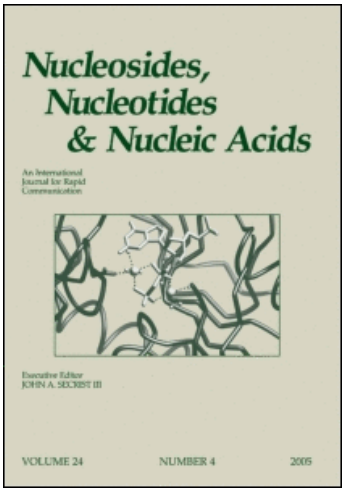

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Activation of Stat1, IRF-1, and NF-κB is Required for the Induction of Uridine Phosphorylase by Tumor Necrosis Factor-α and Interferon-γ

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ACTIVATION OF STAT1, IRF-1, AND NF-κB IS REQUIRED FOR THE INDUCTION OF URIDINE PHOSPHORYLASE BY TUMOR NECROSIS FACTOR-α AND INTERFERON-γ

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□ *Uridine phosphorylase (UPase) has been shown to be induced in various human and murine tumors and could potentially serve as a specific target for the modulation of tumor-selectivity of fluoropyrimidines. However, the signaling mechanisms underlying the regulation of UPase gene expression have not been determined. In this study, we investigated the effects of IFN-γ on the regulation of TNF-α-induced UPase activity and have uncovered the molecular mechanisms of this potentiation, utilizing murine EMT6 breast cancer cells. Our data has shown that IFN-γ can significantly increase UPase mRNA expression and the enzymatic activity induced by TNF-α in a dose-dependent manner, resulting in an enhanced sensitivity to 5-fluorouracil (5-FU) and 5′-Deoxy-5-fluorouridine (5′DFUR). We have previously shown that TNF-α activates NF-κB through increased translocation of NF-κB p65 from the cytoplasm into the nuclei. Exposure to IFN-γ mainly affects nuclear IRF-1 and STAT1 in EMT6, but inhibits NF-κB p65 activity, indicating that the cooperative stimulation was the result of the independent activation of NF-κB, STAT1 and IRF-1 transcriptional factors through binding to their unique sites in the UPase promoter. Notably, the activation of NF-κB and STAT1 in human breast tissues is consistent with UPase activity; signifying their role in the up-regulation of the UPase gene expression in human tumors.*

Keywords TNF-α; IFN-γ; gene expression regulation; NF-κB; STAT-1; IRF-1; promoter analysis

INTRODUCTION

Fluoropyrimidines have been widely used alone or in combination regimens for the treatment of colorectal and breast carcinomas.[1] The first

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generation derivative, 5-fluorouracil (5-FU) requires intravenous administration and has only partial efficacy. Its clinical usefulness is hindered by dose-limiting toxicities, especially on the bone marrow and the gastrointestinal tract. Capecitabine, a new oral prodrug of 5-FU recently approved by the Food and Drug Administration (FDA), is characterized by tumor-selective activation through three sequential enzymatic reactions. First, capecitabine is converted to 5′-deoxy-5-fluorocytidine (5′DFCR) by carboxylesterases, and then to 5′-deoxy-5-fluorouridine (5′DFUR) by cytidine deaminase in the liver. Finally, mainly in tumor tissues, 5′DFUR is activated to 5-FU by thymidine phosphorylase (TPase) and uridine phosphorylase (UPase).[2–6] The higher phosphorolytic activity in human tumor tissues compared to that of the surrounding normal tissue has been suggested to result in the selective activation with consequent improved therapeutic index.

TPase and UPase are important enzymes in the activation of fluoropyrimidines. Several studies have indicated the role of UPase in fluoropyrimidine activation, including the anabolism of 5-FU into 5-fluorouridine with subsequent phosphorylation to 5-fluorouridine-monophosphate by uridine kinase and the phosphorolysis of 5′DFUR into 5-FU.[7–10] 5′DFUR and 5-FU are also substrates for TPase. TPase transforms 5′DFUR into 5-FU through its catabolic activity, and 5-FU into 5-FdUrd through its anabolic activity that can then be converted by thymidine kinase into FdUMP, an inhibitor of thymidylate synthase. Changes in activities of these enzymes could therefore influence the anti-tumor effects of fluoropyrimidines.

