UCN-01 Suppresses E2F-1 Mediated by Ubiquitin-proteasome-dependent Degradation

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ABSTRACT

E2F-1 regulates the transcription of genes required for DNA synthesis. Previously, we have reported that UCN-01 suppresses E2F-1 protein expression without any noticeable effect on its mRNA level in gastric cancer cell line SK-GT5 (Clin. Cancer Res., 4: 2201–2206, 1998). In this study, we investigated the mechanism responsible for the suppression of E2F-1 expression by UCN-01 in SK-GT5 cells. After 24-h exposure to 1 μM UCN-01, E2F-1 protein expression was decreased by >99%. The suppressive effect of UCN-01 could be reversed by ubiquitin-dependent proteasome inhibitors such as calpain inhibitor I and lactacystin. Transfection experiments using expression plasmids encoding full-length E2F-1 or truncated E2F-1 with deletion of the COOH-terminal region (which is required for eliciting ubiquitination and protein degradation) revealed that the expression of truncated E2F-1 was not affected by UCN-01. Other cell-cycle-related and ubiquitin-proteasome-regulated proteins such as p21, p27, and cyclin B1 were not repressed by cycle-related and ubiquitin-proteasome-regulated proteins. These data indicate that UCN-01 suppresses E2F-1 expression mediated by the ubiquitin-proteasome pathway in a specific manner.

INTRODUCTION

The E2F family of transcription factors plays an important role in cell cycle progression from the late G1 into S phase (1, 2). E2F-1, in heterodimeric complex with another protein, DP-1, is normally inactive because it is bound to hypophosphorylated pRb3 (3, 4). When cells progress from the G1 to the S phase, pRb becomes hypophosphorylated and releases the bound E2F-1/DP-1 heterodimer, which subsequently activates the transcription of genes involved in DNA synthesis such as TS and DHFR (1, 5). The loss of functional pRb can give rise to increased free E2F-1 levels, subsequently increased levels of TS and DHFR, and resistance to antimetabolites (6). It has been shown that, in fibrosarcoma cells, overexpression of E2F-1 by genetic transduction leads to up-regulation of TS and FU resistance (7).

Analyses of tumor biopsy samples from patients with metastatic colon cancer reveal that their pulmonary metastases have 4- to 5-fold higher levels of TS gene expression than the hepatic metastases, and this may be the basis of the lack of response of pulmonary metastases to FU treatment (8). Additional studies also demonstrate that elevated TS expression in the metastases of colon cancer correlates with poor survival and an increase in E2F-1 expression (9, 10). These observations indicate that E2F-1 represents a novel target for cancer therapy, and identifying ways of suppressing E2F-1 expression can potentially enhance the effect of cancer chemotherapy.

The regulatory mechanism for E2F-1 gene expression is not yet fully understood. Several lines of evidence have suggested that E2F-1 is controlled through precise temporal control mechanisms involving both transcriptional and posttranscriptional pathways (11, 12). Recently, it has been demonstrated that regulation of E2F-1 protein levels could be mediated through ubiquitin-proteasome-dependent degradation, and that the complex formation with pRb blocks E2F-1 degradation (13, 14).

UCN-01, a protein kinase C/CDK inhibitor, is currently in clinical trials as a potential anticancer agent. It has been shown that UCN-01 causes cell cycle arrest at the G1-S phase, presumably through dephosphorylation of pRb and inhibition of CDK2 activity (15). Additionally, UCN-01 can abrogate G1 checkpoint through inhibition of Chk1 kinase (16). We have shown that UCN-01 suppresses TS gene expression by down-regulation of E2F-1 protein expression without significant change in E2F-1 mRNA levels in human SK-GT5 gastric cancer cells (17). Furthermore, UCN-01 enhances FU-induced apoptosis in a sequence-dependent manner. UCN-01 apparently represents a novel class of agents that can suppress E2F-1 expression and enhance chemosensitivity toward antimetabolites. In this study, 3

3 The abbreviations used are: pRb, retinoblastoma protein; TS, thymidylate synthase; DHFR, dihydrofolate reductase; FU, 5-fluorouracil; CDK, cyclin-dependent kinase; L1L, N-acetyl-l-leucyl-l-leucinal-l-norleucinal; RT-PCR, reverse transcription-polymerase chain reaction.
we have investigated the mechanism responsible for the suppression of E2F-1 expression by UCN-01. We have identified that UCN-01 suppresses E2F-1 expression mediated by the ubiquitin-proteasome pathway in a specific manner.

