Mitogen-activated Protein Kinase Phosphorylates and Targets Inducible cAMP Early Repressor to Ubiquitin-mediated Destruction*

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Inducible cAMP early repressor (ICER) is an important mediator of cAMP antiproliferative activity that acts as a putative tumor suppressor gene product. In this study, we examined the regulation of ICER protein by phosphorylation and ubiquitination in human chorionicarcinoma JEG-3 and mouse pituitary AtT20 cells. We found that cAMP stabilized ICER protein by inhibiting the mitogen-activated protein kinase (MAPK) cascade. Activation of the MAPK pathway increased ICER phosphorylation. ICER phosphorylation was abrogated by inhibition of the MAPK pathway either by cAMP or directly by the MAPK inhibitor PD098059. The MAPKs extracellular signal-regulated kinases 1 and 2 physically interact with ICER and mediated the phosphorylation of ICER on a critical serine residue (Ser-41). A mutant form of ICER in which Ser-41 was substituted by alanine had a half-life 4–5 h longer than its wild-type counterpart. This alteration in stability was due to the inability of the Ser-41-mutant ICER to be efficiently ubiquitinated and degraded via the ubiquitin-proteasome pathway. These results present a novel cell signaling cross-talk mechanism at the cell nucleus between the MAPK and cAMP pathways, whereby MAPK targets a repressor of the cAMP-dependent gene expression for ubiquitination and proteasomal degradation.

The MAPK pathway controls cell growth by regulating gene expression, protein synthesis, nucleotide synthesis, the interaction between cyclins and cyclin-dependent kinases, and protein stability by ubiquitin-mediated proteasomal degradation (Refs. 1 and 2 and references therein). In the classic pathway, activation of MAPK occurs by peptide growth factors that bind to a transmembrane tyrosine kinase receptor. This results in the activation of the small GTP-binding protein Ras that recruits a member of the Raf kinase family (Raf-1, A-Raf, or B-Raf) to the plasma membrane. Raf phosphorylates and activates the MEKs (MEK1 and MEK2), which in turn activates the MAPKs ERK1 and ERK2 by phosphorylation on threonine and tyrosine.

The MAPK pathway is tightly regulated by and cross-communicates with other signaling pathways. The best-characterized signal that regulates the activation of MAPKs is cAMP. cAMP inhibits the growth of fibroblasts cells (3–6), smooth muscle cells (7), and adipocytes (8), at least in part, by blocking the binding of Raf-1 to Ras (3), thus blocking the MAPK pathway. On the contrary, in phaeochromocytoma PC12 cells, cAMP induces activation of MAPK through PKA-induced activation of the Ras-related small G protein Rap1 (9). The activated Rap1 is both a selective activator of B-Raf and an inhibitor of Raf-1. Thus, in cells with little or no B-Raf (e.g. fibroblasts), cAMP inhibits the MAPK pathway.

In addition to cross-talk with the MAPK cascade at the cytosol, the cAMP/PKA pathway has a nuclear component. CREB and CREM are the two best-characterized families of transcription factors that mediate cAMP-driven gene expression (10, 11). Upon phosphorylation by PKA, CREB and CREM induce the transcription of CRE-containing genes. One of the induced proteins, ICER, is itself a product of the CREM gene (12, 13). cAMP strongly induces ICER expression by virtue of an intronic promoter in the CREM gene containing four CREs that in turns confers ICER-negative autoregulation. ICER strongly binds to CREs as a homodimer or heterodimer with CREB or CREM and represses gene expression. ICER consists of a family of four functionally indistinguishable isoforms, termed ICER-I, ICER-II, ICER-Iγ, and ICER-IIγ. In contrast to the rest of the CRE binding factors, ICER is not directly regulated by PKA phosphorylation because it lacks the PKA phosphorylation site.

