Degradation of the inducible cAMP early repressor (ICER) by the ubiquitin–proteasome pathway

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The inducible cAMP early repressor (ICER) is a powerful transcriptional inhibitor that plays an important role in the regulation of the cAMP-dependent transcriptional response in the neuroendocrine system. ICER activity is primarily determined by its intracellular concentration, rather than by post-translational modifications, such as phosphorylation. We investigated the mechanisms that regulate the levels of ICER transcript and polypeptides in cardiocytes, myogenic (C2C12) and pituitary-derived (GH3) cell lines. We show that in primary cardiocytes and GH3 cells ICER was inducible by cAMP but not by membrane depolarization. Moreover, lactacystin, a specific proteasome inhibitor, decreased the rate of ICER degradation. This effect was associated with the accumulation of ICER–ubiquitin conjugates. We conclude that the intracellular levels of ICER are controlled by the ubiquitin–proteasome pathway for protein breakdown.

INTRODUCTION

The transcription of many cellular genes is regulated by changes in cAMP levels in response to extracellular stimuli. This signalling pathway is associated with the protein kinase A (PKA)-dependent phosphorylation of nuclear proteins that belong to the cAMP-response element (CRE)-binding protein (CREB)/activating transcription factor (ATF) family of transcription factors. This family has at least 10 members, all of which bind to a DNA consensus sequence called the CRE (reviewed in [1,2]). The transcriptional response to cAMP is rapid and transient; it peaks around 30–60 min after stimulation and then declines, reaching a nadir after several hours [3]. The attenuation phase of the response is presumably regulated by protein phosphatases involved in the dephosphorylation of CREB [4,5], and by members of the CREB/ATF family of transcription factors that lack activation domains and therefore act as repressors of the cAMP-inducible response [6–8].

The expression of most members of the CREB/ATF multigene family of transcription factors is ubiquitous and non-inducible [1,9], and their activity is regulated by post-translational modifications, such as phosphorylation. The only known exception to this rule is the recently characterized CRE modulator (CREM) gene [2]. This gene is controlled by two promoters, which direct the expression of a family of transcription factors that can either promote or repress the cAMP-inducible transcriptional response. The upstream promoter (P1) controls the transcription of the alternatively spliced α, β, γ and τ isoforms of CREM. The downstream, intronic promoter (P2), contains several CREs and is therefore inducible by cAMP. P2 directs the transcription of the inducible cAMP early repressor (ICER), a potent and specific inhibitor of the cAMP-inducible transcriptional response [6,8,10]. The induction of ICER is transient, because ICER proteins down-regulate the activity of P2 [8]. ICER modulates a number of biological processes, such as circadian control of transcription in the pineal gland [11], expression of cyclin A and cell-cycle progression in fibroblasts [12], and expression of thyroid-stimulating hormone and follicle-stimulating hormone receptors in thyroid gland and Sertoli cells respectively [13,14].

The ICER proteins (molecular mass 12–13.5 kDa) have a modular structure, which consists of a DNA-binding domain and a dimerization domain preceded by a short stretch of 50–60 amino acids. In contrast with other CREB/ATF proteins, ICER polypeptides lack the phosphorylation (P)-box and the activation domain. Therefore, their activity is primarily determined by their intracellular concentration, which depends upon the transcriptional rate of the P2 promoter and the degradation rate of ICER polypeptides [8,12,14].

We reasoned that the elucidation of the mechanism(s) of degradation of ICER proteins would be crucial to understand its biological function. There are multiple pathways for intracellular protein breakdown. In eukaryotes, the ubiquitin (Ub)–proteasome system is a major pathway for selective proteolysis in the nucleus and the cytoplasm. The proteolytic process involves a cascade of enzymic reactions that catalyse the formation of high-energy thioester bonds between Ub and carrier proteins (E1-E2). At the end of the process, Ub is ligated via isopeptide bonding of its C-terminal carboxy group to the ϵ-amino group of a lysine residue(s) of the target protein. This process is catalysed by the E3 Ub–protein ligase. Multiple rounds of Ub conjugation result in the formation of a polyUb chain that is recognized by the 26 S proteasome. This large multi-catalytic proteinase degrades the substrate to small peptides and releases reusable Ub (reviewed in [15,16]).

Although genetic studies in yeast indicate that the Ub–

Abbreviations used: ATF, activating transcription factor; CRE, cAMP-response element; CREB, CRE-binding protein; CREM, CRE modulator; ICER, inducible cAMP early repressor; Ub, ubiquitin; CMV, cytomegalovirus; His_6-Ub, hexahistidine-tagged Ub; IBMX, 3-isobutyl-1-methylxanthine; ACRM, anti-CREMα antibody; GndHCl, guanidinium chloride, PKA, protein kinase A; LLL, carbobenzoxyleucinyl-leucinyl-leucinal-H; LLM, *N*-acetyl-leucinyl-leucinyl-methional-H; NTA, nitrilotriacetic acid.

