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STAT6 Mediates Interleukin-4 Growth Inhibition in Human Breast Cancer Cells[1]

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Abstract

In addition to acting as a hematopoietic growth factor, interleukin-4 (IL-4) inhibits growth of some transformed cells *in vitro* and *in vivo*. In this study, we show that insulin receptor substrate (IRS)-1, IRS-2, and signal transducer and activator of transcription 6 (STAT6) are phosphorylated following IL-4 treatment in MCF-7 breast cancer cells. STAT6 DNA binding is enhanced by IL-4 treatment. STAT6 activation occurs even after IRS-1 depletion, suggesting the two pathways are independent. To examine the role of STAT6 in IL-4–mediated growth inhibition and apoptosis, a full-length STAT6 cDNA was transfected into MCF-7 cells. Transient overexpression of STAT6 resulted in both cytoplasmic and nuclear expression of the protein, increased DNA binding in response to IL-4, and increased transactivation of an IL-4 responsive promoter. In STAT6-transfected cells, basal proliferation was reduced whereas apoptosis was increased. Finally, stable expression of STAT6 resulted in reduced foci formation compared to vector-transfected cells alone. These results suggest STAT6 is required for IL-4–mediated growth inhibition and induction of apoptosis in human breast cancer cells.

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Keywords: IL-4, breast cancer, insulin receptor substrate, STAT6, apoptosis.

Introduction

Interleukin-4 (IL-4) is a pleiotropic immunoregulatory cytokine that modulates cell surface receptor expression and proliferation of B cells [28]. IL-4 acts through a multiunit transmembrane receptor, IL-4R, which is a type II cytokine receptor and lacks intrinsic kinase activity [14]. Binding of IL-4, therefore, activates a signal transduction cascade including the janus kinases Jak1 and Jak3, which phosphorylate the cytoplasmic domain of the receptor as well as downstream signaling molecules. Two main pathways are activated in response to IL-4, the insulin receptor substrate-2 (IRS-2) pathway and the signal transducer and activator of transcription 6 (STAT6) pathway [29]. Activation of IRS-2, and IRS-1 in some cells, leads to association of the p85 subunit of PI-3 kinase and signals for mitogenesis and cell survival [8,43]. Phosphorylation of STAT6 leads to formation of STAT6 homodimers and subsequent translocation to the nucleus. STAT6 binds to specific sequences in the promoters of target genes and activates transcription of specific genes including the IgE receptor in B cells [25].

Interestingly, IL-4R has been identified on the surface of some solid tumors including murine sarcoma and adenocarcinoma cells [31], human renal, melanoma, ovarian [26,27], lung [38], and breast carcinoma cells [27]. In addition, Mat et al. [24] reported that IL-4R may be expressed by as many as 30% of primary breast tumor cells.

In contrast to its proliferative action in hematopoietic cells, IL-4 has been shown to have antitumor activity both *in vivo* and *in vitro*. Tepper et al. [35] found that tumor cells that were engineered to secrete IL-4 failed to form tumors when reintroduced into mice. Similarly, Golumbek et al. [10] found that when IL-4 was transfected into cells derived from a spontaneously arising murine renal carcinoma, expression of IL-4 blocked tumor formation. This observation extended to subsequent challenges with parental, non–IL-4 secreting cells. These results suggested that IL-4 was mediated a host immune response. However, *in vitro* experiments also demonstrated that IL-4 directly inhibited the growth of transformed cells including breast, colon, lung, renal, and melanoma carcinoma cell lines in culture [2,26,27,36,37]. In addition, we have previously shown that IL-4–mediated growth inhibition of breast cancer cells is associated with induction of apoptosis [12]. The signaling pathway responsible for growth inhibition of breast cancer cells has not been fully defined.

The observation that IL-4 treatment inhibits tumor cell growth suggests key molecules in the transduction of the inhibitory signal may be useful in breast cancer therapies. In colon cancer and Burkitt's lymphoma cell lines, it has been suggested that IL-4 induction of STAT1 is responsible for cell growth inhibition [5]. In breast cancer cells, it has been shown that both IRS and STAT6 signaling are required to induce expression of enzymes involved in steroidogenesis [7]. We and other groups have shown that activation of IRS-1 is associated with IL-4–mediated growth inhibition

Abbreviations: ERα, estrogen receptor α; IL-4, interleukin-4; IRS, insulin receptor substrate; SFM, serum-free media; STAT, signal transducer and activator of transcription

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[12,15,34]. In fact, Jackson et al. [15] found that MCF-7 and other breast cancer cell lines express IRS-2, but activate IRS-1 in response to IL-4. Interestingly, reduction of IRS-1 mRNA and protein levels in MCF-7 cells did not effect IL-4–mediated growth inhibition [13]. Therefore, in this study, we hypothesize that STAT6 mediates IL-4–induced growth inhibition and induction of apoptosis. Because STAT6 activation has not been reported in conjunction with IL-4–induced growth inhibition, we first characterized STAT6 activation and DNA binding in human breast cancer cell lines. Next, we characterized the effect of overexpression of STAT6 on cell localization, DNA binding, and transactivation of an IL-4 responsive promoter. Finally, we evaluated the role of STAT6 in IL-4–mediated growth inhibition and apoptosis of MCF-7 breast cancer cells.