ICER has been linked to regulation of cell growth (13–22). ICER blocks cells at the S and G2/M phases of the cell cycle (15). This blockage is the result of the observed inhibition by ICER of various growth-related genes, such as cyclin A (15, 16) and c-fos (14). ICER dramatically inhibits the growth and DNA synthesis of mouse pituitary tumor AtT20 cells and human chorionicarcinoma JEG-3 cells (14). This alteration in cell growth is coupled with a reduced ability of ICER-expressing cells to grow in an anchorage-independent manner and to form tumors in nude mice. In this scenario, ICER has the characteristic of a tumor suppressor gene product that mediates the antiproliferative activity of cAMP. Therefore, ICER represents yet another mechanism for the regulation of cell growth via the cAMP pathway.

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§ The abbreviations used are: MAPK, mitogen-activated protein kinase; ICER, inducible cAMP early repressor; MEK, MAPK kinase; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; CRE, cAMP responsive element; CREB, CRE-binding protein; CREM, CRE modulator; 8-Br-cAMP, 8-bromo-cAMP; EGF, epidermal growth factor.
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This study tested the hypothesis that the MAPK pathway regulates ICER. We found that cAMP stabilizes ICER protein by inhibiting the MAPK cascade. This stabilization is the result of inhibition of ICER phosphorylation by the MAPK Erk and subsequent ICER degradation by ubiquitin-mediated proteolysis. These findings present a novel cross-talk mechanism at the cell nucleus between the cAMP and MAPK pathway. Direct phosphorylation by ERK of a transcriptional repressor represents a previously undiscovered regulation of cAMP-mediated gene expression by the MAPK pathway.

EXPERIMENTAL PROCEDURES

Generation of Cell Lines, Culture Conditions, and Treatments—Several cell clones expressing a phosphorylation-mutant form of ICER-I/II were generated from the established cell line of mouse pituitary tumor AtT20 cells. AtT20 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended. These cell clones were established as described before (14, 15). JEG-3 (human choriocarcinoma) cells were obtained from the American Type Culture Collection and cultured as recommended. Cells were transfected with plasmids by the calcium phosphate co-precipitation technique as described previously (12, 14). JEG-3 and AtT20 cells were used because they were previously used in ICER inducibility experiments and their growth has been shown to be impaired by cAMP treatments (12, 14, 15, 23).

Cells were treated for the indicated times, or as indicated below, with various chemicals as follows: 0.5 mM 8-Br-cAMP (Sigma), 10 μg/ml cycloheximide (Sigma), 100 μg/ml PD098059 (Biomol Inc., Plymouth Meeting, PA), 50 ng/ml EGF (Oncogene Science), 50 μg/ml prosomes inhibitor MG115 (Sigma).

Plasmids—ICER-I/II (pSVICER-I/II), ICER-II (pAla-41), and CREM (pSVCREM) cDNAs used for transient transfection are under the SV40 promoter (12, 15, 30). pFLAGCMV2-hCDC34 (29) was used for transient transfections for the expression of the ubiquitin-conjugating enzyme Cdc34.

Assays—Western blot, RNase protection analyses, and co-immunoprecipitation were performed as described previously (12, 14–16, 23, 29, 35, 36). CREM/ICER specific antisera has been previously characterized and extensively used (12, 14–16, 29). Anti-phospho-p44/42 MAPK (phospho-ERK1&2) monoclonal antibody was purchased from Sigma and used as specified by the vendor. Anti-total-ERK1&2 polyclonal antibody was purchased from Upstate Biotechnology and used as specified by the vendor. Anti-ubiquitin monoclonal antibodies have been previously characterized and were used at a 1:50 dilution as described elsewhere (37). The expression of α-tubulin was determined by Western blot using a monoclonal antibody from Santa Cruz Biotechnology to confirm equal loading of proteins from cell extracts (not shown).

The human ICER riboprobe was generated by reverse transcription-polymerase chain reaction from RNA of JEG-3 cells treated with 8-Br-cAMP to induce the expression of ICER. The oligonucleotides used to amplify human full-length ICER cDNA were CGG GAT CCA ACA TGG CTG TAA CTG GAGA and CGG AAT TCA TGC TGT AAT CAG TTC, and 39 nucleotides of the DNA binding domain (12) was subcloned into pBluescriptII/Blue. All sequences are 5'-3'. For each oligonucleotide, a complementary strand was also synthesized. In some cases, a second mutation was introduced to produce a convenient restriction site for rapid screening. All mutations were confirmed by sequencing.