MATERIALS AND METHODS

Cell Culture, Drug Treatment, Expression Vectors, and DNA Transfection. Early-passage human gastric cancer cell line SK-GT5 was established and characterized as described previously (18, 19). All of the cultures were maintained in standard MEM media supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 20% heat-inactivated normal calf serum (Intergen Co.) at 37°C in a humidified atmosphere of 5% CO2. UCN-01 was graciously supplied by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD). Calpain inhibitor I, LLNL, was purchased from Sigma. Lactacystin was purchased from Calbiochem. The cytotoxicity of each agent for 24-h exposure in SK-GT5 cells was determined by Alamar Blue assay (20), and the concentration causing <20% of growth inhibition was used in this study. Twenty-four h after passage, when cells were ∼50–60% confluent, they were exposed to the indicated drug concentration. Each of these compounds was dissolved in DMSO, and the final concentration of DMSO in the tissue culture media, after addition of the relevant compound, was <0.1%. Cells in control (or no-drug) condition were treated with 0.1% DMSO as a vehicle control. Treatments with proteasome inhibitors such as LLNL and lactacystin were given 1 h before the addition of UCN-01. Cells were checked for Mycoplasma contamination at least every 6 months with a GEN-Probe Mycoplasma rapid detection kit (Fischer Scientific) and consistently tested negative.

The following expression vectors were used for transfection and in vitro coupled transcription/translation: pRcCMV (vector control), pRcCMV-HA-E2F-1 (full-length E2F-1 containing 437 amino acids), and pRcCMV-HA-E2F-1-(1-363) (truncated E2F-1 encoding 363 amino acids and lacking the COOH-terminal 74 amino acids). These plasmids were generously provided by Dr. David M. Livingston (Dana-Farber Cancer Institute, Boston, MA) and were described previously (21). We have confirmed further the coding regions of both pRcCMV-HA-E2F-1 and pRcCMV-HA-E2F-1-(1-363) by DNA sequencing. Plasmid pRcCMV-HA-E2F-1-(1-363) has a stop codon created at codon 364 of E2F-1 and therefore generates a truncated E2F-1 protein lacking the COOH-terminal 74 amino acids. Transfection of exponentially growing SK-GT5 cells with these expression vectors was performed by using Lipofectamine (Life Technologies, Inc.) according to the manufacturer’s instructions. After selection with G418 300 μg/ml for 3–4 weeks, resistant clones were isolated and expanded in the presence of G418.

Western Blot Analysis. SK-GT5 cells were washed twice with cold PBS and then lysed by scraping into a radioimmunoprecipitation assay buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS) containing the protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 25 μg/ml aprotonin, 25 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 1 mM sodium orthovanadate). The lysate was left on ice for 30 min, passed through a 21-gauge needle twice, and then centrifuged at 15,000 × g for 20 min in a microfuge at 4°C. The clarified supernatant was collected, and protein concentration was determined by the Bio-Rad protein assay. Whole cell lysate containing 50 μg of protein from each sample was used in immunoblotting as previously described (17, 22). Human TS protein (a generous gift from Dr. Bruce J. D.nl., Roswell Park Cancer Institute, New York, NY) and HeLa nuclear extract (Upstate Biotechnology) for E2F-1 were used as positive controls. The gels were then electroblotted onto PVDF membranes (Immobilon-P, Millipore). Ponceau S staining of the membranes was performed to assess the equivalence of sample loading and gel transfer and then destaining with water was performed, as described (23). Antibodies purchased from Santa Cruz Biotechnology (unless otherwise specified) were used to detect the proteins of interest: monoclonal E2F-1 (recognizing the first 368 amino acids; Neomarker), monoclonal α-tubulin (Calbiochem), monoclonal cyclin A, monoclonal cyclin B1, polyclonal p21 (Calbiochem), polyclonal p27, polyclonal DP-1, and polyclonal antibodies against human TS and DHFR [both generously provided by Dr. Joseph R. Bertino (Memorial Sloan-Kettering Cancer Center, New York, NY)]. The horseradish peroxidase-conjugated goat antirabbit or antitmouse IgG antibody was used as a secondary antibody. The detection of antibody-binding was performed by using Pierce SuperSignal chemiluminescent detection reagents with the protocols recommended by the manufacturer, and blots were exposed to NEN Renaissance X-ray film with intensifying screens. The linear-range signal intensity of each specific band on the fluorogram was quantitated by a densitometric scanning system, and a comparison of proteins of interest was performed after normalization to α-tubulin protein levels. In some instances, the densitometric scanning of the Ponceau S staining was used as an internal control.