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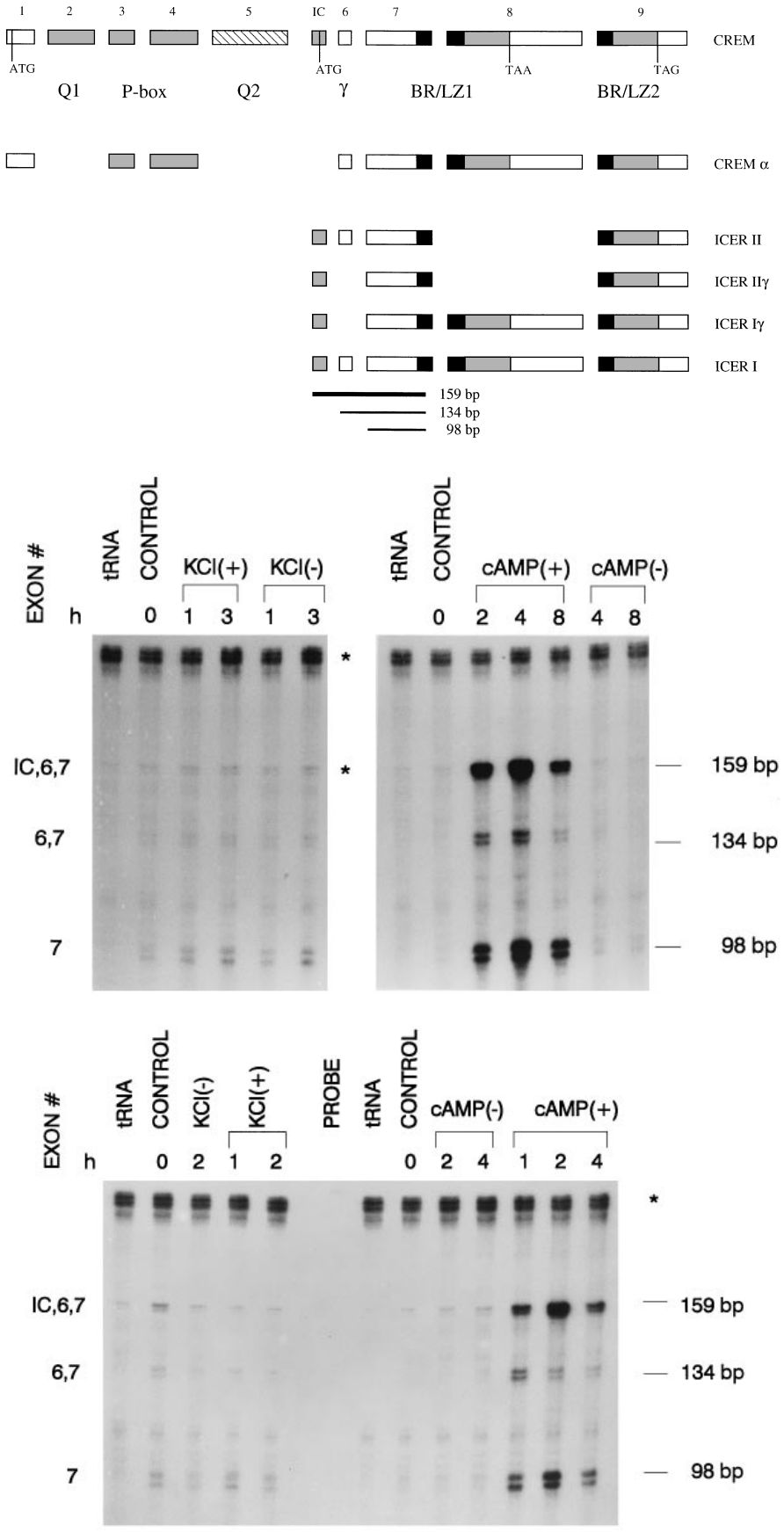

Figure 1 **For legend see opposite.**

proteasome pathway is involved in many cellular processes [16], only a few *in vivo* substrates of this pathway have been identified. They include key regulatory nuclear proteins, such as the yeast transcription factors MATα2 [17] and Gcn4 [18], the oncoproteins c-Jun [19] and c-Fos [20], the tumour-suppressor protein p53 [21] and several cyclins [22]. In the present study, we examined the mechanisms that control the intracellular levels of ICER. We show that ICER expression is induced by cAMP but not by membrane depolarization in primary cardiocytes and GH3 cells. We demonstrate that lactacystin, a specific proteasome inhibitor [23], decreases the rate of ICER degradation and induces the accumulation of ICER–Ub conjugates. These results indicate that the Ub–proteasome pathway is involved in regulating the steady-state levels of ICER.

