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Identification of Tumor-Specific Paclitaxel (Taxol)-Responsive Regulatory Elements in the Interleukin-8 Promoter

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Paclitaxel (Taxol) is a novel chemotherapeutic drug that is effective against breast and ovarian cancers. Although the primary target of paclitaxel is microtubules, its efficacy exceeds that of conventional microtubule-disrupting agents, suggesting that it may have additional cellular effects. Previously, we demonstrated that paclitaxel can induce interleukin-8 (IL-8) gene expression at the transcriptional level in subsets of human ovarian cancer lines. In this as well as the previous report, we present evidence that this ability is not linked to the lipopolysaccharide pathway of IL-8 gene induction. The present study identifies the *cis*-acting elements and *trans*-acting factors involved in this induction by transfecting DNA constructs containing the 5′-flanking region of the IL-8 gene linked to the chloramphenicol acetyltransferase reporter gene into paclitaxel-responsive and nonresponsive ovarian cancer cells (responsiveness refers to the IL-8 response). Paclitaxel only activated the IL-8 promoter in responsive cells. The AP-1 and NF-κB binding sites in the IL-8 promoter are required for activation by paclitaxel; in contrast, a C/EBP site required for IL-8 promoter activation in other cell types is not involved. Gel shift assays demonstrate that paclitaxel causes a marked increase in protein binding to the NF-κB and AP-1 consensus binding sequences in the paclitaxel-responsive ovarian cells, but not the nonresponsive cells. The induction of NF-κB and AP-1 binding is reduced by the addition of protein kinase C inhibitors and cyclic AMP effector, respectively. These results demonstrate a molecular mechanism for cell-specific paclitaxel-induced IL-8 gene expression which may have clinical relevance.

Paclitaxel is a novel antineoplastic drug which has shown promise in the treatment of previously unresponsive breast, ovary, lung, and colon cancers. Paclitaxel is active at nanomolar concentrations and functions by binding to the amino-terminal region of the β-tubulin molecule, thereby promoting and stabilizing the formation of microtubules. However, the therapeutic effectiveness of paclitaxel exceeds that of microtubule-disrupting agents such as colchicine and vinca alkaloids, suggesting that paclitaxel may have multiple effects on cells. Recent studies in mice show that paclitaxel and bacterial lipopolysaccharide (LPS) can induce a broad range of shared bioactivities in macrophages, which includes the following: (i) induction of a series of cytokines, such as interleukin-1α (IL-1α), IL-1β, tumor necrosis factor alpha (TNF-α), and IP-10 (1, 5, 15, 36–38); (ii) internalization of TNF-α receptors (14, 15); (iii) induction of a panel of immediate early genes; (iv) provision of a second signal for macrophage tumoricidal activity and nitric oxide production (25); and (v) induction of the phosphorylation of mitogen-activated protein kinases and activation of the autophosphorylation of *lyn* kinase (15, 22, 36, 37). In addition, LPS-hyporesponsive C3H/HeJ mice do not respond to paclitaxel with the same pattern of phosphorylation and gene induction as other mice do, suggesting that paclitaxel and LPS may share a functionally important signaling transduction pathway (15, 37).

In contrast to its effect on mouse cells, the effect of paclitaxel on human macrophages/monocytes has been more difficult to demonstrate. However, paclitaxel activates IL-8 but not IL-1α or IL-1β or IL-6 transcription in a subset of human ovarian cancer cell lines and in freshly explanted ovarian cancer cells (32). Since ovarian cancer represents a primary type of tumor that is clinically responsive to paclitaxel, these findings may have significant clinical ramifications.

IL-8 is a member of the superfamily of C-X-C chemokines (29), and it is a chemotactic factor for T cells, neutrophils, and basophils (41, 47, 59). IL-8 induction in tumors may be beneficial to the host in that immune cells are attracted to the tumor site. In fact a recent study by Hirose et al. shows that this is indeed the case (23). Alternatively, IL-8 also enhances angiogenesis and may enhance metastasis (51). Regardless of which role IL-8 plays in a clinical setting, understanding how paclitaxel activates the IL-8 gene can elucidate additional molecular targets of paclitaxel and provide an understanding which may lead to better antitumor therapies.

The IL-8 gene is regulated at both the transcriptional and posttranscriptional levels. The former is primarily mediated by multiple *cis* elements, including a CCAAT box, a steroid-responsive element, an HNF-1 element, two IRF-1 elements, an AP-1 sequence, an AP-3 site, a C/EBP sequence, and an NF-κB–NF-IL-6 overlapping sequence (30, 40, 42, 46). Earlier, we demonstrated that paclitaxel can induce IL-8 gene expression in human ovarian cancer cells at the transcriptional level, as determined by nuclear run-on assays (32). In the present study, we investigated the molecular mechanisms responsible for the tumor-specific induction of the IL-8 gene by paclitaxel. The study shows that paclitaxel induces a series of activation steps that lead to IL-8 synthesis; this activation cascade is highly cell specific and is found only in a subset of ovarian carcinoma cells.

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MATERIALS AND METHODS

Cells and reagents. The human ovarian cancer cell lines OVCA 420 (a gift from Robert Bast, Jr., M. D. Anderson, Houston, Tex.) and OVCA 194 (a gift from Oto Martinez-Maza, University of California at Los Angeles, Los Angeles) were maintained as monolayer cultures in DMEM/F12 medium supplemented with 5% fetal bovine serum. The derivation of these cells has been described previously (49). Cells were seeded in 100-mm-diameter plates (Falcon Inc., Plymouth, England) and grown in DMEM supplemented with 5% fetal bovine serum until they reached 60 to 75% of confluence. Immediately before use, the medium was removed and the cells were washed once with sterile phosphate-buffered saline and then treated with paclitaxel or the vehicle, dimethyl sulfoxide (DMSO). Stock paclitaxel was stored at −70°C at a concentration of 30 mM. The final concentration of DMSO never exceeded 0.1%. Paclitaxel was stored at 4°C and warmed to room temperature just prior to use.

