siEGFP) (Fig. 3). Western blot analysis verified that ICER protein levels were reduced by siRNA targeting ICER (Fig. 4). These results strongly suggest that gastrin-induced activation of endogenous
cyclin D1 expression is repressed by ICER, indicating that ICER may play a role in gastrin-mediated cell cycle regulation.

**ICER suppresses gastrin-induced endogenous c-fos expression**

ICER’s potential to function as a negative feed back repressor of c-fos is suggested by a study showing that exogenously over expressed ICER represses cAMP induced activation of a reporter gene driven by the c-fos promoter (41). To examine whether endogenous ICER can modulate gastrin-mediated c-fos transcription in AR42J, we studied the effect of ICER siRNA on gastrin-induced c-fos mRNA levels. Two different siRNAs, each targeting all four ICER splice variants (siICER-1 and siICER-2) were used. The results showed that gastrin induced markedly higher levels of c-fos mRNA in the presence of siRNA against ICER (Fig. 5), indicating that endogenous ICER inhibits endogenous c-fos expression in AR42J cells.

**ICER knockdown enhances gastrin induced CRE-driven gene expression**

The promoters of both cyclin D1 (19) and c-fos (8, 45) are known to contain several binding sites important for their regulation in addition to the CRE promoter elements (14, 32, 41). In order to investigate whether the observed effect of ICER knock down may be associated with the CRE elements in the cyclin D1 and c-fos promoters, we used an artificial transcription-system driven by CRE alone using the reporter plasmid pCREluc. The results showed that siRNA-mediated ICER knockdown leads to a 2-fold enhancement of gastrin-induced CRE-driven transcription compared to cells treated with siRNA against CAT (Fig. 6A). This result suggests that the observed repression by ICER of gastrin-induced cyclin D1 and c-fos expression (Fig. 3 and Fig. 5) is mediated via the CRE promoter elements in these genes.

ICER knock down resulted in approximately 3-fold enhancement of CRE-driven transcription in forskolin-treated cells (Fig. 6B) where the response is mediated via cAMP and protein kinase A
This indicates that endogenous ICER inhibits the PKA pathway known to be involved in gastrin-induced activation of CRE-driven transcription in AR42J (42). Whether ICER also may interfere with protein kinase C and calmodulin-activated pathways involved in gastrin-mediated CRE-driven transcription in AR42J (42) remains to be established.

**ICER represses PACAP-activated transcription via CRE promoter elements**

In order to extend our study to other hormones involved in similar biological responses as gastrin, we included Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) which is a neuropeptide known to regulate, interestingly both the stimulation and inhibition, of proliferation, differentiation and apoptosis in a number of cells (37). PACAP signals via the PKA pathway (3), and we have previously shown that PACAP induces CRE-driven transcription in AR42J (42). Measurements of PACAP-induced CRE reporter plasmid activation in the presence of siRNAs indicated that knockdown of ICER enhanced the PACAP response above the levels seen in cells treated with control siRNA (Fig. 7) ($P < 0.05$). These results show that ICER inhibits CRE-driven transcription induced by PACAP to a similar extent as seen for the gastrin-response, and indicate that ICER may repress also PACAP-mediated gene expression via CRE promoter elements.

**ICER modulates forskolin and PACAP-mediated gene expression in the neuroendocrine cell line RIN5F**

To examine whether our findings on the biological roles of ICER in AR42J may be valid also for other neuroendocrine cells, we performed similar studies in the rat insulinoma cell line RIN5F which is known to express ICER (43). RIN5F does not express the CCK-B (gastrin) receptor (data not shown), but is known to respond to forskolin (43). We found that siRNAs against ICER strongly enhanced CRE-driven transcription induced by either forskolin (Fig. 8A) ($P < 0.05$) or by PACAP (Fig. 8B) ($P < 0.05$). These results indicate that ICER represses PACAP-induced gene expression mediated via CRE promoter elements in RIN5F cells, and that the effect of ICER is linked to cAMP
and the protein kinase A pathway also in these cells. Together the results observed in AR42J and RIN5F underline the role of ICER in neuroendocrine cell biology.

**Gastrin-mediated induction of ICER splice variants show identical time course**

We further examined the time course of expression of the two splice variants ICER I and ICER II. These two variants differ from each other in that ICER II mRNA lacks DNA binding domain I and the untranslated region found in ICER I mRNA (Fig. 1), and thus RT-PCR primers and probes could be designed to specifically detect PCR products representing either ICER I (376 bp) or ICER II (392 bp) (Fig. 9). We found that the levels of both ICER I and ICER II mRNA peaked after 2 h of gastrin stimulation with subsequent rapid decrease (Fig. 10), indicating that transcription and splicing of ICER I and ICER II follow similar time-courses in response to gastrin in AR42J.