In Vitro Translation and Degradation of E2F-1. Full-length and truncated E2F-1 proteins radio labeled with S35 methionine were obtained from expression vectors pRcCMV-HA-E2F-1 and pRcCMV-HA-E2F-1-(1-363), respectively, by using a TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s instructions. Degradation assay was performed based on published method with some modification (24, 25). In brief, cellular extracts from SK-GT5 cells were isolated by lysing cell pellets in 2 volumes of hypotonic buffer [10 mM Tris (pH 7.5), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride] on ice for 10 min before breaking by strokes in a glass homogenizer. The supernatant was collected after centrifugation, and protein concentration was determined by Bio-Rad protein assay. Reactions were set up in a final volume of 150 μl containing the following components: cellular extract at a final protein concentration of 2 μg/μl, 3 μl radiolabeled E2F-1 protein (2% v/v), 10 mM Tris (pH 7.5), 5 mM MgCl2, 5 mM CaCl2, 3 units/ml creatine phosphokinase, 10 mM phosphocreatine, 2 mM ATP, and 1 mM of the protein synthesis inhibitor emetine. The reaction mixtures were incubated at 37°C, and at indicated times, aliquots were removed before terminating reactions in SDS sample buffer. Samples were resolved by SDS-PAGE using 8% polyacrylamide according to the method of Laemmli (26). Specific bands of interest were quantitated with a Fuji BAS 2500 Phosphor Imaging System.
Statistical Analysis. All experiments have been performed at least twice with similar results, and the results of one representative experiment are reported. The curve-fitting analysis in the in vitro E2F-1 protein degradation studies was performed by linear regression.