RESULTS

cAMP Stabilizes ICER Protein—We have previously shown that in JEG-3 and AtT20 cells, cAMP cooperates with ICER to inhibit cellular growth (14, 15). We wanted to study the nature of this cooperation. The regulation of ICER protein and RNA was determined after chronic (constant) or acute (1 h) treatment of JEG-3 and AtT20 cells with 8-Br-cAMP. In the experiment shown in Fig. 1A, it was found that the endogenous ICER protein was induced to similar maximal levels after 3 h in chronically or acutely treated JEG-3 cells. In acutely treated cells, the protein levels of ICER returned to baseline by 12 h after treatment. However, maximal levels of ICER protein were maintained for at least 12 h in the chronically treated cells. This prolongation of ICER protein cannot be attributed to differences in RNA levels, because the induction of ICER RNA was longer in acutely treated cells than in chronically treated cells (Fig. 1B). In both acutely and chronically treated cells, ICER RNA was maximally induced after 3 h. In chronically treated cells, ICER RNA returned to basal levels by 4 h. In acutely treated cells, the induction was maintained until 8 h, possibly as a consequence of a lack of a strong negative auto-regulation (12, 23). These results suggest that cAMP stabilizes ICER protein.

In order to test this hypothesis, the induction of ICER protein was determined in chronically and acutely 8-Br-cAMP-treated cells after inhibition of de novo protein synthesis by treatment with cycloheximide. In a manner similar to that shown in Fig. 1A, ICER protein levels were found to return to basal levels in acutely treated cells but remained elevated in chronically treated cells after 12 h (Fig. 1C). To further test whether cAMP altered ICER protein stability, we compared the half-life of transiently transfected ICER between 8-Br-cAMP-treated cells and untreated cells. We found that the half-life of transfected ICER was 4 h longer in cells chronically treated with 8-Br-cAMP as compared with untreated cells (Fig. 1D). These results demonstrate that the cAMP pathway not only induced ICER RNA and protein levels as demonstrated before (12–18, 29) but also regulated ICER protein stability. In this study, we examined the molecular mechanism of these phenomena and its implications on the regulation of ICER protein.

MAPK Pathway Regulates the Stability of ICER Protein—Because cAMP and MAPK pathways cross-communicate (3–8), we determined whether the MAPK pathway affected the kinetic of ICER protein inducibility by cAMP. JEG-3 cells were acutely treated with 8-Br-cAMP in serum-free medium, and the levels of ICER protein were determined at 9, 12, and 16 h after the addition of serum and in cells maintained in serum-free media. After 16 h, the protein levels of ICER were found to be 5 times higher in cells maintained in serum-free medium than in medium with 10% serum (Fig. 2A). Furthermore, inhibition of the MAPK pathway by PD098059 completely abol-
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A. determination of the protein levels of ICER by Western blot analysis in JEG-3 cells treated either acutely or chronically with 8-Br-cAMP. In the acutely treated cells, the medium containing 8-Br-cAMP was replaced with fresh medium after 1 hr of treatment. At this time, one sample was harvested for total protein extraction (1 hr), and the rest were harvested at the indicated times after treatment. The chronically treated cells were harvested for total protein extraction at the indicated times. 0 hr represents untreated controls. Bottom panel, the levels of ICER protein were quantified by densitometry and presented as fold of induction over the control. Values shown are means ± S.E. (n = 3 independent experiments). Similar results were obtained with AtT20 cells (not shown).

B. determination of the RNA levels of ICER by RNase protection analysis in JEG-3 cells treated chronically or acutely with 8-Br-cAMP as before in A. Bottom panel, the band corresponding to the ICER-protected fragment was quantified by densitometry and presented as fold of induction over the control. Values shown are means ± S.E. (n = 3 independent experiments). Similar results were obtained with AtT20 cells (23).