Levels of transcript and protein from the same gene exhibit distinct kinetics. Protein levels reach maximum some time after the peak of transcript levels, and the decline from maximum levels may follow dissimilar time courses due to different mechanisms governing mRNA- and protein half lives. In the neuroendocrine cell lines JEG-3 and AtT20 ICER protein stability is regulated by cross-talk between the MAPK- and the cAMP signaling pathway (55) where ERK1/2 phosphorylates ICER for degradation by the ubiquitin-proteasome pathway and cAMP antagonizes the MAPK pathway and stabilizes ICER protein. Notably, gastrin-induced ICER expression involves both the cAMP and the MAPK signaling pathways (42, 43), which both may influence the stability of ICER proteins. We found that ICER protein levels peaked at 6 h after start of gastrin stimulation, and then decreased slowly (Fig. 11). Our data suggest that ICER shows similar time course fluctuations at transcript and protein level, albeit with a 4 h delay.
DISCUSSION

Insight into molecular mechanisms of gastrin-mediated regulation of growth and proliferation in neuroendocrine cells can contribute to a better understanding of neuroendocrine tumor biology. In the present study, we show for the first time that the transcriptional repressor ICER modulates gastrin-mediated transcriptional regulation of the cell cycle specific gene cyclin D1. Pradeep and co-workers have recently found that gastrin-induced cyclin D1 transcription is mediated via the β-catenin and CREB signaling pathways, and involves the CRE element in the cyclin D1 promoter (23). Our results show that endogenous ICER represses this transcriptional activation and thus suggest that the activity of cyclin D1 in gastrin-induced cell-cycle progression from G1 to S phase is modulated by ICER. Ectopic ICER expression has previously been reported to block AtT20 cells at the S and G2/M phases of the cell cycle (2), indicating a function of ICER as a tumor suppressor.

Our finding that ICER represses gastrin-induced transcription of the c-fos gene suggests that ICER plays a role in the modulation of gastrin-mediated growth regulation of the neuroendocrine cell line AR42J. Razavi and co-workers have shown that over-expression of exogenous ICER IIy leads to strong inhibition of DNA synthesis and c-fos expression in mouse pituitary tumor cells and human choriocarcinoma cells (26), which is in keeping with our study focusing on endogenous ICER and c-fos gene expression.

Gastric carcinoid tumors arise from proliferating ECL-cells, and elevated levels of plasma gastrin initiate and maintain the neoplastic change in these cells (1, 9, 39). Some studies have suggested that a proportion of gastric adenocarcinomas develop from the ECL cell (47, 49) while others have reported that gastric adenocarcinomas in hypergastrinemic patients show signs of neuroendocrine differentiation (25). Moreover, the majority of patients with poorly differentiated, high grade neuroendocrine carcinomas have hypergastrinemia (29). These studies underline the role of gastrin in the GI-related malignancies and the existence of neuroendocrine differentiation in these
tumors. ICER and CREB have been suggested to play a role in tumorigenesis of neuroendocrine tissues (30). Our results indicate that ICER may play a role in neuroendocrine carcinogenesis involving gastrin and that the underlying mechanisms include modulation of gene expression of cyclin D1, c-fos and other growth related genes. Thus, ICER may act as an anti-oncogene in GI neuroendocrine tumors in a similar manner as has been suggested in prostate tumorigenesis (54). It is also interesting to note that in GI-related malignancies, overexpression of cyclin D1 reflects the severity of gastric cancer and indicates poor prognosis of colorectal cancer (21).

Both PACAP and its receptors are expressed in human neuroendocrine tumors (27, 37), and PACAP has been reported to modulate rat gastric ECL cell proliferation (15), indicating a possible role in neuroendocrine GI tumor cell biology. PACAP induces ICER mRNA transcription in AR42J (data not shown) as well as CRE-driven transcription in the same cell line (42). Our results demonstrate for the first time that ICER is involved in PACAP-induced CRE-mediated gene expression in both AR42J and RIN5F cells. Taken together with the gastrin data, this emphasizes the importance of ICER as regulator of gene expression in neuroendocrine tumor biology.

In conclusion, we have characterized a new role of ICER in gastrin-mediated regulation of genes responsible for growth regulation in neuroendocrine cells. Our findings are in keeping with ICER as a tumor suppressor and indicate new insights into some of the complex mechanisms of tumorigenesis.
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GRANTS

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REFERENCES


FIGURE LEGENDS

Fig. 1. Schematic presentation of ICER, with P2 promoter and exons. Positions and orientations of primers (solid arrows) and probes (dotted arrows) used in RT-PCR are shown. Start site for ICER transcripts (P2) are indicated by a broken arrow, while translational stop codons are shown at end of the DNA binding domain I and II.