The susceptibility of tumor cells to 5-FU or 5′DFUR has been reported to be enhanced by the transfection of TPase.[11] We have shown, with the development of our UPase knockout murine model, that UPase activity directly correlates with the cell sensitivity to fluoropyrimidines.[12] In UPase knockout murine ES cells, the abrogation of UPase has resulted in an 8- and 16-fold increase in the IC50′s to 5-FU and 5′DFUR, respectively, while wild type cells maintain their sensitivity to fluoropyrimidines without any addition of Rib-1-P or its donors.[7] It is therefore expected that TPase or UPase inducers may improve the efficacy of 5-FU or its prodrugs. In fact, some investigations have shown a marked synergistic antiproliferative effect between 5-FU or 5′DFUR and cytokines such as interferon-α and γ (IFN-α and γ), tumor necrosis factor-α (TNF-α), and interleukin-1α (IL-1α). For example, in colon 26 tumor cells, a mixture of TNF-α, IFN-γ, and IL-1α efficiently enhanced 5-FU and 5′DFUR cytotoxicity 2.7- and 12.4-fold, respectively.[13] These three cytokines increased the sensitivity of human cancer cell lines (COLO201, MKN45 and WiDr) to 5-FU or 5′DFUR up to 5-fold or 50-fold.[14] This potentiation with 5-FU or 5′DFUR is the result of induction of TPase and UPase since the mixture of these factors increased UPase activity by 3.7-fold in colon 26 and TPase activity by up to 47-fold in human cell lines.

TPase was found to be identical to platelet-derived-endothelial cell growth factor (PD-ECGF).[15] Compared to normal tissues, TPase overexpression has been found in most tumor cells and may correlate with an increase in neovascularization[16,17] and poor prognosis.[18,19] While the regulation of TPase has been well documented,[20,21] little is known about the regulation of UPase.

Like TPase, the expression level of UPase is higher in various human or murine tumor tissues when compared to normal tissue. Many studies have consistently reported that expression of UPase is higher in various human solid tumors, including breast cancers, colorectal cancer, and oral squamous carcinomas, melanoma tissue and lung adenocarcinomas, compared with adjacent normal tissues.[10] Patients with overexpression of UPase have worse survival than those with lower levels. Therefore, the UPase induction mechanism in tumor cells may be exploited to improve the therapeutic index of fluoropyrimidine-based regimens.

In this report, we investigated in detail the effect of TNF-α/ IFN-γ on the control of UPase gene expression in EMT6 cells. The results presented here demonstrate that the cis-acting elements, NF-κB, IFN-regulatory factor1 (IRF-1) and signal transducers and activators of transcription (STAT-1) in the murine UPase promoter play a critical role in the induction of UPase by the combination of TNF-α and IFN-γ.

EXPERIMENTAL PROCEDURES

Cells and Reagents

EMT6 cells (a murine mammary tumor kindly provided by Dr. S. Rockwell (Yale University, New Haven, CT) were maintained in Waymouth's MB 752/1 medium (GIBCO, New York, NY, USA) with 15% fetal bovine serum. Recombinant murine TNF-α (1×10^7 units/mg) and IFN-γ (1×10^7 units/mg) were purchased from Chemicon, Inc. (Temecula, CA, USA). Rabbit polyclonal antibodies against STAT1, IRF-1, and murine monoclonal antibodies against NF-κB p65, IκBα, and α-Tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Murine monoclonal antibody against β-actin was purchased from Sigma (Sigma Chemicals Co., St. Louis, MO, USA).

Patient Samples

Human breast tumors and adjacent normal tissues were obtained from patients undergoing surgical removal of their malignancy through the Surgical Pathology Department of Yale New Haven Hospital (New Haven, CT,

USA), according to an appropriate clinical protocol approved by the local institutional review board.

RNA Analysis

Total RNAs were extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). mRNA levels were assessed by Northern blot analysis using 15 μg of RNA denatured in formaldehyde/formamide. The UPase levels were normalized to the β-actin level in each lane to correct for the amount of total RNA loaded.

UPase Enzymatic Activity Assay

UPase enzymatic activity was measured by the conversion of uridine to uracil using TLC chromatographic separation using an 85:15:5 mixtures of chloroform, methanol, and acetic acid respectively as described previously.[22]

Purification of UPase Antibody and Western Blot

Rabbit polyclonal antibody against human UPase was obtained in this laboratory and purified using a recombinant human UPase protein affinity column based on Sulfolink Chemistry (Pierce, IL, USA). A 1:500 dilution of purified antibody was used in Western blots.

Human tissue lysates and cell nuclear or cytoplasmic extracts were fractionated by 12% SDS-PAGE and transferred onto nitrocellulose membrane. Blots were developed by incubation with a chemiluminescence substrate (Amersham ECL, GE Healthcare, Piscataway, NJ, USA) and exposed to x-ray film.

In Vitro Cytoxicity Assay

The antiproliferative activity of the fluoropyrimidines was assessed by the XTT cell proliferation kit II (Roche, Indianapolis, IN, USA), after plating 1,000 EMT6 cells/well in 96-well tissue culture plates. Each concentration point was replicated in eight wells and all experiments were conducted in triplicates.