EXPERIMENTAL PROCEDURES

Plasmids and ICER cloning

Cloning and bacterial expression of CREMα cDNA were previously described [24]. The CMV-His_6-Ub expression vector (hexahistidine-tagged Ub driven by the cytomegalovirus promoter) was a gift from D. Bohmann [19]. ICER I cDNA was cloned by PCR amplification of randomly primed cDNA derived from C2C12 cells stimulated with 1 mM 8-Br-cAMP and 250 μM 3-isobutyl-1-methylxantine (IBMX) for 3 h. The following primers were used: M_3 (−), 5′-GCCCTCGAG^{+727}AGAGTTCACAGTCAACAAGGTCCA^{+703}-3′ (numbers correspond to the CREM sequence [6]); I_3 (+), 5′-CGCGGATCCACC^{+1}ATGGCTGTAACTGGAGATG^{+20}-3′ (numbers correspond to the ICER sequence [11]). The PCR product was cloned into pcDNAI-Neo (Invitrogen) as a *Bam*HI/*Xho*I fragment. The sequence was confirmed by dideoxy DNA sequence analysis (Sequenase kit; United States Biochemical).

Cell cultures

GH3 cells and primary neonatal atrial myocytes were cultured as described previously [24]. KCl and 8-Br-cAMP/IBMX treatments for the analysis of ICER mRNA expression were performed as described previously [24].

RNAse protection analysis

Total RNA was isolated by guanidinium isothiocyanate/CsCl centrifugation and subjected to RNAse protection analysis as described previously [24]. The probe (Figure 1, upper panel), containing sequences corresponding to nucleotides 1–159 from ICER I [11] cloned into pBluescript, was generated using the T_3 promoter. Equal loading and quality of RNA was confirmed by ethidium bromide staining of agarose gels and by the use of a control probe generated by *in vitro* transcription of a 143 bp fragment from the rat U3 small nuclear RNA cDNA (results not shown) [24].

Generation of antiserum

Rabbit polyclonal anti-CREMα antibody (ACRM) was produced by sequential immunization with 0.5 mg of gel-purified bacterially expressed CREMα.

In vitro transcription/translation and immunoprecipitation

[^{35}S]Methionine- (NEN-Dupont) labelled proteins were produced *in vitro* in a coupled transcription–translation reaction using reticulocyte lysate system (TNT; Promega) in a total volume of 50 μl. Immunoprecipitations were carried out in RIPA buffer [50 mM Tris, pH 8.0, 150 mM NaCl, 1 % (w/v) Nonidet P-40, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, 0.1 mM EDTA, 0.5 mM PMSF and 0.5 μg/ml of each of the following protease inhibitors: pepstatin A, leupeptin, bestatin, antipain and chymostatin]. The reactions were incubated at 4 °C for 1 h with 5 μl of antiserum, and the immune complexes were collected with Protein A–Sepharose (Sigma), washed four times with RIPA buffer and analysed by SDS/PAGE and fluorography.

Pulse–chase analysis

GH3 cells were incubated for 2 h in methionine- and cysteine-free Dulbecco's modified Eagle's medium. When indicated, 1 mM 8-Br-cAMP and 250 μM IBMX were added to the medium at the beginning of the incubation. For labelling, cells were incubated for 1 h with 150 μCi/ml of [^{35}S]methionine and [^{35}S]cysteine (ICN Translabel) in the same medium. Cells were harvested (0 min), or washed three times in PBS and incubated for various time intervals in complete medium supplemented with 4 mM methionine and 4 mM cysteine. Lactacystin (kindly provided by Dr. S. Omura) was added 18 h before cAMP stimulation at a final concentration of 10 μM, and kept at the same concentration until cell harvesting. Cells were washed twice in ice-cold PBS and lysed in 1 ml of RIPA buffer. Following centrifugation at 1000 ***g*** (3000 rev./min) for 10 min, aliquots from the supernatant containing the same amount of protein were immunoprecipitated with ACRM, resolved by SDS/PAGE and analysed by fluorography.

