Supporting Information

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SI Text

SI Results: I3C Regulation of Cyclin E Protein Processing and the Cyclin E-CDK2 Protein Complex in the Highly Invasive MDA-MB-231 Breast Cancer Cells. Estrogen-unresponsive MDA-MB-231 cells are a highly proliferative and invasive human breast cell line that is representative of late-stage breast cancer where high levels of elastase are considered to be an important marker for disease progression (1–3). This cell line is sensitive to the I3C growth arrest (4) and therefore was used to examine the potential effects of I3C on processing of the 50-kDa cyclin E protein. MDA-MB-231 cells were treated with or without 100 μM I3C throughout a 72-h time course, and Western blots were used to analyze production of the cyclin E protein forms. In the absence of I3C treatment, these cells predominantly produce the lower-molecular-mass 35-/33-kDa forms of cyclin E and accumulate relatively little of the 50-kDa cyclin E protein (Fig. S1*A*). Throughout the time course of I3C treatment, the cellular levels of the 50-kDa cyclin E protein significantly increased with a corresponding decrease in the lower-molecular-weight forms. By 72 h of indole treatment, in which the cells undergo a G_1 cell cycle arrest (4), the 50-kDa cyclin E protein was the predominant form whereas no change was observed in the total cellular level of CDK2.

Cyclin E coimmunoprecipitations were used to examine the composition and function of the CDK2–cyclin E protein complex in 72-h I3C-treated and untreated cells. As shown in Fig. 1*B*, there were no changes in the association of CDK2, p21, or p27 with cyclin E protein regardless of whether the predominant cyclin E was the higher- or lower-molecular-weight form. The native CDK2–cyclin E protein complex that forms in I3C treated cells is larger compared with untreated cells (data not shown) because of the association of the higher-molecular-weight form of cyclin E. Using histone H1 as a substrate, the coprecipitating CDK2 was significantly less active in I3C-treated cells compared with untreated cells (Fig. S1*B*, lower lanes). Thus, consistent with the known effects of the cyclin E protein forms on CDK2 activity (5) in MDA-MB-231 breast cancer cells, the lower-molecular-weight forms of cyclin E are associated with high CDK2 enzymatic activity whereas the 50-kDa cyclin E form is associated with a significant reduction in CDK2 enzymatic activity.

SI Methods

Materials. IMDM, FBS, calcium- and magnesium-free PBS, L-glutamine, penicillin/streptomysin, and typsin-EDTA were supplied by Cambrex/Biowhittaker. I3C and DIM were purchased from LKT Laboratory, and [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from NEN Life Science Products. Insulin (bovine), tamoxifen, tryptophol, human leukocyte elastase, α_1-chymotrypsin, trypsin, thrombin, elastatinal, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (CMK), fibrinogen, gelatin, elastin, and *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MetS) were purchased from Sigma. GenomeWide siRNA for human leukocyte elastase was obtained from Qiagen. Antibodies to cyclin E1, p21, CDK2, CDK4, and actin were purchased from Santa Cruz Biotechnology, and antibodies to human neutrophil elastase were obtained from Labvision. Secondary antibodies conjugated with Texas red fluorescent probes were purchased from Invitrogen.

Cell Culture and Flow Cytometry. MDA-MB-231 cells were cultured in IMDM supplemented with 10% FBS, 2 mM L-glutamine, and 1.25 mL of 20,000 units/mL penicillin and streptomycin. Cells were incubated at 37 °C in humidified air containing 5% CO_2. I3C, DIM, tamoxifen, and tryptophol were dissolved in DMSO (99.9% HPLC grade; Aldrich) at concentrations 1,000-fold higher than the final concentrations used. For flow cytometric analysis of DNA content, MDA-MB-231 cells were plated at 30% confluency on 100-mm tissue culture dishes and then cultured under the conditions described in the text. The cells were harvested and hypotonically lysed in propidium iodide, cell debris was filtered, and then 10,000 nuclei were analyzed for nuclear-emitted fluorescence by using a Coulter Elite Instrument flow cytometer (Coulter XL-3) as we previously described (6). Individual samples were then analyzed with EPICS software, and the data were further subject to numerical analysis using Multicycle and Excel software for graphic interpretation.

Western Blot Analysis. After the indicated treatments, cells were washed once with cold PBS, harvested, and in the appropriate experiments lysed in either immunoprecipitation lysis buffer (250 mM NaCl/0.1% Triton X-100/50 mM Tris·HCl, pH 7.3) or radioimmune precipitation buffer (150 mM NaCl/0.5% deoxycholate/0.1% Nonidet P-40/0.1% SDS/50 mM Tris) containing protease and phosphatase inhibitors (50 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 5 μg/mL leupeptin, and 0.1% NaF), 10 μg/mL β-glycerophosphate, and 0.1 mM sodium orthovanadate. Western blot analyses of eletrophoretically fractionated samples were carried out as previously described (7).