Incorporation of [^{3}H] 5-FU Into Nucleic Acids

Incorporation of radioactive 6-[^{3}H] 5-FU or 6-[^{3}H] 5′ DFUR into nucleic acids was conducted on cells (1×10^5/well) plated in 6-well plates. The amount of incorporated 5′DFUR was calculated by determining the associated radioactivity according to the amount of labeled 6-[^{3}H] 5-FU or

6-[^{3}H] 5′ DFUR relative to the amount of nonlabeled 5-FU or 5′DFUR and expressed in pmol/10^6 cells.

Generation of Deleted and Mutant Promoter Constructs

A series of luciferase expression constructs containing various lengths of the 5′-upstream murine UPase gene in the pGL3-basic vector were generated as previously reported.[23] The Stratagene QuickChange Site-Directed Mutagenesis kit was used to generate mutations in the potential IRF-1-binding site using the p-84 construct as a template (native sequence: 5′-TAAAAATCAAAACAAAACAG**AAA**CTGGCACGGGATAC-3′, mutated sequence: 5′-TAAAAATCAAAACAAAACAG**CCC**CTGGCACGGGATAC-3′.

Transfection and Reporter-Gene Assays

The transfections were conducted in triplicate in 6-well plates on approximately 10^5 cells/well. Two micrograms of indicated mouse UPase promoter constructs and an internal control (pRL-TK plasmid) were co-transfected into cells using Lipofect^{AMINE} reagent (Invitrogen). Firefly luciferase and the control renilla luciferase were detected using a dual luciferase detection kit (Luciferase Assay System, Promega, Madison, WI, USA). Reporter activity was expressed as the ratio of firefly luciferase activity (pGL3-based plasmids) to Renilla luciferase activity (pRL-TK).

Electrophoretic Mobility-Shift Assay (EMSA)

Double-stranded DNA probes containing the NF-κB, putative STAT-1 or IRF-1 binding sites were prepared by annealing the complementary oligonucleotides; NF-κB, 5′-CATTTTTGGGTTTTCCCTTC-3′; STAT-1, 5′GCAGC TTCCCGCAATAAACC and IRF-1, 5′TCAAAACAAAACAGAAACTGGCA-3′. Gel-shift analysis was described previously.[23]

Statistical Analysis

Data shown in the figures indicated the mean ±SD, and was analyzed by the Student's *t* test. Differences resulting in P values < 0.05 were considered to be statistically significant.

RESULTS

TNF-α and IFN-γ Activate UPase Gene Expression and Enzymatic Activity

TNF-α efficiently induces UPase gene expression and enhances cell sensitivity to 5′-DFUR.[10] To determine whether IFN-γ synergistically induces UPase gene expression in the presence of TNF-α in vitro, UPase gene

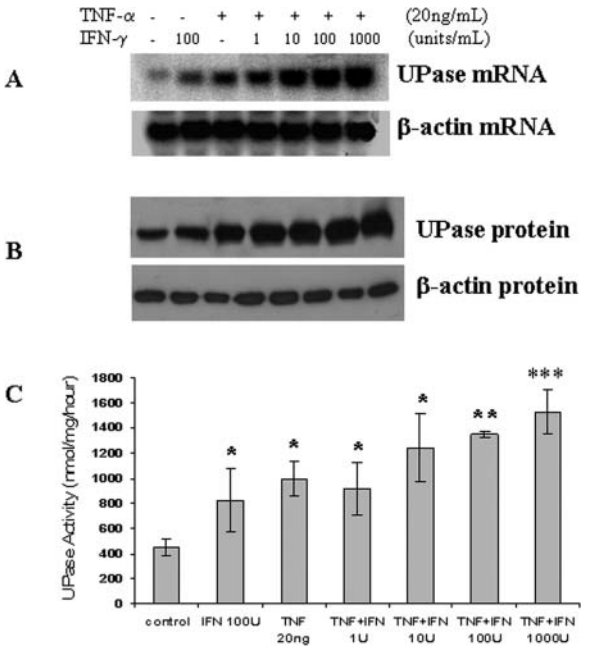

FIGURE 1 UPase induction by TNF-α and IFN-γ. EMT6 cells were incubated with TNF-α (20 ng/mL), IFN-γ (100 units/mL) alone, or TNF-α with increasing concentrations of IFN-γ (1–1000 units/mL) for 24 hours. Total RNA was extracted and analyzed by Northern blot (A). UPase protein levels (B) and enzymatic activity (C) were also determined in the cell lysates. Values are the mean of at least three experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

expression and activity were analyzed in cytokine-treated EMT6 cells employing Northern blot and enzymatic assays. As expected, IFN-γ or TNF-α as single agents resulted in a 1.5-fold or 2.6-fold induction of UPase mRNA levels respectively. Incubation with both IFN-γ and TNF-α significantly increased the induction of UPase mRNA accumulation by 5- 12-fold compared to the nonstimulated cells, and is characterized by an IFN-γ concentration dependent effect (Figure 1A). Consistently, cotreatment with TNF-α and IFN-γ elicited at least an additive effect on UPase protein and enzymatic activity levels (Figures 1B and 1C), clearly indicating that TNF-α and IFN-γ cooperated on the induction of UPase gene expression. The treatments of EMT-6 cells in vitro with TNF-α and IFN-γ alone or in combination resulted in no significant stimulation of thymidine phosphorylase (TPase), uridine kinase (UK), and orotate phosphoribosyltransferase (OPRTase), enzymes also involved in the metabolism of fluoropyrimidines (data not shown).