C. determination of the protein levels of ICER by Western blot analysis in JEG-3 cells after inhibition of de novo protein synthesis. Cells were treated acutely (a) or chronically (c) with 8-Br-cAMP as before. After 2 hr, cells were treated with cycloheximide (+CHX). Bottom panel, the levels of ICER protein were quantified by densitometry and presented as fold of induction over the control. Values shown are means ± S.E. (n = 3 independent experiments). Similar results were obtained with AtT20 cells (not shown).

D. effect of cAMP on ICER protein half-life. Cells were transiently transfected with ICER-IIγ and treated chronically with 8-Br-cAMP (cAMP) or left untreated (control). Values shown are means ± S.E. (n = 3 independent experiments).

**CAMP Antagonizes MAPK Pathway in JEG-3 and AtT20 Cells**—cAMP has been shown to either inhibit or activate the MAPK pathway according to cell type (3–8). Here, we studied the regulation of MAPK pathway by cAMP in JEG-3 and AtT20 cells. Activation of the MAPK pathway was assessed by measuring the level of phosphorylation of the MAPKs ERK1 and ERK2 and by measuring the kinase activity of ERK1 and ERK2 after immunoprecipitation. We observed that activation of ERK1 and ERK2 was strongly inhibited in cAMP-pretreated JEG-3 and AtT20 cells (Fig. 3, A and B). As expected, the MAPK inhibitor PD098059 also inhibited ERK1 and ERK2 activation (Fig. 3, A and B). We then determined the effect of 8-Br-cAMP on the growth of JEG-3 and AtT20 cells. Activation of the cAMP pathway inhibited the growth of both these cell lines (Fig. 3C). JEG-3 cells were more sensitive to the antiproliferative activity of cAMP compared with AtT20 cells. We attribute these differences to the fact that AtT20 cells express low levels of B-Raf (Fig. 3D). B-Raf was shown to modulate cAMP activation of the MAPK pathway in PC12 cells (10). These data imply that JEG-3 and AtT20 cells are appropriate models to study the regulation of ICER protein stability by the MAPK pathway in cells in which cAMP antagonizes the MAPK pathway.

**ICER Phosphorylation Is Induced by Activation of the MAPK Pathway**—Phosphorylation is a common posttranslational modification that may regulate ICER protein stability. ICER does not contain the consensus phosphorylation site for PKA (12) and has not been shown to be phosphorylated as other CREM isoforms (11). The phosphorylation of ICER protein was studied under conditions of high and low MAPK activity. We found that activation of the MAPK pathway by EGF or serum treatment induced the phosphorylation of both endogenous and ectopically expressed ICER protein (Fig. 4). This induction of ICER phosphorylation occurred mostly in a discrete single tryptic phosphopeptide labeled phosphopeptide 3, and exclusively on a serine residue(s). Inhibition of the MAPK pathway by cAMP or PD098059 inhibited the MAPK-induced phosphorylation of ICER on phosphopeptide 3. The finding that basal levels of ICER phosphorylation were high and that inhibition of MAPK did not inhibit these basal levels suggests that ICER might be phosphorylated by other kinases not necessarily related to the MAPK pathway.