Transient transfections and purification of ICER–Ub conjugates

GH3 cells were transfected with the CMV-His_6-Ub expression vector [19] using the Lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. Briefly, cells (50–70 % confluent in 60-mm-diameter dishes) were incubated for 5 h with 3 ml of a mixture containing 25 μl of Lipofectamine and 4 μg of DNA in serum-free Optimem (Gibco BRL), followed by a 18 h incubation in the same medium containing 4 % (v/v) fetal bovine

Figure 1 ICER induction in primary cardiocytes and GH3 cells

Upper panel: schematic presentation of the exon structure of the CREM gene and the ICER isoforms. Each box represents a different exon. The numbers and letters above the boxes correspond to the published order of these exons [8]. Q1 and Q2, the two activation domains; P-box, the kinase-inducible domain; IC, the ICER-specific exon that originates from the intronic promoter (P2); BR/LZ1 and BR/LZ2, the two basic/leucine-zipper regions involved in DNA binding and dimerization. The thick bar represents the cDNA probe used in RNAse-protection assays. The thin bars below the probe indicate the extent of partially protected fragments obtained in the RNAse-protection analysis. The numbers at the right of the bars indicate the size of the fragments. Middle panel: RNAse-protection analysis of CREM transcripts in cAMP-treated and KCl-depolarized primary cardiocytes. For cAMP stimulation, cells were treated with 1 mM 8-Br-cAMP and 0.25 mM IBMX in serum-free medium for the indicated periods of time (right panel). For KCl depolarization, cells were treated for 30 min with osmotically balanced serum-free medium containing 50 mM KCl, and then were washed and incubated in standard medium for the indicated periods of time (left panel). Control cells were maintained in culture in the absence of 8-Br-cAMP/IBMX [cAMP (−)] or KCl [KCl (−)]. The lengths of the protected fragments, determined by using a sequencing reaction of a known template, are indicated on the right. The numbers on the left correspond to the exons encoding the protected fragments. The 159 bp fragment corresponds to ICER transcripts. The 134 bp protected fragment corresponds to exons 6 and 7, and may represent CREMα, β or τ isoforms. The 98 bp protected fragment corresponds to exon 7 and may represent CREMγ or ICERγ isoforms. The asterisks denote non-specific bands. Lower panel: RNAse-protection analysis of CREM transcripts in cAMP-treated and KCl-depolarized GH3 cells. The analysis was performed as described for the middle panel.

serum. The medium was then replaced by Dulbecco's modified Eagle's medium/10 % (v/v) fetal bovine serum, and the incubation was continued for additional 24 h. At a time of 48 h after transfection, cells were treated with or without 8-Br-cAMP/IBMX for 2 h and ^{35}S-labelled for 3 h as described above. Cells were lysed with 1.5 ml of 6 M guanidinium chloride (GndHCl)/10 mM imidazole/0.1 M phospate buffer, pH 8.0, and incubated overnight at room temperature with 100 μl of 50 % (w/v) Ni^{2+}–nitrilotriacetic acid (NTA)–agarose (Qiagen). The Ni^{2+}–NTA–agarose was collected by centrifugation and successively washed three times with 6 M GndHCl/10 mM imidazole/0.1 M phospate buffer, pH 8.0, twice with 1.5 M GndHCl/20 mM imidazole/0.1 M phospate buffer, pH 8.0, and twice with 20 mM imidazole/25 mM Tris, pH 6.8. Bound proteins were eluted by rotating for 30 min at room temperature in 0.5 ml of 100 mM EDTA, pH 8.0, then mixed with 0.5 ml of 2 × RIPA buffer, immunoprecipitated with ACRM and analysed by SDS/PAGE and fluorography.

RESULTS

Excitable cells such as cardiac myocytes and neurons must maintain tight control over their cell excitability and automaticity by regulating the level of expression and properties of the channels expressed in the cell membrane. We showed that in neonatal rat cardiocytes cAMP and membrane depolarization induced a six-fold increase in the steady-state levels of Kv1.5 transcript, whereas in GH3 cells and adult rat cardiocytes cAMP and depolarization induced a 5–6 fold decrease in the steady-state levels of the same transcript. We concluded that cAMP regulates the expression of Kv1.5 in a cell-specific manner [24]. These results prompted us to investigate the pre- and post-transcriptional mechanisms that regulate the steady-state levels of ICER polypeptides in cardiocytes and GH3 cells, since ICER is a likely candidate to regulate the expression of Kv1.5.

Initially, we examined the effect of cAMP and depolarization on the steady-state levels of ICER transcript in primary cardiocytes and GH3 cells using RNAse protection analyses with a probe specific for ICER (Figure 1, upper panel). The results showed that in both primary cardiocytes (Figure 1, middle panel) and GH3 cells (Figure 1, lower panel) cAMP stimulation increased the intensity of a 159 bp fragment that corresponds in size to a transcript containing the ICER-specific exon (IC), exon 6 (γ) and part of exon 7 (Figure 1, middle and lower panels). The induction of ICER was detectable as early as 30 min after cAMP stimulation (results not shown), and peaked after 2–3 h in GH3 cells and 3–4 h in cardiocytes (Figure 1). cAMP stimulation also resulted in a significant increase in the intensity of a 98 bp band, which corresponds to an increase in the steady-state levels of the ICERγ isoforms. A third band (134 bp) with an apparent size that corresponds to exon 6 and part of exon 7 included in the probe most likely represents an artifact of the RNAse-protection assays. Interestingly, in both cardiocytes (Figure 1, middle panel) and GH3 cells (Figure 1, lower panel), depolarization did not increase the steady-state levels of the transcripts coding for ICER. Thus, in contrast with previous observations in which depolarization and cAMP stimulation had a concordant effect on the steady-state levels of several transcripts, including c-*fos*, proenkephalin and Kv1.5 [24–26], here we demonstrated a discordant response. cAMP induced the expression of ICER transcripts, whereas depolarization had no effect on the steady-state levels of ICER transcript.