Cyclin E Immunoprecipitation and Histone Kinase Assays. Cells were lysed for 15 min at 4 °C in immunoprecipitation lysis buffer with protease and phosphatase inhibitors (see above), and samples (500–800 μg of protein) were precleared for 30 min at 4 °C with 30 μL of a 1:1 slurry of protein G-beads (Amersham Pharmacia) with 1 μg of rabbit IgG (Sigma). After a brief centrifugation to remove the precleared beads, 0.5 μg of cyclin E1 antibodies were added to each sample and incubated on a rocking platform at 4 °C for 2 h, and then 30 μL of the 1:1 bead slurry was added to each sample and incubated for an additional 30 min at 4 °C. The beads were than washed 4 times in immunoprecipitation lysis buffer with protease and phosphatase inhibitors and twice with kinase buffer (50 mM Hepes, pH 7.3/5 mM $MnCl_2$/10 mM $MgCl_2$) with protease and phosphatase inhibitors. Half of each immunoprecipitation was subjected to Western blot analysis to confirm the efficiency of the immunoprecipitation. The other half of the immunoprecipitation was assessed for protein kinase activity in the presence of 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) using 0.21 μg of Histone H1 as the substrate as previously described (7).

Elastase Enzyme Kinetics. For the enzyme kinetic analysis of I3C inhibition of human elastase proteolysis of in vitro synthesized cyclin E, the final concentrations of cyclin E (0.12 ng, 0.36 ng, 0.6 ng, 1.2 ng, and 1.8 ng) and of I3C (0 μM, 15 μM, 25 μM, and 50 μM) were varied in the elastase reactions. Because cyclin E proteolysis involves the generation of multiple cyclin E degradation products, the total amount of product formed was indirectly determined by subtracting the amount of product remaining (50-kDa cyclin E) after the enzyme reaction from the total amount of substrate at the beginning of each reaction. Autoradiographic exposures of Western blots were scanned with a UMAX UC630 scanner, and band intensities were quantified by using the NIH Image program. Autoradiographs from a minimum of 3 independent experiments were scanned per time

point. All values represent percentage of control (no inhibitor added).

For the assays employing the chromogenic substrates, 5×10^{-5} units of human neutrophil elastase were used in each in vitro reaction containing increasing concentrations of substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (0.5–5 mM) in the presence of varying concentrations of I3C (0 μM, 15 μM, 25 μM, and 50 μM). Substrates and inhibitors were all dissolved in DMSO, and the 200-μL in vitro reactions were incubated in 100 mM Tris·HCl (pH 7.5) and 250 mM NaCl. for 10 min at 37 °C. The release of *p*-nitroaniline was monitored at 410 nm. Specific elastase activity was expressed in the amount of *p*-nitroaniline produced per minute of reaction time.

Double reciprocal plots were generated by using EnzymeKinetics Pro (ChemS), which generated the Lineweaver–Burk plots. A nonlinear regression analysis was used to accurately calculate the K_m and V_{max} because small changes in the initial velocity can lead to large errors in the reciprocal of velocity used in the double reciprocal plots. For the determination of the inhibitory coefficient (K_i), the reaction velocity was measured at a fixed concentration of substrate (1.2 ng) in the presence of various I3C concentrations. A Dixon plot was generated by graphing the reciprocal of the velocity vs. inhibitor concentrations from 2 different concentrations of substrate (0.6 ng and 1.2 ng of cyclin E). The vertical line dropped from the point where the lines intersect each other down to the inhibitor axis determines the calculated negative K_i.

Zymographic Determination of Enzyme Activity. For the modified zymographic procedure (8), purified human leukocyte elastase, gelatin, or fibrinogen was mixed in nondenaturing 4× protein loading buffer and incubated at room temperature for 15 min. Samples were loaded onto a 10% polyacrylamide gel (with SDS) containing elastin (0.01 g per gel), gelatin (0.005 g per gel), or fibrinogen (0.01 g per gel) for the assessment of elastase, trypsin/chymotrypsin, and thrombin enzymatic activity, respectively. The gels were washed with 2.5% Triton X-100 for 30 min followed by incubation at 37 °C with 1× Zymogram developing buffer (Invitrogen) for 25 h. For enzyme inhibition assays, individual lanes were incubated with the indicated molecules that were diluted in the developing buffer to the stated concentrations. The gels were stained in 30% methanol/10% acetic acid containing Coomassie Brilliant Blue R-250 and destained in the same solution without the dye. Clear bands against the dark blue background indicate degradation of the substrate (elastin, gelatin, fibrinogen) by the proteases used in the assay.