Fig. 2. Gastrin-mediated activation of cyclin D1 and c-fos. AR42J cells were treated with gastrin (10 nM) for 15 min to 24 h before RNA isolation at indicated time points. Endogenous gene expression was assayed by measuring mRNA levels of cyclin D1 (A) and c-fos (B) by real-time RT-PCR. Fold induction levels were calculated using the ΔΔCt-method (16) where the expression levels were normalized to the level of β-actin expression, and gastrin-treated samples were compared to untreated cells. The results shown represent one of three independent experiments performed. The PCR was performed in triplicates and the mean ± SD values are shown (n=3). Data shown in Fig. 9 confirm that the Real Time RT-PCR analyses were specific for the given targets.

Fig. 3. ICER siRNA enhances gastrin induced cyclin D1 expression. AR42J cells were transiently transfected with siRNA against ICER, CAT or EGFP. 24 h after transfection, the cells were treated with gastrin (10 nM) for 24 h. Real Time RT-PCR was performed to measure endogenously expressed cyclin D1. Fold induction levels were calculated as described in Fig. 2. The results shown represent one of two independent experiments performed. The PCR was performed in triplicates and the mean ± SD values are shown (n=3).

Fig. 4. Verification of ICER protein knockdown by siRNA. AR42J cells were transiently transfected with siRNAs against ICER or CAT. 24 h after transfection, the cells were treated with gastrin (10 nM) for 6 h before the cells were harvested for Western blot analysis. A, detection on western blot with polyclonal antisera towards CREM (ICER) and Akt. The latter for loading
control. B, ICER protein levels in siICER transfected cells were compared to siCAT transfected cells, and quantified by computer software in KODAK Imager 2000S. The results are representative of three independent experiments performed, and the mean ± SD values is shown (n=3).

Fig. 5. siICER enhances gastrin induced c-fos expression. AR42J cells were transiently transfected with siRNAs targeting ICER, CAT or EGFP. 24 h after transfection, the cells were treated with gastrin (10 nM) for 1 h. Real Time RT-PCR was performed to measure endogenously expressed c-fos. Fold induction levels were calculated as described in Fig. 2. The results represent one of four independent experiments performed. The PCR was performed in triplicates and the mean ± SD values are shown (n=3).

Fig. 6. ICER inhibits gastrin- and forskolin-mediated activation of CRE promoter elements. AR42J cells were transiently transfected with pCREluc in combination with siRNAs against ICER or CAT and treated with A, gastrin (10 nM) or B, forskolin (10 µM) for 6 h. Fold induction was compared to untreated cells. The mean ± SD values of triplicate experiments are shown (n=3). (*P < 0.05 vs control)

Fig. 7. ICER modulates PACAP-mediated gene expression. AR42J cells were transiently transfected with pCREluc in combination with siRNAs targeting ICER or CAT and treated with PACAP (200 nM) for 6 h. The results are representative of three independent experiments. The mean ± SD values of quadruplicate measurements are shown (n=4). (*P < 0.05 vs control)

Fig. 8. ICER modulates forskolin and PACAP-mediated gene expression in RIN5F. RIN5F cells were transiently transfected with pCREluc in combination with siRNA against ICER or CAT and treated with A, forskolin (10 µM) or B, PACAP (200 nM) for 6 h. The results are representative of three independent experiments. The mean ± SD values of quadruplicate measurements are shown (n=4). (*P < 0.05 vs control)
Fig. 9. RT-PCR products visualized on a 1% agarose gel. The bands represent ICER I (376 bp), ICER II (392 bp), c-fos (152 bp), cyclin D1 (163 bp) and β-actin (73 bp).

Fig. 10. Kinetics of ICER I and ICER II mRNA levels. AR42J cells were treated with gastrin (10 nM) for 15 min to 24 h before RNA isolation. Real Time RT-PCR analysis was performed to measure endogenously expressed ICER I and ICER II, respectively. Fold induction levels were calculated as described in Fig. 2. The results shown are representative of three independent experiments. The mean ± SD values of triplicate measurements are shown (n=3). Data shown in Fig. 9 confirm that the Real Time RT-PCR analyses were specific for the given targets.