To analyse the expression and stability of ICER polypeptides, we generated a polyclonal antibody (ACRM) against the full-length CREMα polypeptide expressed in bacteria (Figure 1, upper panel). This antibody should therefore recognize all known CREM and ICER isoforms. Indeed, ACRM precipitated *in vitro* translated ICERγ (Figure 2, top, lane 3), whereas preimmune serum failed to precipitate ICERγ (Figure 2, top, lane 2). An excess of recombinant CREMα protein blocked the precipitation of ICERγ (Figure 2, top, lane 4), indicating that the immunoprecipitation was specific. In additional immunoprecipitation experiments, ACRM specifically recognized *in vitro* translated CREMα and CREMSα, a polypeptide originated from an internal AUG initiation codon (results not shown).

We then used ACRM to characterize the expression of ICER in GH3 cells stimulated with 8Br-cAMP and IBMX. Crude cell lysates from cells labelled with [^{35}S]methionine and [^{35}S]cysteine were immunoprecipitated with ACRM. The results confirmed that cAMP stimulation induced the expression of four ICER isoforms (Figure 2, bottom, lane 2), as predicted by the alternative use of DNA binding and dimerization domains and by the inclusion or exclusion of the γ domain (Figure 1, upper panel).

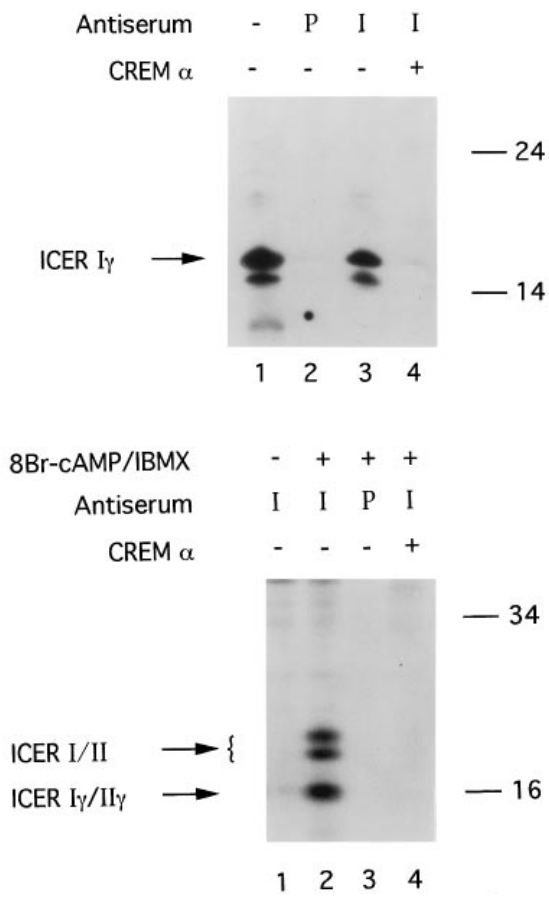

Figure 2 Detection of *in vitro* and *in vivo* produced ICER by ACRM

Top, immunoprecipitation of ^{35}S-labelled *in vitro* translated ICER Iγ. Lane 1, total translate; lane 2, control immunoprecipitation with preimmune serum; lanes 3 and 4, immunoprecipitation with ACRM (antiserum), alone (lane 3) or in the presence of 5 μg/ml of bacterially expressed CREMα (lane 4). Immunopellets were analysed by SDS/PAGE and fluorography. P, preimmune serum; I, immune serum. Bottom, immunoprecipitation of ^{35}S-labelled ICER proteins from cAMP-stimulated GH3 cells. Cells were incubated for 2 h with 8-Br-cAMP/IBMX in methionine- and cysteine-free medium, and labelled for 1 h with ^{35}S Translabel in the same medium; and cell lysates were immunoprecipitated with ACRM (antiserum), alone (lane 2) or in the presence of 5 μg/ml of bacterially expressed CREMα (lane 4). Lane 1, control of non-stimulated cells immunoprecipitated with ACRM; lane 3, control immunoprecipitation with preimmune serum. Immunopellets were analysed by SDS/PAGE and fluorography.