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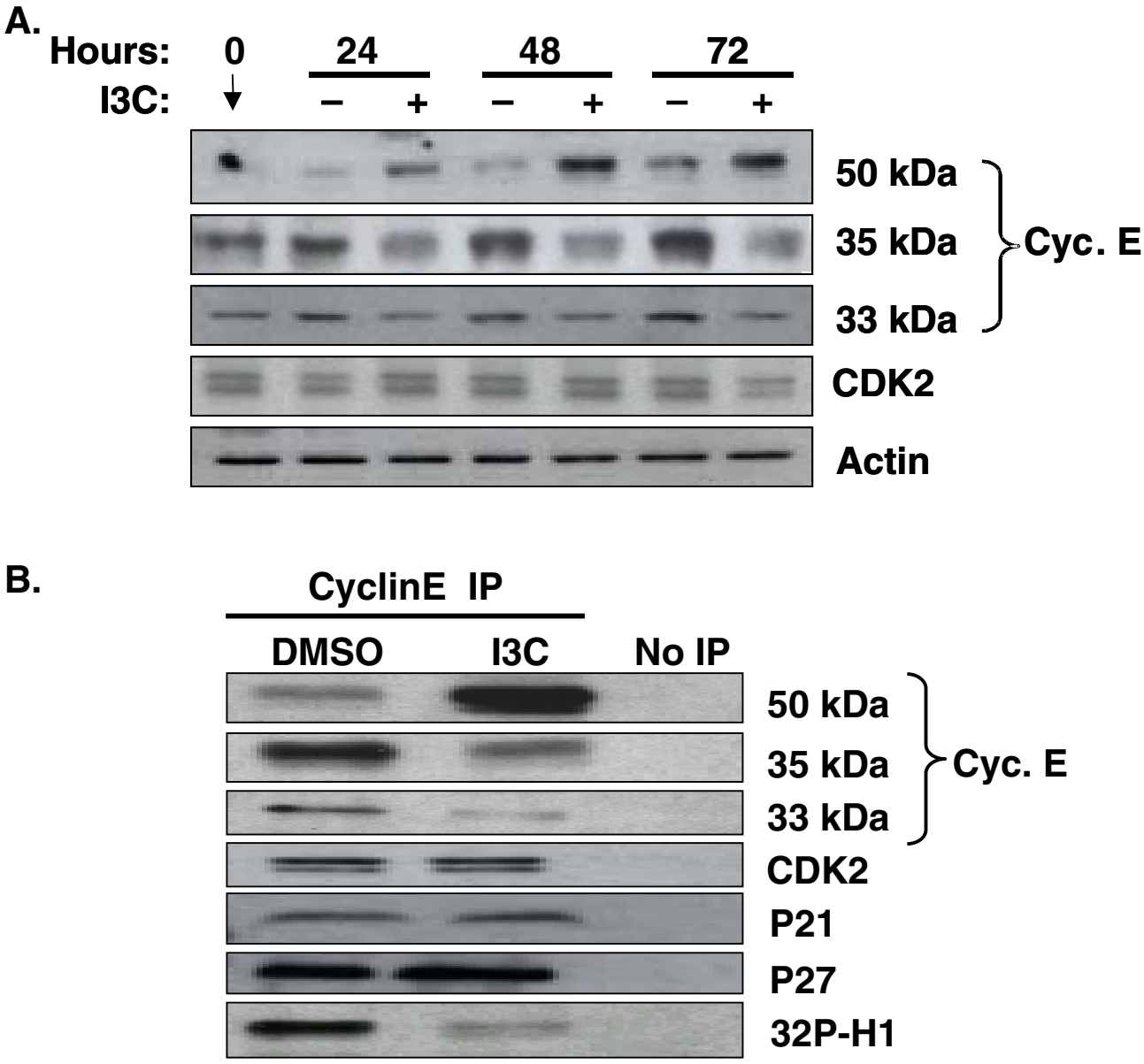

Fig. S1. Effects of I3C on the intracellular processing of the 50-kDa cyclin E protein, and composition and function of the cyclin E protein complex. (*A*) MDA-MB-231 cells were treated with or without 100 μM I3C for the indicated times, and cell extracts were electrophoretically fractionated and analyzed by Western blots for the presence of the 50-kDa cyclin E and the hyperactive 35-/33-kDa forms of cyclin E. The same blot was stripped and reprobed for CDK2 and actin. (*B*) Cells were treated with or without 100 μM I3C for 72 h, and the cyclin E was immunoprecipitated from each sample by using anti-cyclin E antibodies. The ''No IP'' lanes represent an immunoprecipitation using a nonspecific IgG. Half of each sample was fractionated by SDS/PAGE, and Western blots were analyzed for cyclin E, CDK2, p21, and p27. The other half of the immunoprecipitated cyclin E was assayed for the associated CDK2 kinase activity using histone H1 as a substrate in the presence of [γ-^{32}P]ATP.