Plasmid constructs. The plasmids pUXCAT-546, pUXCAT-133, pUXCAT-98, and pUXCAT-85 were as described previously (42). Additional mutant chloramphenicol acetyltransferase (CAT) plasmids were generated by inserting the corresponding double-stranded oligonucleotides into pUXCAT-133. Briefly, pUXCAT-133mAP-1 contains a mutated AP-1 binding site, which was generated by oligonucleotide site-directed mutagenesis (changing the AP-1 site from TGACTCA to TATCTCA) using −133 IL8CAT as the wild-type template. pUXCAT-133mNF-κB contains a mutated NF-κB site with GGAATTTCCT changed to TAACTTTCCT. pUXCATmC/EBP contains a mutated C/EBP site with CAGTTGCAAATCGT changed to AGCTTGCAAATCGT (57). Plasmid pUXCAT3XHLAκB contains three tandem repeats of the NF-κB binding site from positions −189 to −177 of the HLA-B7 gene (46). Plasmid pUXLUC2X(−126/−120) contains two copies of the IL-8 AP-1 binding site (TGACTCA) from the IL-8 promoter (45) inserted upstream of the IL-8 enhancer-less core promoter (−50 to +44 bp) linked to a firefly luciferase gene. Plasmid pUXLUC3XColAP-1 contains three copies of the AP-1 binding site from the collagenase promoter linked to the luciferase reporter gene. The construction of the remaining plasmids within the pUXCAT series has been reported elsewhere (42). Briefly, the sequences from −94 to −80 and from −80 to −71 of IL-8 were synthesized in triplicate and then were inserted into −50CAT to create pUXCAT3X(−94/−80) and pUXCAT3X(−80/−71), respectively. Plasmid mut MEKK was obtained from Channing J. Der (University of North Carolina).

Transfection and CAT assay. Cells from the human ovarian cancer cell lines OVCA 420 and OVCA 194 were transfected by the calcium phosphate method (8). Four hours after transfection, the cultures were glycerol shocked and replenished with fresh medium. Trypsin was added after 2 to 3 h to release the cells. These cultures were subdivided and plated into two 100-mm-diameter dishes (3×10^6 cells per dish), which were incubated overnight at 37°C and 5% CO_2. The cultures were then treated with DMSO or paclitaxel for 24 h prior to extract preparation. CAT enzymatic activity was assayed as described elsewhere (44, 46). To address variations in transfection efficiency, each construct was tested with at least two separate plasmid preparations in three independent experiments. These multiple experiments should have eliminated experiment-to-experiment variations in transfection efficiency.

Nuclear extract preparation and EMSA. Nuclear extracts from OVCA 420 cells were prepared as previously described (13) by using a modification of Dignam's procedure (13). Electrophoretic mobility shift assays (EMSAs) were performed as described previously (24, 56). Four to six micrograms of nuclear extracts was incubated with 2 μg of poly(dI-dC) and 50,000 to 100,000 cpm of ^{32}P-end-labeled DNA probe in a 20-μl reaction volume. After incubation for 20 min at room temperature, the complex was subjected to electrophoresis in a 6% nondenaturing polyacrylamide gel containing 1× Tris-glycine.

Oligonucleotides. The IL-8 NF-κB binding site was created by annealing the oligonucleotide 5′-ATCGTGGAATTTCCTCTGACA-3′ to its complementing strand. Other binding sites were as follows (point mutations are underlined): IL-8 mutant NF-κB (mNF-κB), 5′-ATCGTTAACTTTCCTCTGACA-3′; IL-8 C/EBP, 5′-CATCAGTTGCAAATCGTGGA-3′; IL-8 mutant C/EBP (mC/EBP), 5′-CATAGCTTGCAAATCGTGGA-3′; IL-8 AP-1, 5′-AGTGTGATGACT CAGGTTTG-3′; IL-8 mutant AP-1 (mAP-1), 5′-AGTGTGATATCTCAGG TTTG-3′ (45).

Antibodies. Anti-p65 and anti-p50 antisera were obtained from Albert Baldwin (Lineberger Cancer Center, University of North Carolina at Chapel Hill). The anti-p65 antiserum was raised against a C-terminal peptide (LLSGDEDFSSI ADMDFSALLSQISS) of NF-κB. Anti-p50 antibody was raised against an N-terminal peptide of the p50 subunit. The polyclonal immunoglobulin Gs against Sp1, p52, and c-Jun were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.).

EMSA supershift. The nuclear extract for antibody supershift was prepared according to the method of Dignam et al. (13) from OVCA 420 cells grown in 100-mm-diameter plates to a density of 2×10^6. Briefly, the cells were resuspended in a volume of buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM dithiothreitol [DTT]) five times the volume of the packed cells and then stored on ice for 10 min. The cells were spun down at $209 \times g$ in a microcentrifuge at 4°C for 10 min, and the pellet was resuspended in twice the volume of buffer A and homogenized by 10 strokes with a Dounce homogenizer. The nuclear pellets were resuspended in buffer C (20 mM HEPES, 20% glycerol, 0.6 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5 mM DTT) followed by 10 strokes with the Dounce homogenizer. The clear supernatants were obtained by centrifugation at maximum speed ($14,300 \times g$) in a microcentrifuge for 30 min. The supernatants were dialyzed in buffer D (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) for at least 5 h. Antibody supershift experiments were performed by preincubating 1 μg of antibody for 2 h on ice with each binding reaction mixture prior to the addition of the DNA probe.

Northern blot. Total RNA was isolated from paclitaxel- and DMSO-treated cell lines by the guanidinium isothiocyanate-CsCl method as described previously (9). Three to five micrograms of purified RNA were loaded into each lane of a denaturing agarose gel (50). IL-8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were hybridized to Northern blots as described previously (52).

IL-8 ELISA. IL-8 was measured as previously described (32). Briefly, a goat anti-human IL-8 antibody (R&D, Minneapolis, Minn.) was used as the coating antibody, rabbit polyclonal anti-human IL-8 antiserum (Endogen) was used as the primary antibody, and alkaline phosphatase-conjugated goat anti-rabbit antibody (Capel) was used as the secondary reagent (10). The enzyme-linked immunosorbent assay (ELISA) is sensitive at the range of nanograms per milliliter.

TABLE 1. LPS-induced IL-8 secretion in paclitaxel-nonresponsive OVCA 194 cells

Treatment[a]	Amt	IL-8 (ng/ml)[b]
LPS	1 ng/ml	8.07
Paclitaxel	30 μM	0.37
DMSO[c]		0.43

[a] Cells were treated for 6 h.
[b] Measured by ELISA.
[c] Concentration never exceeded 0.1%.

RESULTS

A sequence spanning positions −133 to +44 of the IL-8 gene promoter is sufficient to mediate activation by paclitaxel. A previous report from our group showed that paclitaxel induces IL-8 secretion in a subset of human ovarian cancer cells, with the OVCA 420 and OVCA 429 cell lines as prime examples of paclitaxel-responsive cell lines and OVCA 194 and OVCA 494 as examples of nonresponsive lines. Nuclear run-on assays indicate that paclitaxel selectively induces the expression of the IL-8 gene at the transcriptional level (32). IL-8 induction in human ovarian cancers is likely distinct from the cytokine (e.g., IL-1 and TNF-α) induction by paclitaxel observed in murine macrophages (1, 5, 15, 36–38). For the latter, the induction is tightly linked to the LPS response. Murine macrophages defective in LPS response do not activate cytokine genes in response to paclitaxel. In human tumor cells, we have previously shown that a colon carcinoma line which is defective in LPS responsiveness nonetheless produced IL-8 when incubated with paclitaxel (32). Evidence for the converse is now presented in this report, where OVCA 194, which does not produce IL-8 in response to paclitaxel, can respond to LPS treatment with IL-8 synthesis (Table 1). These results clearly show a dichotomy in the LPS and paclitaxel responses with respect to IL-8 induction.