RESULTS AND DISCUSSION

Ubiquitin-proteasome-mediated proteolysis controls the destruction of many cellular regulatory proteins in a timely fashion (27). Many regulators of cell cycle machinery, such as cyclins and CDK-inhibitors, have been shown to be regulated by the ubiquitin-proteasome pathway during the progression of cell cycle (28). Similarly, E2F-1, a transcriptional factor that controls G1 progression and the transcription of a group of S-phase genes, has also been shown to be regulated through the ubiquitin-proteasome pathway (14, 21). In this study, we examined whether proteasome inhibitor LLnL could reverse the suppressive effect of UCN-01 on E2F-1 expression. As shown in Fig. 1, Western blot analyses for E2F-1, TS, and α-tubulin (as internal control) were performed in SK-GT5 cells exposed to DMSO vehicle, 1 μM UCN-01, or 50 μM LLnL plus 1 μM UCN-01 for 24 h. LLnL prevented the UCN-01-mediated decline of E2F-1, and TS expression remained unchanged. Cell cycle analysis revealed LLnL could not prevent G1 arrest by UCN-01 (data not shown). This suggests that the effect of UCN-01 on E2F-1 is not primarily related to G1 arrest. Similar results were observed with a more specific proteasome inhibitor, lactacystin, and in human HCT-116 cells (data not shown). These data indicate that UCN-01 represses E2F-1 expression by promoting proteolysis through a ubiquitin-proteasome-dependent pathway. Ubiquitin-proteasome-dependent degradation of E2F-1 has been shown to rely on the availability of its COOH-terminal sequence (21). To examine the contribution of E2F-1 COOH-terminal sequences to its ubiquitin-proteasome-dependent degradation by UCN-01, we transfected SK-GT5 cells with the following expression vectors: pRcCMV (vector only control), pRcCMV-HA-E2F-1 encoding full-length E2F-1 protein, and pRcCMV-HA-E2F-1-(1-363) (encoding a truncated E2F-1 protein lacking COOH-terminal 74 amino acids). Western blot analyses for E2F-1 using an antibody recognizing both the full-length and the truncated E2F-1 were performed in transfected cells treated with DMSO vehicle or 1 μM UCN-01 for 24 h. As shown in Fig. 2, the E2F-1 wild-type transfected clone overexpressed E2F-1 >20-fold when compared with the vector-only-transfected clone. However, the overexpression of E2F-1 was repressed in the presence of UCN-01 in a fashion similar to the vector-transfected clone. In the truncated E2F-1 transfected clone, UCN-01 treatment did not alter the levels of the truncated E2F-1 protein. Furthermore, in the truncated E2F-1-transfected clone, a longer exposure of the fluorogram clearly showed a similar degree of decrease in the levels of the native full-length E2F-1 protein when compared with the vector-only transfected clone (data not shown). These results demonstrate that the COOH-terminal sequences of E2F-1 protein are essential for the ubiquitin-proteasome-dependent degradation by UCN-01, and UCN-01 can repress E2F-1 even in E2F-1-overexpressing cells. Targeting of E2F-1 for ubiquitination requires the COOH-terminal region containing the activation/pRb-binding domains, and the binding of pRb to this region blocks cellular ubiquitination machinery from recognizing E2F-1 (14). When pRb is phosphorylated, it no longer binds to E2F-1. Therefore, it has been shown that a mutant form of pRb, which cannot be phosphorylated, is much more efficient in stabilizing E2F-1 than its wild-type counterpart (21). It has been reported that UCN-01 causes dephosphorylation of pRb in human epidermoid carci-
noma A431 cells (15). We found in SK-GT5 cells that pRb was similarly dephosphorylated by UCN-01 and that p16 was not detectable (data not shown). Therefore, the phosphorylation status of pRb most likely does not play a role in the ubiquitin-proteasome-dependent degradation of E2F-1 by UCN-01.

Many cell cycle-related proteins such as p21, p27, cyclin A, and cyclin B have been shown to be regulated by ubiquitin-proteasome pathway (28). To examine whether UCN-01 also activated ubiquitin-proteasome-dependent degradation of E2F-1 by UCN-01.

Many cell cycle-related proteins such as p21, p27, cyclin A, and cyclin B have been shown to be regulated by ubiquitin-proteasome pathway (28). To examine whether UCN-01 also activated ubiquitin-proteasome-dependent degradation of other cell cycle-related proteins, Western blot analyses for E2F-1, p21, p27, cyclin A, cyclin B, and DP-1 were performed in wild-type E2F-1-overexpressing SK-GT5 cells treated with DMSO (vehicle control), 1 μM UCN-01, and 50 μM LLnL plus 1 μM UCN-01 for 24 h. All of the proteins studied, except DP-1, have been shown to be regulated by ubiquitin-proteasome pathway. As shown in Fig. 3, the levels of both p21 and p27 proteins increased after UCN-01 treatment, and similar results were observed in human A431 cells by Akiyama et al. (15). The levels of both p21 and p27 proteins showed additional increases in the presence of LLnL and UCN-01. Cyclin B1 demonstrated degrees of induction similar to p21 and p27 under these conditions. These data for p21, p27, and cyclin B1 are consistent with the notion that all of the three proteins can be regulated by the ubiquitin-proteasome system. Both cyclin A and DP-1 were repressed upon UCN-01 treatment, but returned to baseline levels when LLnL was added to UCN-01. The levels of cyclin A mRNA by RT-PCR correlated with the levels of E2F-1 and cyclin A proteins (data not shown). Although cyclin A can be regulated through the ubiquitin-proteasome pathway, it has been shown also to be regulated by E2F-1 through transcriptional activation (5). Therefore, the repression of cyclin A expression by UCN-01 could occur as a result of E2F-1 suppression. Taken together, these data indicate that UCN-01 mediates the ubiquitin-proteasome-dependent degradation of E2F-1 in a specific manner.