TNF-α Significantly Enhances the Sensitivity to 5-FU and 5′DFUR in EMT6 Cells in the Presence of IFN-γ

To further determine the effect of INF-γ on induction of UPase by TNF-α, we examined the antiproliferative activity of 5-FU and 5′DFUR and their

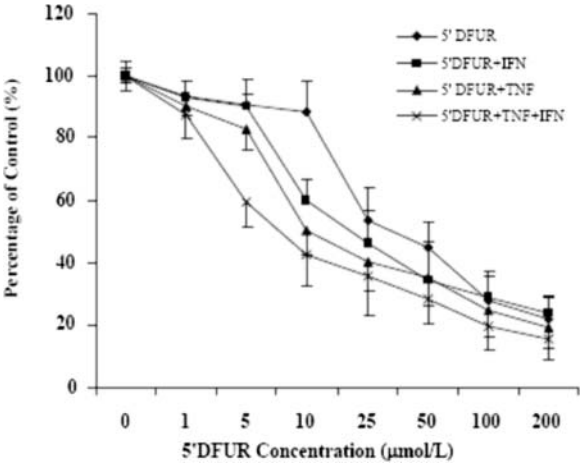

FIGURE 2 Enhancement of 5′DFUR antiproliferative activity by TNF-α and IFN-γ in EMT6 cells. Cells were exposed to TNF-α and/or IFN-γ for 24 hours and then incubated with 5′-DFUR for additional 72 hours. Growth inhibition was calculated in comparison to untreated cells.

incorporation into RNA in EMT6 cells. Individual treatment with TNF-α or IFN-γ did not significantly affect the antiproliferative activity of 5-FU, but their combined pretreatment led to a 2.3-fold reduction in 5-FU IC50 (data not shown). Moreover, the sensitivity of cells pretreated with TNF-α or IFN-γ to 5′DFUR resulted in 2.5-fold and 1.7-fold decreases in IC50, respectively, whereas the combination of these two cytokines decreased the IC50 by 4.6-fold compared to 5′DFUR alone treated cells (Figure 2). These differences in sensitivity were consistent with an increased incorporation of 5-FU and 5′DFUR mainly into RNA. The anabolism of 5-FU was only slightly affected by TNF-α or IFN-γ as single agents. The co-treatment of TNF-α and INF-γ, however, resulted in 1.8-fold increased incorporation of 5-FU into RNA (data not shown). Pretreatment with TNF-α or IFN-γ alone and their combination increased the incorporation of 5′DFUR into RNA by 2.3-fold, 2.8-fold, and 8.5-fold, respectively, compared to 5′DFUR-treated cells (Figure 3). Nevertheless, when the pretreated cells were simultaneously exposed to 5-FU or 5′DFUR in the presence of the specific UPase inhibitor BAU (50 μM), the increased sensitivity to 5-FU or 5′DFUR was abolished (data not shown), indicating that the enhanced sensitivity is limited to the potentiation of UPase by TNF-α and IFN-γ.

Localization of UPase Promoter Elements Required for the Induction by TNF-α and IFN-γ

To investigate the potentiating effect of IFN-γ and TNF-α on the induction of UPase gene expression, we defined the requirement of putative cis-acting elements in the UPase promoter employing deleted or mutated

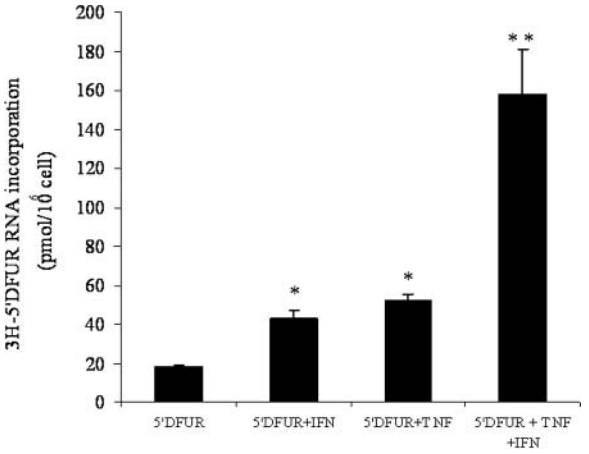

FIGURE 3 Incorporation of radiolabeled 5′DFUR into RNA. EMT6 cells were treated with TNF-α (20 ng/mL) and/or IFN-γ (100 units/mL) for 24 hours and then incubated with radiolabeled 5′-DFUR (30 μmol/L) for additional 4 hours. Total RNA was extracted and the amount of 5′-DFUR radioactive incorporation determined. * $P < 0.05$, ** $P < 0.01$, compared to the control.