**ICER Is Phosphorylated by the MAPK ERK1 on Serine 41**—Because EGF activates the mitogen-activated protein signaling pathway (25) and inhibition of this pathway by cAMP and PD098059 reduces ICER phosphorylation (Fig. 3, A and B), we investigated whether ICER is a substrate for the MAPKs. The fact that PD098059 blocks the MAPK pathway by inhibiting MAPK kinase MEK phosphorylation of the MAPK ERK (24) suggested to us that one of these kinases directly phosphorylates ICER. From the outset, we suspected that MEK was not a suitable candidate, because in the experiment shown in Fig. 4, we found that ICER was uniquely phosphorylated on serine residues. To test which kinase was capable to phosphorylate ICER protein in vitro, we performed a kinase assay using bacterially purified ICER-IIγ protein (12) as a substrate and purified ERK1 and constitutively active MEK1 proteins (26). We found that ERK1 and not MEK1 phosphorylated ICER-IIγ (Fig. 5A). ERK1 phosphorylated ICER-IIγ on serine residues and on tryptic peptide 3 as found before in JEG-3 cells. Further...
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**Fig. 2. Regulation of ICER protein stability by the MAPK pathway.** A, determination of the protein levels of ICER by Western blot analysis in JEG-3 cells in the presence or absence of serum. After 16 h in serum-free medium, cells were treated for 1 h with 8-Br-cAMP to induce endogenous ICER protein levels. 3 h after being washed with serum-free medium, the cells were cultured with (+) or without (−) 10% fetal bovine serum. Cells were harvested for total protein extraction at the indicated times after treatment. 0 hr represents untreated control. Bottom panel, the levels of ICER protein were quantified by densitometry and presented as fold of induction over the control. Values shown are means ± S.E. (n = 3 independent experiments). Similar results were obtained with AtT20 cells (not shown). B, determination of the protein levels of ICER by Western blot analysis in JEG-3 cells after inhibition of the MAPK pathway. After 16 h in serum-free medium, cells were treated (+) for 1 h with 8-Br-cAMP (cAMP) to induce endogenous ICER protein levels. 3 h after being washed, cells were cultured with (+) or without (−) 10% fetal bovine serum (serum) and with (+) or without (−) the MAPK inhibitor PD098059 as indicated. Cells were harvested 16 h after treatment for total protein extraction. Bottom panel, the levels of ICER protein were quantified by densitometry and presented as fold of induction over the control. Values shown are means ± S.E. (n = 3 independent experiments). Similar results were obtained with AtT20 cells (not shown). C, effect of PD098059 on ICER protein half-life. Cells were transiently transfected with ICER-II and treated with PD098059 or left untreated (control). Values shown are means ± S.E. (n = 3 independent experiments).

Furthermore, using co-immunoprecipitation experiments, we demonstrated that endogenous ICER and ERK1&2 proteins physically interact with each other in JEG-3 cells (Fig. 5B). A kinase assay performed in this immunocomplex confirmed that ICER is phosphorylated by ERK1&2 exclusively on serine residues and on tryptic peptide 3. We believe that the ICER-MAPK interaction is mediated via two putative MAPK docking motifs (27) in ICER protein at amino acids 51–53 (sequence, RRKR) and 68–72 (sequence, RRKKG).

To determine which serine residue(s) was phosphorylated by ERK, we performed site-directed mutagenesis of all five individual serine residues present in ICER-II protein (Fig. 5C). We termed these mutants ICER-II (Ala-35), ICER-II (Ala-38), ICER-II (Ala-41), ICER-II (Ala-80), and ICER-II (Ala-104). We found that substitution of serine 41 to alanine (ICER-II (Ala-41)) at the putative MAPK phosphorylation site was sufficient to completely abolish the phosphorylation of ICER-II (Fig. 5D) on tryptic peptide 3. EGF was not capable of inducing phosphorylation of ICER-II (Ala-41) at tryptic peptide 3, demonstrating that EGF specifically phosphorylates ICER on serine 41. EGF, in turn, induced the phosphorylation of ICER-II (Ala-35) and the other three mutants (not shown). The fact that serine 41 is conserved among all four isoforms of ICER (12, 13) suggests that serine 41 is also a phosphorylation site for MAPK in the other isoforms. Other tryptic peptides were found to be marginally phosphorylated but not induced by activation of the MAPK pathway, suggesting that ICER may be phosphorylated by kinases other than MAPK.