To delineate the mechanism by which paclitaxel induces IL-8 gene transcription, paclitaxel-responsive promoter elements in the IL-8 promoter were identified in this study. This was done by transfecting OVCA 420 cells with plasmid constructs containing the CAT reporter gene driven by the wild type and deletion mutants of the IL-8 promoter (Fig. 1A). Twenty-four hours posttransfection, cells were stimulated with DMSO (the diluent for paclitaxel) or paclitaxel. The full-length promoter was reproducibly activated by paclitaxel. Deletion of sequences upstream of position −133 decreased the constitutive promoter activity but had little effect on paclitaxel induc-

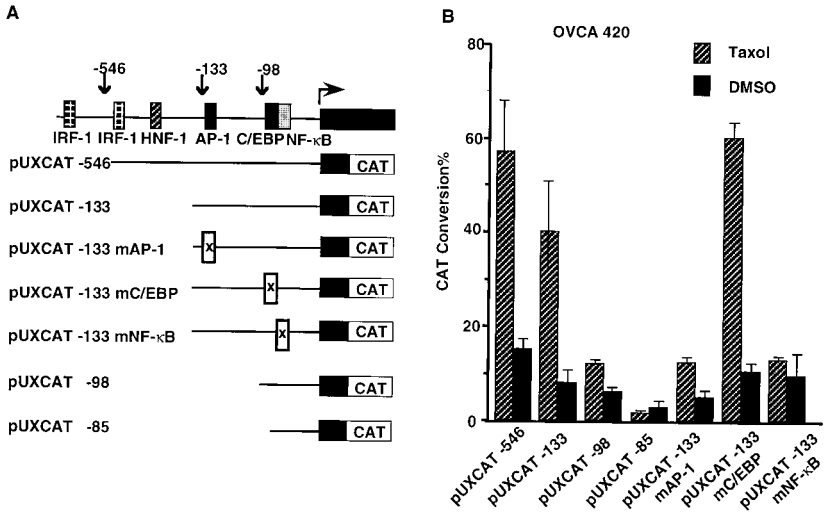

FIG. 1. Sequences within −133 to −85 kb of the IL-8 promoter are necessary for activation by paclitaxel. (A) Potential binding sites for transcription factors present in the region are indicated by the various shaded boxes. Deletion constructs were cloned into the pUXCAT plasmid (open box) as shown. Also, several mutant DNAs (pUXCAT-133mAP-1, pUXCAT-133mC/EBP and pUXCAT-133mNF-κB) were synthesized by oligonucleotide site-directed mutagenesis and then inserted into −133pUXCAT as described in Materials and Methods. (B) After transfection with the appropriate plasmids, the OVCA 420 cells were treated with DMSO or 30 μM paclitaxel (Taxol) as indicated for 24 h. Cell extracts were prepared and CAT assays were performed as described in Materials and Methods. These results represent the averages of three independent experiments. Error bars indicate standard errors.

ibility (Fig. 1B). In contrast, deletion of sequences upstream of −98 significantly reduced inducibility by paclitaxel, and deletion of sequences upstream of −85 abolished all induction. This maps the region from −133 to −85 as a paclitaxel-responsive region which is likely to contain individual paclitaxel-responsive regulatory elements.

To identify the *cis*-acting elements in the −133 to −85 region of the IL-8 promoter which served as a paclitaxel-responsive regulatory element, site-directed mutant constructs were prepared and tested. The results shown in Fig. 1B indicate that mutation in the AP-1 (pUXCAT-133mAP-1) or the NF-κB site (pUXCAT-133mNF-κB) resulted in a significant reduction of inducibility by paclitaxel. The C/EBP site was shown to act cooperatively with NF-κB to induce IL-8 gene transcription in response to IL-1, TNF, and tetradecanoyl phorbol acetate (TPA) (42). Mutation of this C/EBP site (pUXCAT-133mC/EBP) had no effect on paclitaxel-induced CAT activity. These results indicate that the AP-1 and NF-κB binding sites of the IL-8 promoter are specifically important for induction by paclitaxel in OVCA 420 cells.

The various IL-8 promoter constructs were also tested in the paclitaxel-nonresponsive OVCA 194 cell line (32). The results demonstrate that paclitaxel had no effect on either the full-length −546 or the −133 deletion construct (Fig. 2). This lack of effect on the IL-8 promoters in OVCA 194 cells correlates precisely with the lack of IL-8 chemokine induction in this cell line (32). This analysis was extended to the OVCA 429 cell line, which also responds to paclitaxel with IL-8 synthesis, and the nonresponsive OVCA 494 cell line as previously reported (32). As shown in Table 2, paclitaxel induced the IL-8 promoter in the OVCA 429 line but not the OVCA 494 line. This indicates that IL-8 cytokine induction by paclitaxel is reflected at the promoter level and that IL-8 promoter analysis in paclitaxel-responsive versus paclitaxel-nonresponsive lines is useful in elucidating the underlying mechanism of tumor-specific, paclitaxel-activated gene expression.

AP-1 and NF-κB sites of the IL-8 gene are sufficient to mediate paclitaxel-induced gene activation. The IL-8 gene fragment spanning positions −133 to +44 contains three prominent DNA-protein interaction sites for the transcription

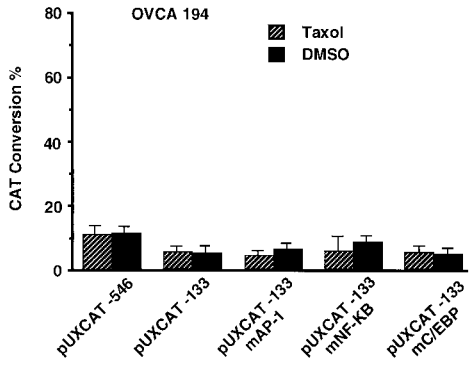

FIG. 2. Nonresponsive OVCA 194 cells did not show significant induction of CAT activity by paclitaxel (Taxol) treatment for any of the constructs shown in Fig. 1. The results were compiled from three independent experiments. Error bars indicate standard errors.

TABLE 2. Paclitaxel activation of the IL-8 promoter[a] in four ovarian carcinoma cell lines

Cell line	Fold induction[b]
Responsive	
OVCA 420	5.2
OVCA 429	4.5
Nonresponsive	
OVCA 194	1.2
OVCA 494	1.0

[a] pUXCAT-133 used for transfection.
[b] Fold induction of paclitaxel-treated cells normalized to CAT activity of DMSO-treated cells. Results represent averages of three experiments.