reporter constructs.[23] Within the proximal 1619-bp region of the UPase promoter, we have previously reported a NF-κB p65 binding site (-1339/-1320 bp),[10] as well as a putative STAT-1 (-1052/-1044 bp) and an IRF-1 (-35/-23 bp) binding site. These elements most likely mediate the induction of UPase by TNF-α and IFN-γ. In order to test this hypothesis, a series of deletion or mutation constructs with or without the putative elements were transfected into EMT6 cells (Figure 4A). A significant stimulation (2.8-fold)

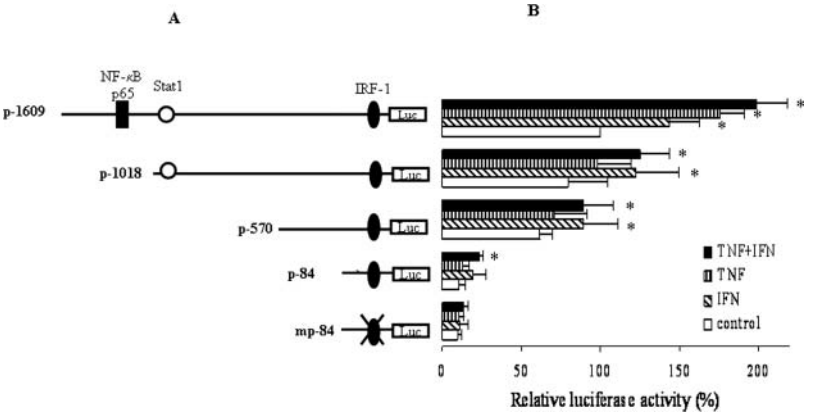

FIGURE 4 Functional NF-κB and putative STAT1, IRF-1 binding sites in the UPase promoter region and their role in TNF-α and IFN-γ induced promoter activity. A) Location of NF-κB and putative STAT1, IRF-1 elements in the UPase promoter and the UPase/luciferase gene constructs used in the transient transfection analysis. B) Luciferase activity of different constructs after cytokine induction. Activity levels were normalized to that of untreated p-1619 cells.

of reporter activity by TNF-α and IFN-γ in combination was observed in EMT6 cells transfected with the p-1619 plasmid, while treatment with TNF-α and IFN-γ alone resulted in 1.8-fold and 1.5-fold increased promoter activity respectively, demonstrating that the two cytokines stimulate the murine UPase promoter in at least an additive manner. Deletion of the UPase promoter up to -1081 bp (p-1081) resulted in a reduced effect by the TNF-α and IFN-γ combination. A further deletion of the promoter to -84 bp (p-84) significantly decreased both levels of basal and induced activity. However, the mixture of TNF-α and INF-γ still led to an increased induction in p-84, while plasmid mp-84, which IRF-1 binding sites was mutated, was not responsive to either TNF-α and IFN-γ or IFN-γ alone treatment (Figure 4B), indicating these three transcription factors NF-κB, STAT-1, and IRF-1 may play an important role in the synergistic induction of UPase by TNF-α and INF-γ.

TNF-α and INF-γ Activate the Inducible Nuclear Proteins Binding to UPase Promoter Elements

We next tried to determine whether a potentiating effect could be observed in the binding of transcription factors to their respective consensus sequences. Nuclear extracts were isolated from EMT6 cells after 24-hour exposure to cytokines, and equal amounts of proteins were incubated with the radiolabeled UPase probes containing p65, STAT-1, or IRF-1 binding sites, respectively. As we reported previously, TNF-α alone significantly stimulates NF-κB p65 activity in EMT6 cells, while IFN-γ does not. In these experiments, IFN-γ treatment resulted in decreased NF-κB interaction with the UPase promoter element (Figure 5A, Lane 2). TNF-α moderately induced the formation of a p65 specific protein-DNA complex, while co-treatment with IFN-γ increased NF-κB binding. (Figure 5A, Lanes 3 and 4). Formation of the TNF-α and IFN-γ induced complex was highly specific to the sequence contained in the NF-κB 65 probe, because the addition of 100-fold excess of unlabeled NF-κB 65 probe as a competitor inhibited complex formation (Figure 5A, Lane 5), while the addition of a 100-fold excess of unlabeled nonspecific competitor had no effect (data not shown). To verify that the induced complexes contained NF-κB and to verify the subunit in the complexes, an antibody supershift was performed. The addition of anti-p65 antibody resulted in a decreased mobility of the specific complex (Figure 5A, Lane 6), but not STAT-1 and IRF-1 antibodies (data not shown), confirming that p65 was responsible for complex formation and not physical interaction between NF-κB and STAT1 or IRF-1.