Materials and Methods

Materials

MCF-7 cells were provided by C. Kent Osborne (Baylor College of Medicine, Houston, TX) and were maintained in improved minimal essential medium (IMEM, Gibco, Bethesda, MD) plus phenol red supplemented with 5% fetal bovine serum (Summit, Ft. Collins, CO). IL-4 was a gift from Satwant Narula (Schering-Plough Research Institute, Kenilworth, NJ). IRS-1 and IRS-2 antibodies were from Upstate Biotechnology (Lake Placid, NY), STAT6 Western blotting antibody was from New England Biolabs (Beverly, MA), and STAT6 antibody used in immunoprecipitation and supershifting was from Santa Cruz Biochemicals (Santa Cruz, CA). All other reagents were from Sigma unless otherwise noted. Mouse STAT6 (mSTAT6) cDNA [42] and pKB350luc IL-4–responsive luciferase construct [40] were kind gifts from Dr. Michael Berton (University of Texas Health Science Center, San Antonio, TX). Antisense STAT6 (asSTAT6) was created by subcloning mSTAT6 into pCDNA3.1 in the reverse orientation. Dr. Paul B. Rothman (Columbia University Health Sciences, New York, NY) kindly provided the ΔSTAT6(645) construct [23].

Western Blots and Immunoprecipitation

Total cellular protein was extracted using a buffer containing 50 mM Tris–HCl pH 7.4, 2 mM EDTA, 1% NP-40, 100 mM NaCl, 100 mM Na orthovanadate, 100 μg/ml leupeptin, 20 μg/ml aprotonin, and 10^{-7} M phenylmethylsulfonyl (PMSF). Fifty micrograms of total protein or 250 μg of protein immunoprecipitated overnight with antibody followed by 4 hours incubation with protein A agarose was separated by 8% SDS-PAGE.

Following transfer of proteins to nitrocellulose, the membrane was incubated in 5% milk–TBST (0.15 M NaCl, 0.01 Tris–HCl, pH 7.4, and 0.05% Tween 20) and then immunoblotted with anti–IRS-1 (1:1000), anti–IRS-2 (1:100), or anti-STAT6 (1:2500). HRP-conjugated goat anti-rabbit secondary antibody was added at 1:2000 for IRS blots and HRP-conjugated donkey anti-mouse secondary antibody at 1:5000 was added for STAT6 immunoblotting. Proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

Gel Shift

Nuclear extracts were prepared by lysing cells first in a hypotonic buffer consisting of 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 5 μg/ml aprotonin, and 0.5 mM PMSF. Nuclear pellets were then briefly centrifuged and resuspended in one half volume low-salt buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 20 mM KCl, 25% glycerol, 5 mM EDTA, 0.5 mM DTT, 20 μg/ml leupeptin, 5 μg/ml aprotonin, and 0.5 mM PMSF) and one half volume high-salt buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.12 M KCl, 25% glycerol, 5 mM EDTA, 0.5 mM DTT, 20 μg/ml leupeptin, 5 μg/ml aprotonin, and 0.5 mM PMSF) for 30 minutes on ice. Extracts were centrifuged for 30 minutes at 4° and the protein concentration of the resulting extracts was determined. Ten micrograms of nuclear proteins were then added to a reaction mixture containing 6 μl buffer A (10 mM HEPES, pH 7.9, 1.5 nM $MgCl_2$, 0.2 mM PMSF, and 0.5 mM DTT) containing 0.5 μg poly DI/DC, and 12 μl of sample extracts to volume in buffer C (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 100 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT). Two microliters of labeled probe corresponding to the STAT6 binding site from the FcγR1 gene promoter (AGCTTGTATTTCCCAGAAAAGGGATC) was added at room temperature for 15 minutes. Samples were run on 4% native acrylamide gels in 0.5% Tris–boric acid–EDTA buffer for approximately 3 hours. Gels were then dried and exposed to film overnight.