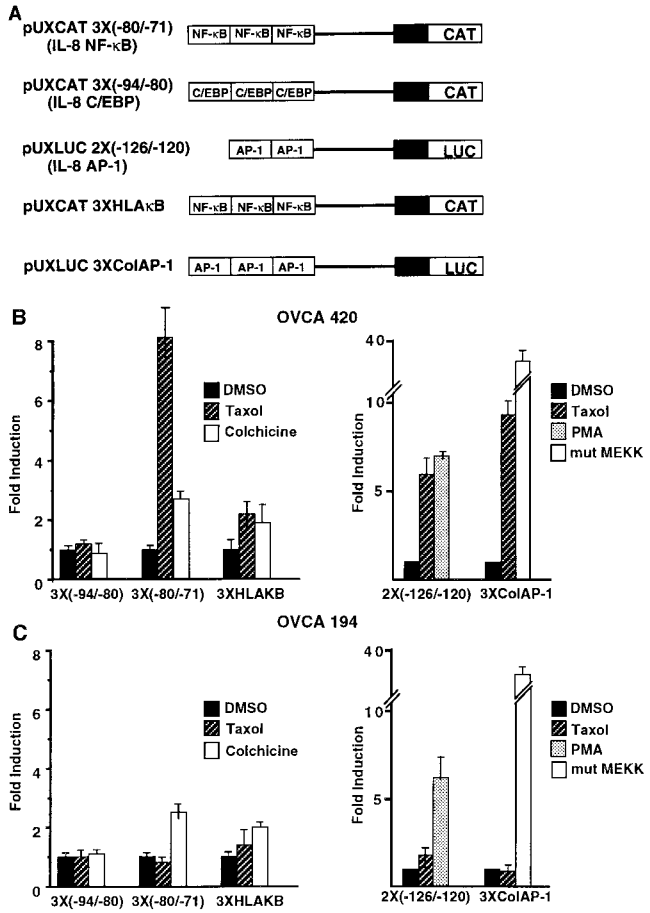

FIG. 3. Paclitaxel-induced IL-8 gene expression is specific for the NF-κB region of the IL-8 gene. (A) The constructs used are diagrammed. Three copies of the *cis*-acting elements corresponding to either the NF-κB or the C/EBP site of the IL-8 gene, two copies of the AP-1 site from the IL-8 gene, three copies of the NF-κB site from the HLA-B7 gene, and three copies of the AP-1 site from the collagenase gene were linked to the minimal promoter region of the IL-8 gene, generating the indicated plasmids. (B) Transfection, cytokine treatment, and CAT assays were performed as described in Materials and Methods. Paclitaxel (Taxol) induces CAT or luciferase expression in OVCA 420 cells transfected with pUXCAT 3X(−80/−71) (left) and pUXLUC 2X(−126/−120) (right). A positive control for AP-1 activation, PMA, induces luciferase activity sevenfold more than DMSO. An activated form of MEKK (mu^+ MEKK) induces luciferase by 35-fold. (C) Unresponsive OVCA 194 cells did not respond to paclitaxel with any of the promoter constructs. The results are taken from three independent experiments.

factors AP-1, C/EBP, and NF-κB. To further determine which sites are sufficient for activation by paclitaxel, several constructs were used (Fig. 3A). Each construct contains three tandemly repeated copies of one of the following elements: the NF-κB site from the IL-8 gene, the C/EBP site from the IL-8 gene, the NF-κB site from the HLA-B7 gene, or two copies of the AP-1 binding site from the IL-8 gene. Each was linked to the minimal IL-8 promoter spanning −50 to +44 bp and to the CAT reporter gene. All constructs were tested in the paclitaxel-responsive OVCA 420 and the nonresponsive OVCA 194 cells.

The construct pUXCAT3X(−80/−71), which contains three tandem repeats of the NF-κB sites from the IL-8 gene, generated an eightfold stimulation by paclitaxel. In contrast, colchicine treatment of OVCA 420 cells resulted in only a slight increase in expression (Fig. 3B). A construct that contains three copies of the NF-κB site from the human HLA B-7 gene was marginally activated by treatment with paclitaxel and colchicine (Fig. 3B and C). This indicates that the κB sequence in the IL-8 promoter is preferred for paclitaxel induction. The induction of pUXCAT3X(−80/−71) is cell-specific because paclitaxel did not activate this construct in OVCA 194 cells. Again, colchicine treatment of OVCA 194 produced a small increase in CAT expression which approximates that observed in OVCA 420 cells (compare Fig. 3B and C). This indicates that the colchicine effect is not cell line-specific while the paclitaxel effect is cell line-specific. Construct pUXCAT3X (−94/−80), which contains three copies of the C/EBP site from the IL-8 gene, did not exhibit paclitaxel inducibility in either OVCA 420 or OVCA 194 cells.

A construct, pUXLUC2X(−126/−120), which contains two tandemly repeated copies of the AP-1 element derived from the IL-8 gene linked to the luciferase reporter gene, was also introduced into OVCA 420 and OVCA 194 cells. As shown in Fig. 3B and C, paclitaxel treatment enhanced luciferase activity in OVCA 420 cells by 5.5-fold but it did not activate this construct in OVCA 194 cells. As a positive control, phorbitol myristate acetate (PMA), which is known to activate the AP-1 transcription factor, was transfected into the cells, and it enhanced luciferase expression by sevenfold in both OVCA 420 and OVCA 194 cells (Fig. 3B and C). A second construct, pUXLUC3XColAP-1, which contains three copies of the AP-1 binding site from the collagenase promoter, was also selectively activated in OVCA 420 but not OVCA 194. Another positive control, an activated form of MEKK (mut MEKK) known to activate the AP-1 transcription factor, was transfected into cells, and it enhanced luciferase expression by 35-fold in both OVCA 420 and OVCA 194 cells (Fig. 3B and C). This indicates that AP-1 can be equally activated in both cell lines by PMA and MEKK, but the cell lines exhibit differential responses to paclitaxel. These results demonstrate that the AP-1 and NF-κB sites of the IL-8 gene are important for cell line-specific activation of paclitaxel-induced gene expression. In contrast, the C/EBP site (42), essential for IL-8 induction by TNF-α, IL-1, and TPA in other cell types, is not involved in this form of paclitaxel responsiveness.