EMSA was also performed to confirm whether the STAT-1 transcription factor binds to the UPase promoter. As shown in Figure 5B, both TNF-α and IFN-γ significantly induced the formation of STAT-1 complex, while the co-stimulation of IFN-γ and TNF-α did not enhance the formation of

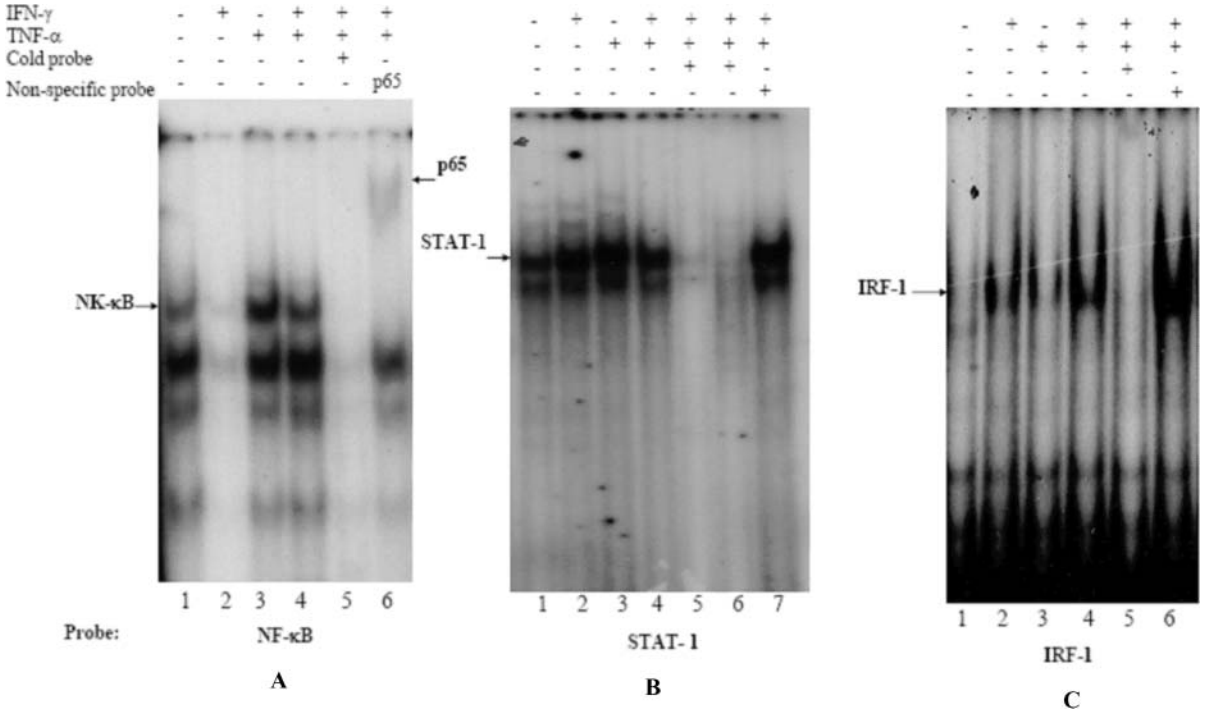

FIGURE 5 Specific binding of NF-κB, IRF-1, and STAT1 to their response elements in the UPase gene promoter. EMSAs were performed with the probes containing NF-κB, IRF-1, and STAT1, respectively, and nuclear extracts (10 μg) prepared from EMT6 cells unstimulated or stimulated with TNF-α and/or IFN-γ for 24 hours. Competition analysis of each probe was carried out in the presence of 100× -fold molar excess of unlabelled or nonspecific probes. For supershift analysis, NF-kBp65 antibody was preincubated with nuclear protein before addition of probe. A) NF-κB; B) STAT1; C) IRF-1.

the STAT-1 complex. Next, we evaluated the induction of IRF-1 by treatment with TNF-α and IFN-γ. A complex from nuclear extracts treated with IFN-γ was observed, but not from TNF-α (Figure 5C, Lanes 2, 3). TNF-α and IFN-γ together did not potentiate the formation of the specific complex (Figure 5C, Lane 4). The induced complex was specific, as it was inhibited by the addition of unlabeled IRF probe to the binding reaction (Figure 5C, Lane 5), but not by nonspecific competitor (Figure 5C, Lane 6). Incubation of the nuclear extracts with anti-IRF-1 antibody prior to the addition of the labeled IRF-1 probe prevented the formation of the specific protein-DNA complex, but incubation of nuclear extracts with anti-NF-κB and STAT1 antibodies did not (data not shown). This indicated that this complex contained IRF-1, but was not physically interacting at the IRF-1 element with NF-κB and STAT1 in the additive activation of UPase by TNF-α and IFN-γ in EMT6 cells.