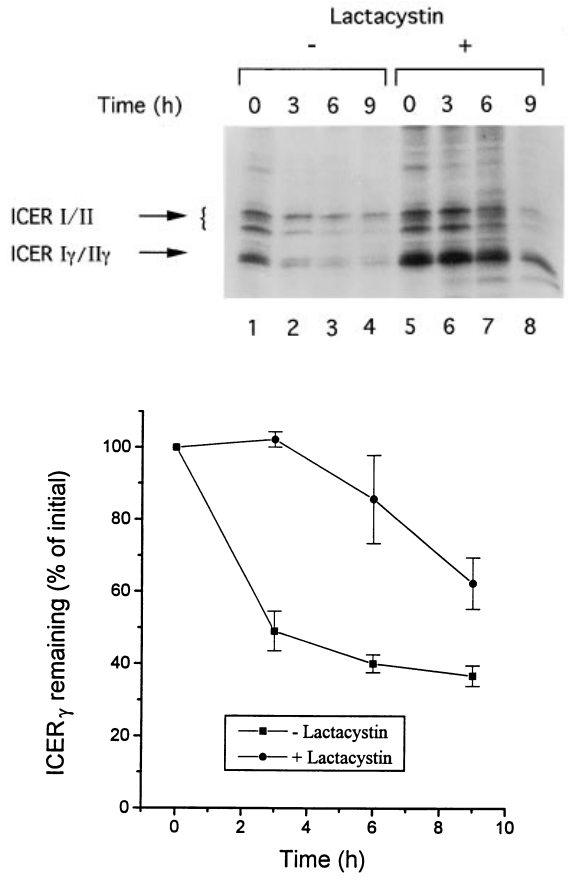

Figure 3 Effect of lactacystin on ICER stability

Top, GH3 cells were incubated for 18 h in complete medium alone (lanes 1–4) or with 10 μM lactacystin (lanes 5–8), followed by cAMP stimulation, ^{35}S-labelling (see the legend of Figure 2) and chase with complete medium containing excess unlabelled methionine and cysteine for the indicated periods of time. Cell lysates were immunoprecipitated with ACRM, and the immunopellets were analysed by SDS/PAGE and fluorography. Bottom, densitometric analysis of the bands corresponding to ICERγ isoforms from the top panel. Each point represents the average of two independent experiments.

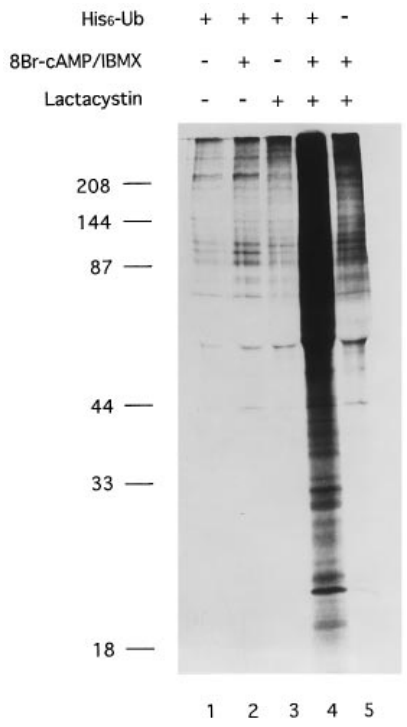

Figure 4 Ubiquitination of ICER *in vivo*

CMV-His$_6$-Ub-transfected (lanes 1–4) or control (lane 5) GH3 cells were pretreated without (lanes 1 and 2) or with (lanes 3–5) 10 μM lactacystin for 18 h, incubated for 2 h with (lanes 2, 4 and 5) or without (lanes 1 and 3) 8-Br-cAMP/IBMX and ^{35}S-labelled for 3 h in the same medium. Ubiquitinated proteins were isolated from cell lysates by precipitation with Ni^{2+}–NTA–agarose, eluted with 100 mM EDTA, pH 8.0, and immunoprecipitated with ACRM. The immunopellets were analysed by SDS/PAGE and fluorography. The sizes of molecular-mass standards are shown on the left.

The two ICERγ isoforms migrated as a closely spaced doublet that could be visualized by shorter exposure of the autoradiogram (see Figure 3). Control experiments confirmed that, in the absence of cAMP, ICER isoforms were not detectable (Figure 2, bottom, lane 1). ICER polypeptides were not precipitated by preimmune serum (Figure 2, bottom, lane 3), and preincubation of ACRM with bacterially produced CREMα blocked the immunoprecipitation of the four ICER isoforms (Figure 2, bottom, lane 4).