Paclitaxel activates DNA binding by the AP-1 factor. To investigate the *trans*-acting factors that are involved in the activation of IL-8 by paclitaxel, we performed EMSA using a probe spanning positions −130 to −114 of the IL-8 promoter, which includes the AP-1 binding site at −126 to −120 bp (Fig. 4A). Paclitaxel treatment for 30 min induced strong binding to this DNA (compare Fig. 4A, lanes 1 and 2) comparable to that induced by PMA (Fig. 4A, lane 3). This binding was specifically competed by an excess of wild-type unlabeled AP-1 (Fig. 4A, lanes 4 to 6) but not by an oligonucleotide containing point mutations which abolished AP-1 binding (Fig. 4A, lanes 7 to 9). To determine if the complex contains AP-1, anti-Jun antibody was used in a supershift assay. The antibody specifically shifted and diminished this DNA-protein complex, while a control normal rabbit serum had no effect (Fig. 4A, lanes 10 to 11). Taken together, the cold competition and supershift assays indicate that the DNA-protein complex formed is likely AP-1 (Fig. 4A).

Paclitaxel induced DNA-binding by NF-κB but not by C/EBP. Previous reports have described the cooperative binding of NF-κB and C/EBP to adjacent binding sites in IL-1-, TNF-α-, and TPA-stimulated fibrosarcoma cells (42, 53). To determine whether paclitaxel also induces IL-8 expression through the synergistic stimulation of NF-κB and C/EBP, EMSA analyses were performed with NF-κB and C/EBP DNA probes. Incubation of nuclear extracts from paclitaxel-stimulated OVCA 420 cells with the IL-8 NF-κB probe resulted in in-

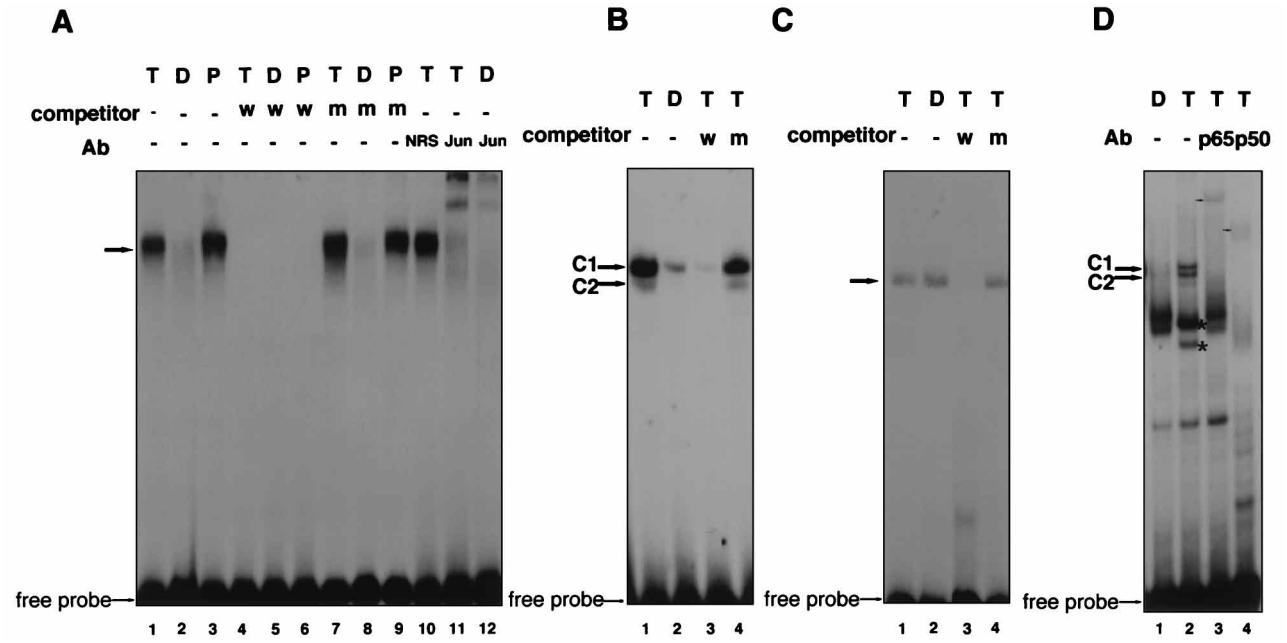

FIG. 4. Gel mobility shift assay. (A) Characterization of nuclear factors binding to the −130 to −114 segment of the IL-8 gene. A ^{32}P-labeled DNA fragment was mixed with 4 μg of nuclear protein extract prepared from OVCA 420 cells stimulated with paclitaxel (T) (30 μM) (lanes 1, 4, 7, 10, and 11), DMSO (D) (lanes 2, 5, 8, and 12), and PMA (P) (1 μg/ml) (lanes 3, 6, and 9) for 30 min in 20 μl of binding reaction buffer in the absence and presence of an unlabeled wild-type (w) DNA fragment (lanes 4 to 6) and a mutant (m) DNA fragment (lanes 7 to 9). For the supershift study, 1 μl of antibody (Ab), either normal rabbit serum (NRS) (lane 10) or anti-c-Jun (lanes 11 and 12), was incubated with nuclear extracts and reaction buffer on ice for 1 h prior to the addition of the radiolabeled probe. The arrow indicates the DNA-protein complexes. (B) A ^{32}P-labeled DNA fragment spanning positions −85 to −64 (−80 to −71 in the IL-8 gene contains the NF-κB binding site) was mixed with nuclear extracts from OVCA 420 cells treated with either paclitaxel (30 μM) (lanes 1, 3, and 4) or DMSO (lane 2) for 30 min in the absence (lanes 1 and 2) or presence of an unlabeled wild-type DNA fragment (lane 3) and a mutant competitive DNA fragment (lane 4). (C) A ^{32}P-labeled DNA fragment spanning positions −97 to −78 (−94 to −81 in the IL-8 gene spans the C/EBP binding site) was mixed with nuclear extracts from OVCA 420 cells treated with either paclitaxel (30 μM) (lanes 1, 3, and 4) or DMSO (lane 2) for 30 min in the absence (lanes 1 and 2) or presence of an unlabeled competitive wild-type DNA fragment (lane 3) and a mutant DNA fragment (lane 4). The arrow indicates DNA-protein complexes. (D) Analysis of the components of complexes C1 and C2 by supershift with antibodies directed against p50 and p65. OVCA 420 cells were treated with either DMSO (lane 1) or paclitaxel (30 μM) (lanes 2 to 4) for 30 min. Nuclear extracts were prepared, and EMSA was performed with the NF-κB element as the probe in the presence of either anti-p50 or anti-p65 as indicated. Asterisks mark bands that appeared only in paclitaxel-treated cells which may represent degraded p65 or p50.

creased formation of two DNA-protein complexes. DMSO-treated OVCA 420 cells gave rise to a faint complex with lower mobility (Fig. 4B, lane 2). Both complexes were specifically inhibited by an excess of the unlabeled NF-κB fragment but not by a mutant NF-κB fragment (Fig. 4B, lanes 3 and 4). Nuclear extracts from paclitaxel-treated OVCA 420 cells incubated with the C/EBP probe showed no increase in binding over DMSO-treated extracts (Fig. 4C, lanes 1 and 2). The complex was specifically competed by the C/EBP probe but not by the mC/EBP fragment (Fig. 4C, compare lanes 3 and 4). This indicates that paclitaxel-induced proteins bind the NF-κB site but not the C/EBP site, which is in agreement with the functional data shown in Fig. 1 and 3.