Fig. 11. Kinetics of ICER protein levels. AR42J cells were treated with gastrin (10 nM) for 15 min to 24 h, and protein was analyzed in the flow-through from RNA purification using QIAGEN RNeasy RNA isolation kit. Equal amounts of protein (18 μg) were analyzed. A, detection on western blot using polyclonal antisera towards CREM and Akt. The latter for loading control. B, relative quantities of ICER protein quantified by computer software in KODAK Imager 2000S. The results are representative of three independent experiments. The mean ± SD values of triplicate Western blots are shown (n=3).
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Fig. 2. Gastrin-mediated activation of cyclin D1 and c-fos. AR42J cells were treated with gastrin (10 nM) for 15 min to 24 h before RNA isolation at indicated time points. Endogenous gene expression was assayed by measuring mRNA levels of cyclin D1 (A) and c-fos (B) by real-time RT-PCR. Fold induction levels were calculated using the \( \Delta \Delta \text{Ct} \) method (16) where the expression levels were normalized to the level of \( \beta \)-actin expression, and gastrin-treated samples were compared to untreated cells. The results shown represent one of three independent experiments performed. The PCR was performed in triplicates and the mean ± SD values are shown (n=3). Data shown in Fig. 9 confirm that the Real Time RT-PCR analyses were specific for the given targets.
Fig. 3. ICER siRNA enhances gastrin induced cyclin D1 expression. AR42J cells were transiently transfected with siRNA against ICER, CAT or EGFP. 24 h after transfection, the cells were treated with gastrin (10 nM) for 24 h. Real Time RT-PCR was performed to measure endogenously expressed cyclin D1. Fold induction levels were calculated as described in Fig. 2. The results shown represent one of two independent experiments performed. The PCR was performed in triplicates and the mean ± SD values are shown (n=3)
Fig. 4. Verification of ICER protein knockdown by siRNA. AR42J cells were transiently transfected with siRNAs against ICER or CAT. 24 h after transfection, the cells were treated with gastrin (10 nM) for 6 h before the cells were harvested for Western blot analysis. A, detection on western blot with polyclonal antisera towards CREM (ICER) and Akt. The latter for loading control. B, ICER protein levels in siICER transfected cells were compared to siCAT transfected cells, and quantified by computer software in KODAK Imager 2000S. The results are representative of three independent experiments performed, and the mean ± SD values is shown (n=3).
Fig. 5. siICER enhances gastrin induced c-fos expression. AR42J cells were transiently transfected with siRNAs targeting ICER, CAT or EGFP. 24 h after transfection, the cells were treated with gastrin (10 nM) for 1 h. Real Time RT-PCR was performed to measure endogenously expressed c-fos. Fold induction levels were calculated as described in Fig. 2. The results represent one of four independent experiments performed. The PCR was performed in triplicates and the mean ± SD values are shown (n=3).
Fig. 6. ICER inhibits gastrin- and forskolin-mediated activation of CRE promoter elements. AR42J cells were transiently transfected with pCRE-luc in combination with siRNAs against ICER or CAT and treated with A, gastrin (10 nM) or B, forskolin (10 μM) for 6 h. Fold induction was compared to untreated cells. The mean ± SD values of triplicate experiments are shown (n=3). (*P < 0.05 vs control)
Fig. 7. ICER modulates PACAP-mediated gene expression. AR42J cells were transiently transfected with pCREluc in combination with siRNAs targeting ICER or CAT and treated with PACAP (200 nM) for 6 h. The results are representative of three independent experiments. The mean ± SD values of quadruplicate measurements are shown (n=4). (*P < 0.05 vs control)
Fig. 8. ICER modulates forskolin and PACAP-mediated gene expression in RIN5F. RIN5F cells were transiently transfected with pCREluc in combination with siRNA against ICER or CAT and treated with A, forskolin (10 μM) or B, PACAP (200 nM) for 6 h. The results are representative of three independent experiments. The mean ± SD values of quadruplicate measurements are shown (n=4). (*P < 0.05 vs control)
Fig. 9. RT-PCR products visualized on a 1% agarose gel. The bands represent ICER I (376 bp), ICER II (392 bp), c-fos (152 bp), cyclin D1 (163 bp) and β-actin (73 bp).
Fig. 10. Kinetics of ICER I and ICER II mRNA levels. AR42J cells were treated with gastrin (10 nM) for 15 min to 24 h before RNA isolation. Real Time RT-PCR analysis was performed to measure endogenously expressed ICER I and ICER II, respectively. Fold induction levels were calculated as described in Fig. 2. The results shown are representative of three independent experiments. The mean ± SD values of triplicate measurements are shown (n=3). Data shown in Fig. 9 confirm that the Real Time RT-PCR analyses were specific for the given targets.
Fig. 11. Kinetics of ICER protein levels. AR42J cells were treated with gastrin (10 nM) for 15 min to 24 h, and protein was analyzed in the flow-through from RNA purification using QIAGEN RNeasy RNA isolation kit. Equal amounts of protein (18 μg) were analyzed. A, detection on western blot using polyclonal antisera towards CREM and Akt. The latter for loading control. B, relative quantities of ICER protein quantified by computer software in KODAK Imager 2000S. The results are representative of three independent experiments. The mean ± SD values of triplicate Western blots are shown (n=3).