We next performed pulse–chase experiments to examine the stability of ICER polypeptides in GH3 cells. The cells were stimulated with 8Br-cAMP and IBMX, and radiolabelled with [^{35}S]methionine and [^{35}S]cysteine. The labelling period was followed by a chase period with excess of non-radioactive methionine and cysteine. Immunoprecipitation experiments with ACRM demonstrated that ICERγ isoforms were rapidly degraded, with a half-life of 3 h (Figure 3, top, lanes 1–4; Figure 3, bottom). Interestingly, there was a difference in the rate of degradation of the two ICER isoforms containing the γ domain (compare ICER I with ICER II; Figure 3, top, lanes 1–4): the slowest migrating isoform was reproducibly more stable than the three more rapidly migrating ICER isoforms.

The Ub–proteasome pathway is involved in the selective turnover of several key regulatory proteins, such as transcription factors and proteins that control the cell cycle [16]. The rapid degradation of ICER creates an instant switch that can precisely regulate the cAMP-induced transcriptional response. We therefore tested whether the proteasome pathway is involved in the degradation of ICER. Initially we tested whether lactacystin, a highly selective proteasome inhibitor [23,27], would inhibit the degradation of ICER. The results showed that 10 μM lactacystin significantly inhibited the degradation rate of all ICER isoforms (Figure 3, top, lanes 5–8; Figure 3, bottom). Densitometric analysis revealed that, in the presence of the inhibitor, the half-life of the ICERγ isoforms was greater than 9 h (Figure 3, bottom). To confirm our previous observations, we tested the effect of additional protease inhibitors on the turnover of ICER. Recently, several peptide aldehydes have been used as protease inhibitors to evaluate the participation of various proteolytic pathways in protein turnover in intact cells [28]. Two of these peptides, carbobenzoxyleucinyl-leucinyl-leucinal-H (LLL) and *N*-acetyl-leucinyl-leucinyl-methional-H (LLM), exhibit similar inhibitory activity against calpains and lysosomal cathepsins, but LLL is a much more potent proteasome inhibitor than LLM [28,29]. Our results showed that LLL completely blocked ICER degradation at a concentration of 20 μM, whereas LLM was not effective even at a concentration of 50 μM (results not shown). These results confirm the notion that the proteasome is the major

proteolytic system involved in the degradation of ICER in GH3 cells.

The proteasome is known to be involved in Ub-dependent and -independent proteolysis [16]. To discriminate between these two possibilities, we examined whether ICER can undergo polyubiquitination in GH3 cells. We used an approach similar to that developed by Treier et al. [19] to investigate ubiquitination of c-Jun, which involves isolation of Ub conjugates in the presence of 6 M GndHCl in order to inactivate isopeptidases. We transiently transfected GH3 cells with cDNA encoding His_6-Ub and, after cAMP stimulation and metabolically labelling, we recovered total His_6-Ub conjugates from cell lysates by precipitation with Ni^{2+}–NTA–agarose. ICER-His_6-Ub conjugates were then isolated from the Ni^{2+}–NTA–agarose eluates by immunoprecipitation with ACRM, and analysed by SDS/PAGE and fluorography. Figure 4 (lane 4) shows that lactacystin treatment followed by cAMP stimulation resulted in the accumulation of a typical histidine-tagged, Ub-conjugated ICER ladder [19,30]. The ladder consists of distinct low-molecular-mass products and a high-molecular-mass smear that are detectable only in His_6-Ub-transfected cells. This pattern corresponds to ICER proteins linked to a variable number of Ub residues. ICER–His_6-Ub conjugates were not detectable in His_6-Ub-transfected cAMP-stimulated cells that were not pretreated with lactacystin (Figure 4, lane 2), indicating that their steady-state levels were below the detection threshold of this assay. His_6-Ub-transfected non-cAMP-stimulated cells both in the presence (lane 3) and absence (lane 1) of lactacystin, and non-transfected cells (lane 5), were used as controls and represent the background noise of this assay. Collectively, the inhibition of ICER degradation by lactacystin and the accumulation of ICER–Ub conjugates in the presence of this inhibitor indicate that ICER is degraded by the proteasome–Ub pathway.

DISCUSSION

Excitable cells maintain tight control of their properties by controlling the transcription of the genes coding for voltage-gated ion channels [24]. We previously reported that cAMP regulates the expression of Kv1.5, a voltage-gated potassium channel, in primary cardiocytes and GH3 cells at the transcriptional level [24]. The attenuation of the cAMP-induced transcriptional response is believed to be regulated by several mechanisms [4–8]. Recently, it has been reported that cAMP induces the expression of a family of potent transcriptional antagonists, ICER, from an intronic promoter of the CREM gene [8]. The induction of ICER transcripts is rapid and independent of *de novo* protein synthesis. Tissue distribution of ICER revealed that these proteins play a crucial role in inhibiting the cAMP-dependent signal-transduction pathway in the neuroendocrine axis [8,11,13,14]. We report that ICER is also inducible in primary cardiocytes and myogenic cell lines and confirm previous observations regarding ICER expression in GH3 cells [8]. We speculate that ICER participates in regulating the expression of genes, such as Kv1.5, that control membrane potential and cell excitability in cardiac myocytes.