To further characterize the κB complex, supershift experiments using specific antisera were performed. The preparation of nuclear extract for the assay shown in Fig. 4B was not optimal for supershift analysis, and a modified protocol was necessary. Nuclear extracts of paclitaxel-treated OVCA 420 cells were first dialyzed in buffer D containing 20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT and then incubated with binding buffer and antibodies against the p50 and p65 subunits of human NF-κB as described in Materials and Methods (Fig. 4D). Antibody against NF-κB p50 abolished the formation of both complexes C1 and C2 (Fig. 4D, compare lanes 4 and 2) and resulted in a faint supershifted band (Fig. 4D). Anti-p65 antibodies also reduced the C2 complex, blocked formation of the C1 complex, and produced a supershifted band. In a similar experiment performed with antibodies against other members of the Rel family (e.g., p52) or an unrelated antibody (e.g., Sp1), supershifted bands were not observed (data not shown). It is possible that C1 may represent a p50-p65 heterodimer product and C2 may represent a slightly degraded p50-p65 heterodimer product. Two other bands appeared only in paclitaxel-treated cells and were eliminated by anti-p65 antibodies and diminished by anti-p50 antibodies. They may also be degraded NF-κB products. It is important to note that paclitaxel did not induce AP-1 or NF-κB binding in the paclitaxel-nonresponsive cell line OVCA 194 (data not shown).

Mechanism of paclitaxel activation of AP-1 and NF-κB. To explore the pathways responsible for paclitaxel activation of AP-1 and NF-κB, OVCA 420 cells were preincubated for 2 h with protein kinase inhibitors and agonists prior to treatment with paclitaxel. The cells were then harvested, and extracts were prepared for EMSA. Protein kinase C (PKC) inhibitors (staurosporine and calphostin), tyrosine kinase inhibitor (genistein), and PKA activator (forskolin) were used. The results show that preincubation of OVCA 420 cells for 2 h with 10 nM staurosporine nearly abolished the paclitaxel induction of NF-κB binding activity (Fig. 5A, lane 3). Five nM calphostin C partially reduced NF-κB binding activity, whereas 25 μM forskolin or genistein had little effect (Fig. 5A). On the other hand, preincubation of OVCA 420 cells for 2 h with forskolin almost completely inhibited paclitaxel-induced AP-1 binding (Fig. 5B). Calphostin C minimally reduced AP-1 activity, but genistein had no obvious effect. Lastly, none of these inhibitors

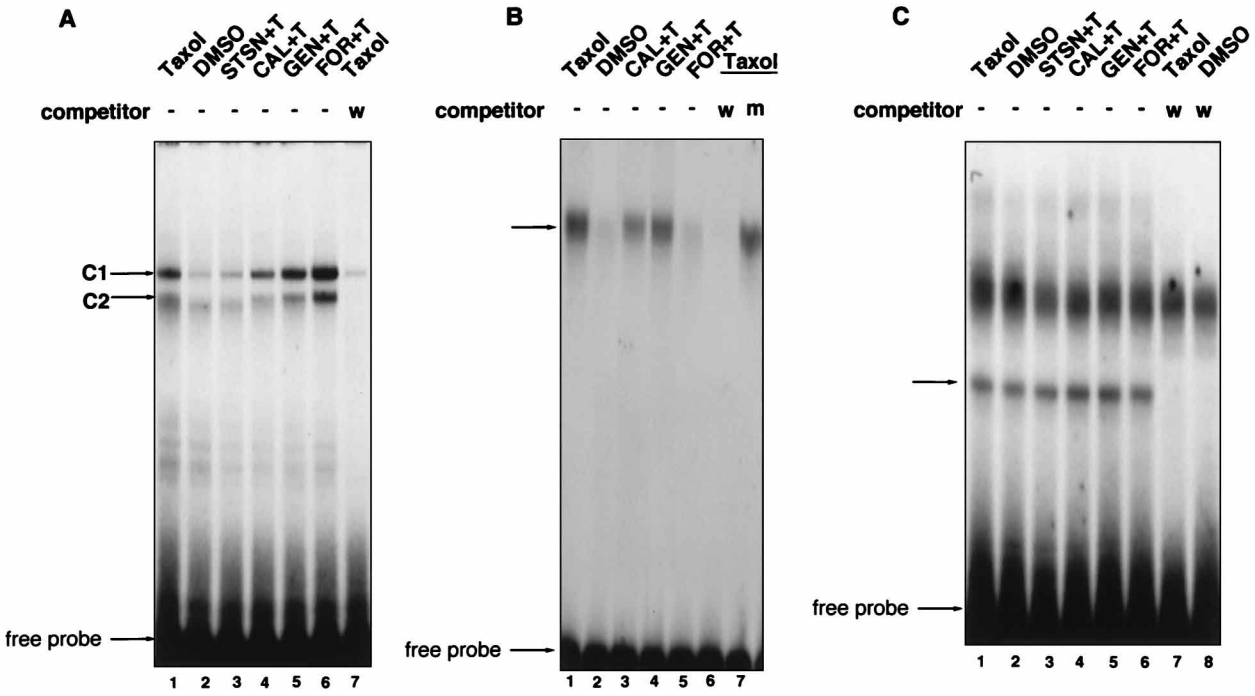

FIG. 5. (A) Role of selected protein kinases in the paclitaxel (Taxol) activation of NF-κB. OVCA 420 cells (5×10^6/sample) were preincubated with staurosporine (STSN; 10 nM) (lane 3), calphostin (CAL; 5 nM) (lane 4), genistein (GEN; 25 μM) (lane 5), and forskolin (FOR; 25 μM) (lane 6) for 2 h. The cells were stimulated with paclitaxel (T; 30 μM) for 30 min, and the nuclear extracts were collected. NF-κB binding activity was examined by EMSA with a double-stranded oligonucleotide probe comprising the NF-κB sequence of the IL-8 promoter. Nuclear extracts from DMSO-treated OVCA 420 cells were used as a control (lane 2). The specificity of binding was examined by incubation of nuclear extracts with unlabeled wild-type (w) NF-κB oligonucleotide (lane 7). (B) Role of different protein kinases in the paclitaxel activation of AP-1. The same nuclear extracts as in panel A were incubated with the AP-1 sequence of the IL-8 gene. The specificity of binding was determined in the presence of unlabeled wild-type AP-1 (lane 6) or mutant (m) AP-1 oligonucleotide (lane 7). (C) Role of different protein kinases in the paclitaxel activation of C/EBP. A ^{32}P-labeled C/EBP sequence of the IL-8 gene was used as the probe for EMSA analysis. The specificity of binding was assessed by competition with unlabeled wild-type C/EBP oligonucleotide (lanes 7 and 8). Unlabeled arrows indicate specific DNA-protein complexes.

affected C/EBP activity (Fig. 5C). Collectively, these results suggest that both PKC and PKA can contribute to the paclitaxel activation of NF-κB and AP-1, respectively. The differential effects of these inhibitors on binding by NF-κB, AP-1, and C/EBP indicate that these inhibitors are not causing a generalized change in the level or activity of DNA-binding proteins but rather a factor-specific change.