**Mutation on MAPK Phosphorylation Site Lengthens the Half-life of ICER**—Phosphorylation did not affect ICER activity as a transcriptional repressor, binding to a consensus CRE or nuclear localization on transient transfection experiments (not shown). Phosphorylation may therefore regulate ICER half-life because cAMP stabilizes ICER protein (Figs. 1 and 2) and blocks ICER phosphorylation (Fig. 4). To test this hypothesis, the half-life of the wild-type ICER-II was compared with the half-life of the mutant ICER-II (Ala-41). JEG-3 cells were transiently transfected with plasmid containing the corresponding cDNAs and treated or not with 8-Br-cAMP. We found that the half-life of transfected ICER-II (Ala-41) was at least 5 h longer than the wild-type form (Fig. 6). In addition, the half-life of ICER-II from cells treated with 8-Br-cAMP was similar to the half-life of ICER-II (Ala-41) from cells treated or not treated with 8-Br-cAMP. To further study the effect of Ser-41 phosphorylation on ICER-II...
Fig. 4. Regulation of ICER phosphorylation by the MAPK pathway. Phosphorylation of the endogenous ICER is induced by activation of the MAPK pathway. A. After 16 h in serum-free medium, JEG-3 cells were treated as follows: with EGF or 10% fetal bovine serum (serum) for 10 min, with 8-Br-cAMP 30 min prior to EGF (cAMP + EGF), or with PD098059 30 min prior to EGF (PD + EGF). Phospho-ICER from labeled cells was immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis. The amount of radioactivity on the bands corresponding to phospho-ICER was determined in counts per min (CPM) before subjected to tryptic phosphopeptide map (PPM). The identity of the major phosphopeptides (phosphopeptide 2 and 3) was confirmed by mixing 100 cpm of endogenous immunoprecipitated phospho-ICER from EGF-treated and serum-treated cells in a single map (mixture). Phosphopeptide 2 was found to be an oxidation isomer of phosphopeptide 3; therefore, they represent the same tryptic peptide (not shown). The EGF and serum treated samples were subjected to phosphoamino acid analysis (PAA). The identity of the phosphoamino acid was determined by co-migration with nonradioactive phosphoserine, phosphothreonine, and phosphotyrosine. As loading control for protein stability, the half-life of ICER-II/H9253 was compared with the half-life of ICER-II/Ala-41) was about 4 h longer than the half-life of ICER-II/Ala-41) in the stable clones. Treatment with 8-Br-cAMP did not affect ICER-II/Ala-41) half-life but lengthened ICER-II/Ala-41) half-life by 4 h. These data suggest that ICER phosphorylation at serine 41 by the MAPK pathway targeted endogenous ICER protein stability.

Phosphorylation at Serine 41 Targets ICER for Degradation by the Ubiquitin-Proteasome Pathway—It was recently shown that the half-life of ICER was regulated by degradation via the proteasome-ubiquitin pathway (28, 29). In this study, we found that ICER stability is regulated by phosphorylation. Thus, it is likely that ICER phosphorylation regulates ICER ubiquitination and subsequent degradation by the proteasome-ubiquitin pathway. Using stable clones of the AtT20 cells, we tested the ability of ICER-II/Ala-41) and ICER-II/Ala-41) to be ubiquitinated. ICER and ubiquitinated ICER were detected by Western blot after the cells were treated with the proteasome inhibitor MG115 in order to cause accumulation of polyubiquitinated peptides (Fig. 7A). Wild-type ICER-II/Ala-41) was found to be efficiently ubiquitinated and accumulated as shown before (28, 29). However, the expression of ubiquitinated intermediates was not altered in two independent AtT20 cell clones expressing different levels of ICER-II/Ala-41). The identity of the ubiquitinated intermediate of ICER-II/Ala-41) was confirmed by Western blot analysis after immunoprecipitation with ICER antibodies. To test ICER-II/Ala-41) ubiquitination in another system, JEG-3 cells were transiently transfected with the cDNAs for ICER-II/Ala-41) and ICER-II/Ala-41), and ubiquitination was assessed after treatment with MG115 as before. Consistent with the previous findings, ICER-II/Ala-41) was not efficiently ubiquitinated as the wild-type ICER-II/Ala-41) (Fig. 7B). Inhibition of the proteasome did not cause a significant accumulation of the ubiquitinated intermediates in ICER-II/Ala-41)-transfected cells. Furthermore, we did not observe a significant effect of MG115 on the accumulation of the nonubiquitinated ICER protein. This may be due to the strong expression of the transfected cDNAs that are driven by an SV-40 promoter. These data suggest that the phosphorylation of ICER at Ser-41 targets ICER for ubiquitination.