Membrane depolarization can induce the transcription of immediate early genes, such as c-*fos*, and other genes, such as proenkephalin and Kv1.5 [24–26]. This induction is mediated by a Ca^{2+}/calmodulin-dependent protein kinase, which phosphorylates CREB on the same residue as PKA [31], explaining the concordant transcriptional response of several genes to cAMP and depolarization. We demonstrated that the expression of Kv1.5 responds concordantly to cAMP and KCl depolarization in primary cardiocytes and GH3 cells [24]. In contrast, ICER responds discordantly to cAMP and depolarization. We speculate that this differential response may contribute to establishing different long-term programmes of gene expression in response to CREB phosphorylated by PKA versus CREB phosphorylated by calmodulin kinase.

ICER is the only known member of the CREB/ATF family of transcription factors that is cAMP-inducible and lacks a phosphorylation domain [2,8]. Its transcriptional activity, including the repression of its own promoter (P2), is therefore determined by the intracellular concentration of the four ICER polypeptides [8,12,14]. We reasoned that ICER degradation might be an important mechanism to limit the negative effect of ICER on the cAMP-inducible transcriptional response. Thus the identification of the proteolytic system(s) involved in ICER degradation would be essential to understand its function in living cells. We hypothesized that the Ub–proteasome pathway would be responsible for ICER turnover. Experiments designed to demonstrate directly the participation of this pathway should involve the detection of ICER–Ub conjugates. The detection of Ub–protein conjugates is often difficult, because these reaction intermediates are labile and their steady-state levels are very low [17–20]. Indeed, only 0.1–1 % of c-Jun is polyubiquitinated in HeLa cells [19]. Recently, Treier et al. [19] circumvented these problems by the overexpression in cells of His_6-Ub, which allowed them to isolate His_6-Ub conjugates by nickel chromatography in the presence of 6M GndHCl, followed by immunochemical methods to determine the presence of c-Jun in those conjugates. A similar approach was used to demonstrate polyubiquitination of the cystic fibrosis transmembrane conductance regulator [30] and STAT 1 (signal transduction and activator of transcription) [32]. The main advantage of this approach is that the strongly denaturing conditions used in the cell-lysis buffer inactivate isopeptidases that could disassemble the labile Ub conjugates during their isolation. Using a similar experimental approach, we determined that the intracellular levels of ICER in GH3 cells are regulated by Ub-dependent proteolysis, as indicated by the accumulation of ICER–Ub conjugates in cAMP-stimulated GH3 cells treated with lactacystin.

Of particular interest is the fact that the rate of degradation of ICER is likely to be regulated in a cell-specific manner. In Sertoli cells, stimulation with follicle-stimulating hormone induces a rapid and transient induction of ICER transcript, whereas elevated levels of ICER polypeptides persist for 36 h [14]. These data, together with our finding that the Ub system is involved in ICER breakdown, suggest that cells may control the levels of ICER proteins by a selective regulation of specific components of the Ub pathway. The involvement of the Ub–proteasome pathway in ICER turnover suggests a role of this proteolytic system in the regulation of biological processes in which the cAMP-induced transcriptional response is regulated by ICER. Specifically, ICER is known to be involved in processes such as circadian control of transcription in the pineal gland [11] and regulation of hormonal responses in the neuroendocrine axis [13,14]. Desdouets et al. [12] have recently proposed that ICER controls cell-cycle progression by regulating the expression of cyclin A. Thus Ub-mediated destruction of ICER may represent a novel control step at specific points in the cell cycle, in addition to the previously characterized role of the Ub pathway in the degradation of cyclins and the cyclin-dependent kinase inhibitor p27 [22,33].

These results highlight the role of the Ub–proteasome pathway in regulating the steady-state levels of a polypeptide that is a key inhibitor of the cAMP-induced transcriptional response. cAMP plays an important role in regulating the transcription of voltage-gated K^+ channels, such as Kv1.5, and therefore in cell excitation.

Thus the Ub–proteasome pathway may regulate cell excitation either by controlling the degradation of ion channels [30] or by regulating the level of transcription factors that may control the expression of proteins such as voltage-gated potassium channels. This latter form of regulating the expression of ion channels may represent a novel mechanism of controlling cell excitation.

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