Taken together, these results indicate that NF-κB, STAT1 and IRF-1 are independently induced in response to TNF-α and IFN-γ, respectively, and activate the murine UPase promoter with no physical binding to each other.

Induction and Nuclear Translocation of Transcription Factors by IFN-γ and TNF-α is Not Synergistic

To provide further evidence of the potentiating effects of IFN-γ and TNF-α on the induction and nuclear translocation of the transcription factors, nuclear and cytoplasmic extracts from EMT6 cells stimulated with TNF-α and IFN-γ were immunoblotted with antibodies against p65, STAT1, and IRF-1. Under normal conditions NF-κB is sequestered in the cytoplasm by its inhibitory protein (IκBs). A variety of stimuli promote the degradation of specific IκB proteins, resulting in the release and nuclear translocation of NF-κB. Consistent with the EMSA results reported above, NF-κB p65 was induced by TNF-α stimulation and translocated into the nuclear compartment (Figure 6). The treatment of IFN-γ alone resulted in nuclear accumulation of IRF-1 and STAT-1, with no effect on the nuclear translocation of NF-κB p65. No synergism was observed after combination treatment in the activation and translocation of NF-κB p65, IRF-1, and STAT-1.

Cytokines and other factors capable of stimulating NF-κB or STAT1 expression may induce UPase expression in tumor tissues. We investigated the correlation between NF-κB, IRF-1, and STAT-1 expression levels and induction of UPase in human breast cancer specimens. As reported in Figure 7, NF-κB p65 and STAT-1 expression in tumor tissues is higher than in their adjacent normal tissues; however, no IRF-1 expression was detected (data not shown). NF-κB p65 and STAT-1 levels are consistent with increased UPase protein levels and enzymatic activity in tumor tissue and showed a positive correlation in the human specimens. Our data indicate that the activation of the transcription factors NF-κB and STAT-1 is indeed involved in the UPase upregulation observed in many tumor types.

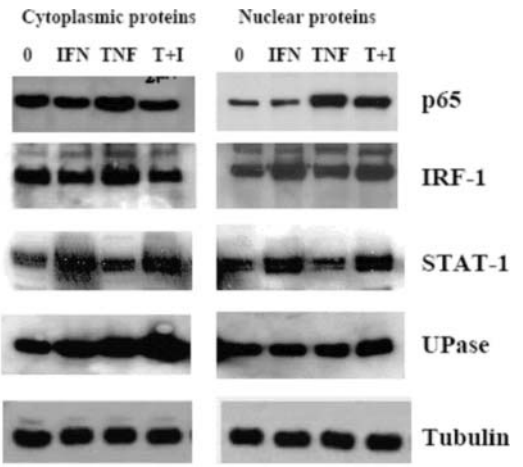

FIGURE 6 Activation of transcription factors NF-κB, IRF-1, and STAT1 by TNF-α and IFN-γ. EMT6 cells were treated with TNF-α (20 ng/mL), and/or IFN-γ (100 units/mL) for 24 hours. Cytoplasmic (50 μg) and nuclear extracts (30 μg) were evaluated for NF-kBp65, IκBα, IRF-1, STAT1, and UPase expression by Western blot analysis.

DISCUSSION

In this article, we have evaluated the effects of TNF-α and IFN-γ on UPase gene regulation in EMT6 cells and explored the intracellular molecular mechanisms by which these two cytokines may act cooperatively to regulate gene expression. The results demonstrate that TNF-α and IFN-γ together

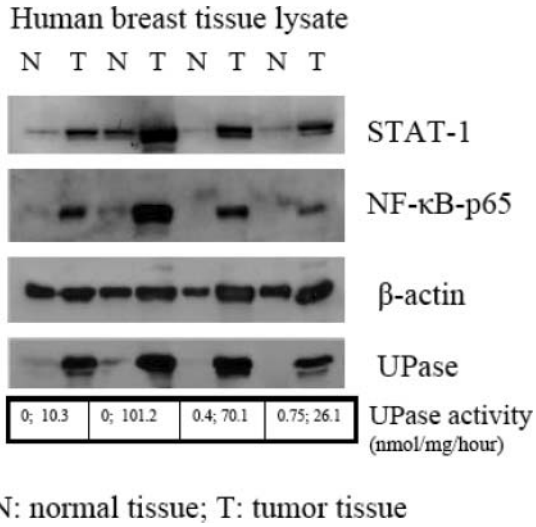

FIGURE 7 NF-kBp65 and STAT1 expression levels are correlated with UPase activity in human breast cancer tissues. Tumor tissue (T) and paired adjacent normal tissue extracts (N) (50 μg) were analyzed by Western blot and by UPase enzymatic activity.

potentiate the induction of UPase mRNA expression and enzymatic activity and enhance 5-FU and 5′DFUR antiproliferative activity. Furthermore, this potentiating effect required independent activation of IRF-1 and STAT1 binding activity by IFN-γ and NF-κB p65 binding activity by TNF-α; these transcription factors cooperate by interaction with NF-κB, IRF-1 and STAT-1 sites in the UPase promoter.