Among the CREM isoforms, ICER has been uniquely shown to be a substrate of the ubiquitin-conjugating enzyme CDC34 (29). ICER physically interacts with and becomes ubiquitinated by CDC34, to be degraded by the proteasome (29). To further understand the effect of Ser-41 phosphorylation on ICER ubiquitination and proteasomal degradation, JEG-3 cells were transiently co-transfected with the cDNAs for CDC34, with ICER-II/Ala-41) or ICER-II/Ala-41), and with CREM7. As shown before (29), CDC34 caused ICER-II/Ala-41) to be degraded (Fig. 7C). However, expression of CDC34 did not affect the expression of ICER-II/Ala-41) protein at any of the two concentrations tested. The expression of the internal control, CREM7, was not affected by CDC34 expression, as shown before for other CREM isoforms (29). These data suggest that ICER phosphorylation on Ser-41 is necessary to target ICER for degradation by the ubiquitin-conjugating enzyme CDC34.

To determine whether the MAPK and cAMP pathways regulated ubiquitination of endogenous ICER, we examined ubiquitination of ICER in JEG-3 cells after acute induction of ICER by 8-Br-cAMP, in the presence or in the absence of serum (Fig. 7D). We found that serum induced the accumulation of ubiquitinated ICER after inhibition of the proteasome. Inhibition of the MAPK pathway by PD098059 or chronic treatment with 8-Br-cAMP abolished the serum-induced ubiquitination of ICER. These data demonstrate that activation of the MAPK pathway targeted endogenous ICER for ubiquitination.

DISCUSSION

In this study, we discovered a novel mechanism of cell signaling cross-talk at the cell nucleus between the MAPK and cAMP pathways. We found that activation of the MAPK pathway by growth factors targeted the transcriptional repressor (12) and putative tumor suppressor ICER (14) for degradation by the proteasome-ubiquitin pathway. MAPK-directed ICER degradation may result in the alleviation of transcriptional...
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**Fig. 7. Effect of phosphorylation on ICER ubiquitination.** Western blot analysis of ICER proteins after treating (+) or not (−) the cells with the proteasome inhibitor MG115 (PI). A, an AtT20 stable cell clones expressing ICER-IIy(Ala41) and two independent AtT20 stable cell clones expressing ICER-IIy(Ala41). Right panel, Western blot analysis of ubiquitinated ICER protein (Wantubi) after immunoprecipitation with CREM/ICER-specific antibodies (IP/ICER) from an ICER-IIy-expressing AtT20 clone. B, transiently transfected JEG-3 cells with ICER-IIy or ICER-IIy(Ala41). Control cells were transfected with an empty vector. C, Western blot analysis of ICER and CREM proteins from cells transiently co-transfected with ICER-IIy or ICER-IIy(Ala41) and 5 μg (+) or 10 μg (++) of a mammalian expression plasmid encoding for the ubiquitin-conjugating enzyme CDC34 (12). Cells were co-transfected with CREM as an internal control (12). D, Western blot analysis of endogenous ICER and ubiquitinated ICER proteins from JEG-3 cells treated (+) or not (−) as indicated. Cells were incubated for 16 h in serum-free medium, before treatments. Acutely treated cells (ac) were incubated for 1 h with S-Br-cAMP (cAMP) in serum-free medium. The cells were washed and fed with fresh serum-free medium. 1 h later, the cells were treated (+) or not (−) with the proteasome inhibitor MG115 (PI). 30 min later, the cells were treated (+) or not (−) with the MAPK inhibitor PD98059 (PD). After 30 min, 10% serum was added (+) or not (−). After 2 h, cells were harvested. Chronically treated cells (ch) were incubated with S-Br-cAMP in serum-free medium for the full 5 h and treated with proteasome inhibitor and serum as were the acutely treated cells. The relative migration of protein molecular mass markers is indicated (kDa). Also indicated are CREM, ICER, and several polyubiquitinated forms of ICER (*, **, ***, and ICER-Ub). The relative mobility for the immunoglobulin heavy and light chains is indicated (Ig).