For additional confirmation that UCN-01 regulated E2F-1 expression by activating E2F-1 proteolysis, the degradation of full-length and truncated E2F-1 was studied in a cell-free system. Cellular protein extracts were obtained from exponentially growing SK-GT5 cells treated with 1 μM UCN-01 for 24 h. Radiolabeled full-length and truncated E2F-1 proteins were generated as described in “Materials and Methods.” At indicated times, aliquots were removed before terminating reactions in SDS sample buffer and subjected to SDS PAGE (A). Specific bands of full-length E2F-1 (●) and truncated E2F-1 (○) proteins were quantitated with a Fuji BAS 2500 PhosphorImaging System and plotted as a percentage of initial protein over time (B). Lines, the results of best-fit analysis determined by linear regression.

In ubiquitin-proteasome-dependent proteolysis, proteins are targeted for degradation by covalent ligation to ubiquitin, a 76-amino acid protein (27, 29). First, the ubiquitin-activating enzyme (E1) forms a high-energy thiolester bond with ubiquitin requiring the hydrolysis of ATP. Subsequently, ubiquitin is transferred to form a thiolester linkage with one of many ubiqui-
uitin-conjugating enzymes, Ubcs or E2s. The final transfer of activated ubiquitin to the target protein requires the action of a ubiquitin-protein ligase, E3. The formation of multibiquitin chains in the target protein facilitates efficient recognition of the substrate by the proteasome. Some E3s act only as substrate recognition factors. Although some components of ubiquitin-proteasome pathway have been identified for the degradation of certain cyclins, such as cyclin B (30, 31), none of them have been identified for E2F-1. It is plausible that UCN-01 may activate certain components of the ubiquitin-proteasome pathway specific for E2F-1, and that subsequently enhances the degradation of E2F-1.

E2F-1 must heterodimerize with DP-1 to interact with pRb. The full range of potential interactions among E2F-1, DP-1, and pRb has not been fully explored. As shown in Fig. 3, UCN-01 suppresses DP-1, and LLN-L reverses the suppression. DP-1 has been shown to be cell cycle-regulated, but it is not known whether it is regulated by the ubiquitin-proteasome pathway or by E2F-1. The down-regulation of DP-1 by UCN-01 may contribute to the instability of E2F-1 by attenuating the availability of DP-1 for heterodimerization with E2F-1. We are currently in the process of investigating the mechanism responsible for the altered DP-1 expression by UCN-01 and exploring the interaction among DP-1, E2F-1, and pRb in the presence of UCN-01.

Transcriptional repression of E2F-1 has been shown to account for the inhibition of cell cycle progression by retinoic acid and IFN-α (32, 33). Although the action of UCN-01 is similar to those two compounds in terms of inhibition of cell cycle progression, its mechanism of E2F-1 suppression is unique. Retinoic acid has been shown to mediate the ubiquitin-proteasome-dependent degradation of cyclin D1 in normal bronchial epithelial cells (25). Akiyama et al. (15) have shown that UCN-01 suppresses cyclin D1 protein expression in A431 cells. We have also observed repression of cyclin D1 protein expression by UCN-01 in SK-GT5 cells (data not shown). Whether this is mediated by ubiquitin-proteasome-dependent degradation or through another mechanism is currently under investigation in our laboratories.

E2F-1 clearly represents a novel target for anticancer therapy. Its downstream S-phase genes, such as TS, DHFR and ribonucleotide reductase, are important anticancer drug targets. Therefore, identification of a compound like UCN-01, which exerts a E2F1-suppressive effect, bears important clinical implications. Previously, we have shown that UCN-01 enhanced FU-induced apoptosis through the suppression of TS as a result of the down-regulation of E2F-1 (17). A Phase I trial using the FU and UCN-01 combination based upon these preclinical data is currently underway. Additional study from our laboratories also indicates that UCN-01 can enhance methotrexate-induced apoptosis through suppression of DHFR4. With the ability to suppress E2F-1, UCN-01 can provide a novel way to enhance cellular sensitivity toward antimetabolites.

REFERENCES

4 C-T. Hsueh and G. K. Schwartz, unpublished observations.