Effect of kinase inhibitors on IL-8 synthesis. To determine if the effects of staurosporine and forskolin on transcription factor binding can be extended to paclitaxel-induced IL-8 cytokine synthesis, Northern blot analysis and ELISA were performed. Staurosporine added for 2 h prior to the addition of paclitaxel caused a decrease of paclitaxel-induced IL-8 transcript. A significant decrease in IL-8 transcript was also observed in forskolin-treated OVCA 420 cells. Genistein minimally influenced IL-8 transcript levels (Fig. 6A). As a control for loading, the levels of GAPDH were examined and they were identical. Consistent with the results of Northern blots, forskolin and staurosporine dramatically reduced paclitaxel-induced IL-8 cytokine expression as measured by an ELISA while genistein had a minimal effect on IL-8 expression (Fig. 6B). These results indicate that the effects of these modulators are manifested at the level of transcription factors, which then correlate with IL-8 chemokine induction.

DISCUSSION

IL-8 is produced by various human somatic cells, including monocytes/macrophages, fibroblasts, keratinocytes, mesangial cells, vascular endothelial cells, and several types of tumor cell lines. Previously, we found that a promising chemotherapeutic agent, paclitaxel, transcriptionally induced IL-8 in a subgroup of ovarian carcinomas (32). This finding has significant thera-

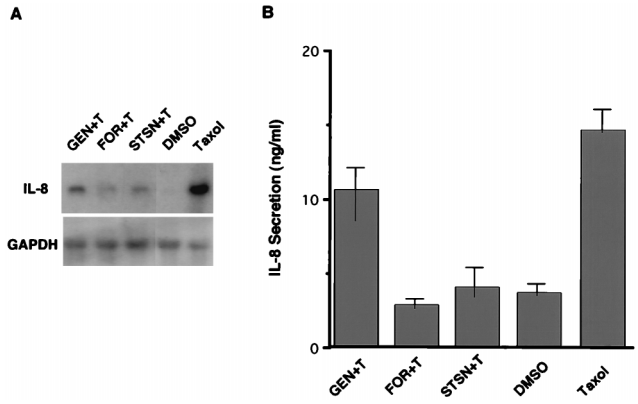

FIG. 6. Effects of protein kinase inhibitors on IL-8 expression and protein secretion in OVCA 420 cells pretreated with staurosporine (STSN; 10 nM), forskolin (FOR; 25 μM), and genistein (GEN; 25 μM) for 2 h and then exposed to 30 μM paclitaxel (T or Taxol). (A) OVCA 420 cells pretreated with the indicated pharmacologic agents and then further stimulated with paclitaxel (30 μM) for 4 h. Total RNA (5 μg per lane) was analyzed by Northern analysis. Blots were probed sequentially for IL-8 and GAPDH. (B) Supernatants were analyzed for IL-8 protein by ELISA as described in Materials and Methods. Pretreatment with staurosporine (10 nM) and forskolin (25 μM) blocks paclitaxel-induced IL-8 protein. Results are expressed as the means ± standard errors of triplicate experiments.

peutic implications and provides an important model to elucidate the mechanism by which paclitaxel induces gene expression, which has not been previously elucidated.

Our primary goal is to determine the mechanism by which paclitaxel activates the IL-8 promoter in human ovarian carcinomas. It has been shown that a variety of agents, such as IL-1, phorbol esters, TNF-α, and the hepatitis B virus X protein, can activate IL-8 gene expression (34, 42). These analyses reveal that the sequence from −94 to −71 bp, consisting of C/EBP and NF-κB binding sites, is required for responsiveness to these inducers. In contrast to previous reports (42, 53), we now report that paclitaxel induction of IL-8 is independent of an intact C/EBP site. Rather, IL-8 gene activation by paclitaxel appears to be mediated by the AP-1 site in conjunction with the NF-κB site. The requirement for a similar region for IL-8 transcription by cytokines has been observed in human lung epithelial cell lines (43) and a human gastric cancer cell line, MKN 45 (57). These results suggest that different sets of nuclear transcription factors may be responsible for the regulation of IL-8 gene transcription in a cell type- and inducer-specific manner. Interestingly, lung carcinoma, similarly to the ovarian carcinoma studied here, is clinically responsive to paclitaxel. It will be critical to determine if the molecular utilization of NF-κB and AP-1 by subsets of tumor cells correlates with a clinical phenotype.

One noteworthy observation is the differential response of various ovarian carcinomas to paclitaxel treatment. Previously, we noted that approximately 50% of ovarian carcinoma lines synthesize IL-8 upon paclitaxel treatment (32). This observation has been extended to primary freshly explanted ovarian cancer cells (32). Analysis of ovarian cancer cell lines which represent each of the two phenotypes discussed in this report shows that the nonresponsive phenotype exemplified by OVCA 194 and OVCA 494 cells is correlated with the lack of IL-8 promoter activation. Further analysis of OVCA 194 cells traced this to a lack of AP-1 and NF-κB activation by paclitaxel. There are at least two possible explanations for the difference between responsive and nonresponsive cells. It is possible that nonresponsive cells are deficient in the appropriate signals necessary for the activation of distinct sets of transcription factors. This includes an array of activation cascades controlling the relevant transcription factors, such as appropriate expression, modification, and cellular localization. In the case of NF-κB, different IκB inhibitors also have to be considered. Alternatively, nonresponsive cells may not receive the signal transmitted upon paclitaxel binding. Elucidating why some cancer cells respond to paclitaxel and how distinct sets of transcription factors are involved in IL-8 gene expression in different types of ovarian cell lines will be important in understanding paclitaxel's mode of action. Correlating this molecular difference with variations in clinical responsiveness may provide a molecular explanation for the therapeutic pathways of paclitaxel.