between the MAPK and cAMP pathway might have important implications in the hormonal response of endocrine and neuroendocrine cells. For instance, ICER was found to be strongly induced by luteinizing hormone but not induced by follicle-stimulating hormone in ovarian granulosa cells (40). Follicle-stimulating hormone and luteinizing hormone have different effects upon granulosa cell growth; the former appears to maintain and/or stimulate cell proliferation, whereas the latter appears to inhibit growth. Despite these disparate effects on granulosa cell growth, the actions of both follicle-stimulating hormone and luteinizing hormone are mediated by cAMP (41). Therefore, it is surprising that ICER was found to be preferentially induced by luteinizing hormone in these cells. Thus, it is possible that, depending on the cell type or developmental status of a cell, ICER regulation by the cross-talk between the MAPK and cAMP pathway might be opposed. In this study, we examined ICER regulation in cells in which MAPK and cAMP pathways might be antagonistic. A yet to be tested hypothesis is that cells such as PC12, in which cAMP activates the MAPK pathway, cAMP would increase ICER phosphorylation by ERKs and would subsequently direct ICER for proteasomal degradation.

We have previously shown that ICER has antioncogenic characteristics (14, 15). Therefore, the regulation of ICER protein stability by MAPK phosphorylation may have important consequences in cell transformation. The MAPK pathway is activated in a large number of human tumors, mainly due to constitutive activation by oncogenic Ras. Based on our results, one could postulate that ICER protein might be targeted to degradation in tumors in which ras is mutated. For instance, the MAPK pathway has been found to be constitutive activated in advanced prostate cancer (31). We have recently discovered that ICER protein expression in epithelial cells of human prostate tumors was significantly lower than in normal prostate epithelial cells (42). Furthermore, in two prostate tumor cell lines, LNCaP and DU 145, ICER protein expression was found to be lower than in nontransformed prostate cells and uninducible by cAMP. In yet another prostate cancer cell line, PC-3, ICER protein was abnormally regulated by cAMP. These observed lower levels of ICER protein expression in human prostate tumors and in prostate tumor cell lines are not completely understood. In view of the results described in this report, we hypothesize that ICER protein might be targeted to proteasomal degradation in prostate cancer cells. MAPK regulation of ICER might have far-reaching consequences in human cancer because the ras oncogene is mutated in over 30% of all human cancers (38) and the MAPK kinase pathway is regulated by other signaling pathways targeted to deregulation in other human cancers (39).

CREM-mutant mice have been generated by homologous recombination, resulting in the mutant male mice being sterile due to postmeiotic arrest at the first step of spermatogenesis (32, 33). However, the CREM-null mice have not been reported to be tumor-prone. Judging by the apparent pleiotropic function of CREM, such specific phenotype is surprising. We observed that the homozgous mutant mice lacked activators and repressors such as CREM and ICER. Therefore, the phenotype cannot be attributed to the lack of expression of a specific CREM isoform. However, we suspected that the balance mechanism controlling CREM-mediated gene expression in these animals may be affected in two opposite directions, which would result in mutual cancellation without other apparent phenotypic differences observed. However, redundancy in the CREM-null mice could be the culprit. It is possible that many of the functions of the CREM are taken over by CREB or other CRE-binding proteins that function as transcriptional activators. Because the CREB-null mice shows impaired fetal T cell development and perinatal lethality (34), this hypothesis cannot be tested with this mouse model. Based on the differences in the presence of ICER in these two genes, we propose the development of ICER-null mice to clarify these discrepancies.
and to be used as an animal model to further study the anti-oncogenic characteristics of ICER.

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