Several studies have reported functional synergy between TNF-α and IFN-γ in promoting gene expression and immune response in numerous cell types (see discussion in Cheshire et al.[24]) through a variety of mechanisms. First, the potentiation of TNF-α and IFN-γ may be exerted by the independent activation of distinct transcription factors. For example, TNF-α often uses NF-κB as the primary effector in target cells.[25] IFN-γ elicits gene expression by primarily activating the Janus kinase/STAT pathway which in turn triggers production of IRF-1.[26] The transcription factors NF-κB, IRF-1, and STAT may bind to their unique promoter sites and then regulate the expression of genes containing TNF-α and IFN-γ-response elements in their promoter.[27] Second, the transcription factors could interact physically on the promoter, thus forming a complex with higher avidity to the recognition sites than the individual transcription factors.[12,28] NF-κB and IRF-1 have been shown to physically interact and synergistically induce major histocompatibility class I gene expression.[29] In addition, IFN-γ also synergizes with TNF-α by activating NF-κB in several cell lines. IFN-γ alone can activate NF-κB by a Janus kinase-1-mediated STAT-1-independent mechanism.[30]

Our data clearly showed that TNF-α can strongly induce NF-κB p65 in cytoplasm and its accumulation in nuclei. However, in our experiment, IFN-γ decreased NF-κB binding to its response element in the UPase promoter (Figure 5A) and had no effect on its translocation into nuclei in EMT-6 cells (Figure 6). Overall the negative effect of IFN-γ on NF-κB would also explain why UPase gene expression and activity did not significantly increase in EMT-6 cells treated with IFN-γ alone.

In the IFN-γ signaling pathway, activation of JAK1/JAK2 leads to phosphorylation of latent STAT1, followed by their subsequent dimerization, nuclear translocation, and site-specific DNA binding to various genes critically involved in different pathologies correlated to the inflammatory process, such as IRF-1. IRF-1 in turn can activate the expression of several genes including IFN-α and PKR genes. Our experiments have shown that IFN-γ significantly increased STAT1 expression levels and nuclear translocation in EMT6 cells. Similarly, IRF-1 also accumulated in nuclear extracts after treatment by IFN-γ. TNF-α alone or in combination did not activate nuclear translocation of STAT1 or IRF-1. In summary, the activity of IFN-γ and TNF-α on murine UPase upregulation mainly occurs through cooperation between IFN-γ activating STAT1/IRF-1 and TNF-α activating NF-κB.

The elucidation of UPase transcriptional regulation reveals similarities to the induction of TPase expression that is controlled by the transcription

factors Sp1 and STAT1.[31–33] A recent report also indicated that the post-transcriptional regulation of TPase mRNA stability in response to interferons[21,32] is the result of the ERK-mediated cytoplasmic accumulation of hnRNP K that stabilizes the mRNA of TPase, increasing its half-life and thereby increasing the protein levels of Tpase.[34]

As we have previously indicated,[10] we found that the basal activity of TPase in murine EMT-6 cells is barely detectable, and the TNF-α and interferon treatments have no effect on its activity level, indicating that TPase expression is not cytokine-inducible in this cell line. Therefore, the increased antiproliferative effect of 5-FU and 5′-DFUR in these cells has to be entirely ascribed to the induction of UPase.

A combination of 5-fluorouracil (5-FU) and cytokines has been proposed for various malignancies, and satisfactory results have been obtained in some studies.[31,34] It is known that certain radiotherapy and chemotherapeutics, such as taxanes and cyclophosphamide, upregulate UPase or TPase enzymes[35–41] through induction of cytokines such as TNF-α and IFN-γ. Here, we have elucidated that UPase expression was significantly increased after TNF-α and IFN-γ treatment. Therefore, it is possible that UPase upregulation would be triggered by increases in the tumor concentrations of these immune mediators in response to chemotherapy. Also, UPase induction in tumor tissues may partially contribute to the selective cytotoxicity and tumor-specific effect associated with capecitabine treatment since this 5-FU prodrug is activated by the enzymatic activity of pyrimidine phosphorylases. Therapeutic strategies utilizing fluorinated nucleosides may benefit from combined targeting of the UPase promoter. Also, determining UPase expression levels within certain tumors may allow improved and personalized treatment regimens.

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