It has been documented that microtubule-depolymerizing agents, such as nocodazole and colchicine, can activate several genes, including IL-1β (19, 35) and urokinase-type plasminogen activators, in specific cells (6, 31). Therefore, we wish to determine if the effects of paclitaxel on IL-8 genes are related to its effects on microtubules. A construct composed of three tandemly repeated copies of the NF-κB element from the IL-8 gene was significantly activated by paclitaxel and much less activated by colchicine (Fig. 3). An equal activation of this construct by colchicine was also observed in the nonresponsive OVCA 194 cells, indicating that the colchicine effect is not cell restricted. In contrast, the induction of NF-κB by paclitaxel was only found in the OVCA 420-responsive line.

To further test if the effect of paclitaxel on IL-8 induction is related to its effect on microtubules, OVCA 420 cells were treated with colchicine and vinblastine. Neither drug had any effect on either IL-8 synthesis or NF-κB activation by paclitaxel (data not shown). Thus, the activation of NF-κB by paclitaxel may not be solely due to changes in microtubules. We are aware that colchicine and vinca alkaloids cause microtubule depolymerization while paclitaxel prevents depolymerization, although the end result of all three treatments is the disruption of microtubule function. It is possible that these different modes of action for colchicine versus paclitaxel may account for the difference in IL-8 induction. However, paclitaxel promoted microtubule polymerization and disrupted the depolymerization of microtubules nearly identically in OVCA 194 cells and OVCA 420 cells, which differ in their IL-8 response (53a). This dissociates the effects of paclitaxel on IL-8 induction and microtubules. In addition, our comparison of IL-8 gene induction and cytotoxicity in OVCA 420 cells by using 12 structurally related paclitaxel analogs (54) revealed 3 analogs that induced IL-8 synthesis in the ovarian lines, but tubulin stabilization was observed for all 12 analogs. Recent studies of murine macrophages indicate that the effects of paclitaxel on TNF-α gene expression or nitric oxide (NO) production are distinct from its well-known effects on microtubules (7, 27, 37, 38). Structure-activity relationship studies show that changes in the structure of paclitaxel do not alter cytotoxicity but prevent TNF-α gene expression (7, 27). Furthermore, docetaxel (Taxotere), a semisynthetic taxoid which is more potent than paclitaxel as an inducer of microtubule bundling, did not induce TNF-α gene activation and production (38). These composite results strongly suggest that paclitaxel may have an additional antitumor effect in addition to its effects on microtubules.

This study also suggests that paclitaxel-induced activation of IL-8 expression in human ovarian cells is associated with the intracellular signaling pathway. This conclusion is supported by the following observations. The addition of specific kinase inhibitors or adenyl cyclase activators inhibited paclitaxel-induced NF-κB and AP-1 binding to cognate DNA, respectively, and caused a correlative diminution of IL-8 gene expression. Although the signaling pathway leading to NF-κB activation is still not entirely understood, a report by Perera et al. showed that paclitaxel could induce the translocation of NF-κB to the nucleus of murine macrophages in a manner mimicking LPS (48). Several kinases have been proposed in the activation of NF-κB, including the double-stranded-RNA-activated kinase (PKR), which phosphorylates IκBα in vitro (28); Raf-1, which targets IκBα (33), leading to the activation of gene expression through a κB site (20); and PKC ζ subspecies, which acts as a regulator of NF-κB (4, 12, 16). Finally, many different inducers of NF-κB, such as ultraviolet light, LPS, and proinflammatory cytokines, may affect the stress-activated protein kinases or their regulators, and these kinases may also play a role in IκB phosphorylation (2). It will be of interest to test if paclitaxel affects any of these kinases.

The downregulation of AP-1 activity by forskolin is consistent with several earlier observations showing cross-talk between the cyclic AMP (cAMP) and AP-1 (3, 11, 26, 58). The AP-1 transcription factor binds to 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (TRE) palindromic sequences. TREs are similar to cAMP-responsive elements (CREs), and Jun and Fos are structurally similar to CRE-binding proteins (CREBs), which employ cAMP as a second messenger. Thus, the two main signal transduction pathways have closely related nuclear effectors which could possibly cross talk. CRE modulator proteins and CREBs can bind to a TRE and therefore compete with Jun for binding (39). Forskolin, which activates

the CREs by increasing intracellular cAMP levels, has been shown to inhibit AP-1 activity and to revert AP-1-dependent transformation (17, 18). The molecular mechanism by which forskolin inhibits AP-1 may involve interaction between CREBs and Jun protein, thereby inhibiting basal and induced AP-1 activity (39). It is likely that forskolin inhibits paclitaxel-induced IL-8 in ovarian carcinoma cells by similarly modulating AP-1 activity.

We have previously investigated proinflammatory cytokine expression in a series of cell lines and recent explants of human ovarian cancer cells (32), and we have found that paclitaxel induced secretion of IL-8, but not IL-6, IL-1α, or IL-1β, in a subgroup of human ovarian cancer cell lines; thus, this effect is cytokine specific. Paclitaxel did not induce IL-8 in breast carcinoma, endometrial stromal, or T-lymphocyte or human primary monocyte cultures, revealing another aspect (cell specificity) of this phenomenon. Furthermore, a recent report from our group noted that paclitaxel alone enhanced IL-1β mRNA levels and secretion in human primary monocytes as long as the cells were cultured in the more physiologically autologous human serum but not in fetal calf serum (55). All these studies indicate a complicated web of gene regulation that is significantly altered by paclitaxel, and the composite effects may determine the outcome of patient responsiveness to the chemotherapy.

In summary, this report shows that paclitaxel induces the NF-κB and AP-1 transcription factors, which in turn upregulate the IL-8 gene in a subgroup of human ovarian cancer cell lines. From a broader perspective, the activation of AP-1 and NF-κB most likely affects the expression of myriad target genes. Identifying these genes and understanding their role in tumor growth or inhibition will be crucial in understanding the antitumor mechanism of paclitaxel. From a biological standpoint, IL-8 may be produced locally by paclitaxel-treated ovarian cancer cells and lead to the infiltration of T lymphocytes and neutrophils into tumor sites. Such a mechanism would suggest that leukocyte infiltration induced by IL-8 in human ovarian cells may play a role in initiating immune responses to tumor cells or may control tumor cell growth and metastasis (21, 51). A recent report from one of our groups shows that the introduction of IL-8 into hamster ovarian tumor cells reduced tumorigenicity in nude mice in association with neutrophilic infiltration (23). The role of IL-8 in human ovarian cancer cell growth in nude mice is currently being investigated. On the other hand, IL-8 also promotes angiogenesis, where it is possible that IL-8 induction by paclitaxel may have differential effects on the growth of primary tumors and the spreading of secondary growth. Regardless of which scenario takes place, this induction is likely to have significant clinical implications.

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