Immunofluorescence

MCF-7 cells were transfected overnight with 1 μg of pcDNA or mSTAT6 using Fugene transfection reagent (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The next morning, 10,000 cells from each transfected dish were seeded into two wells of an eight-well chamber slide and allowed to express for 48 hours. Slides were fixed with 70% methanol and allowed to air dry for 15 minutes. Slides were then rehydrated in PBS–0.1% BSA for 15 minutes followed by blocking with anti-mouse immunoglobulin for 30 minutes at room temperature. Ten micrograms per well of STAT6 antibody (New England Biolabs) was added for 30 minutes at room temperature, followed by three 5-minute washes with PBS–0.1% BSA. Ten micrograms per well fluorescein-conjugated anti-mouse secondary antibody was added for 30 minutes at room temperature followed by three 5-minute washes with PBS–0.1% BSA. Slides were prepared with Crystal mount (Biomeda, Foster City, CA) and covered with glass coverslips before examination by fluorescent microscopy.

Luciferase Assays

COS-7 cells were plated in triplicate in DMEM+5% FCS at 2.5×10^5 cells in six-well plates and transfected the next day. Briefly, cells were washed once with PBS, and transiently cotransfected with 1 μg of pKB350luc construct plus 0.1 μg of pSVβ-gal using Fugene transfection reagent

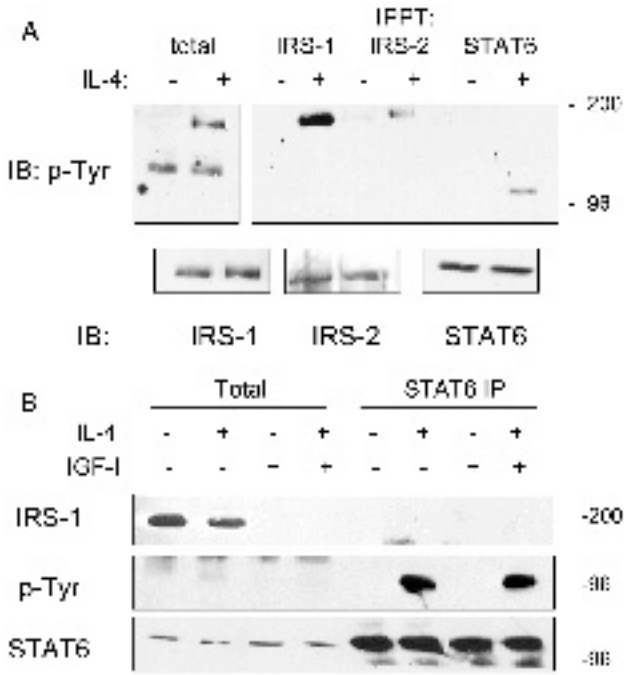


Figure 1. *IL-4 treatment results in phosphorylation of IRS-1, IRS-2, and STAT6 in MCF-7 breast cancer cells. (A) MCF-7 cells were untreated (−) or treated with IL-4 (10 ng/ml) (+) for 10 minutes. Total cell lysates (total) or immunoprecipitate lysates (IRS-1, IRS-2, and STAT6) separated by SDS-PAGE and then immunoblotted with anti-phosphotyrosine antibody. Total cell lysates were also immunoblotted with antibodies to IRS-1, IRS-2, and STAT6 to show the amount of representative protein in the lysates (lower panels). Data shown are representative of three independent experiments. (B) To degrade IRS-1, MCF-7 cells were incubated in SFM in the absence (−) or presence (+) of IGF-I for 24 hours. Cells were then treated with IL-4 (10 ng/ml) for 10 minutes and levels of IRS-1 and STAT6 in total cell lysates were determined by immunoblotting. Phosphorylated STAT6 was detected by immunoprecipitation with anti-STAT6 antibody followed by anti-phosphotyrosine immunoblotting. Data shown are representative of three independent experiments.*

(Boehringer Mannheim) according to the manufacturer's instructions. For some experiments, increasing amounts of mSTAT6 cDNA plus empty vector to normalize the total amount of DNA were transfected along with 0.1 μg of pSVβ-gal using Fugene transfection reagent. Transfected DNA was left on cells overnight. The next morning, media were changed to control medium or medium containing 50 ng/ml IL-4 for 24 hours. Cells were harvested and luciferase activity was measured using the luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. β-Gal activity was measured as described by Rouet et al. [33]. To correct for differences in transfection efficiency, luciferase values were divided by the appropriate β-gal value in the same sample to achieve relative luciferase units.

Single-Cell Proliferation Assay

The single-cell proliferation assay used in this study was previously described [16,21]. MCF-7 cells were plated in DMEM+5% FCS at 2.5×10^5 cells per well in six-well plates and transfected the next day. Cells were washed once with PBS, and transiently cotransfected with 5 μg of each expression construct plus 0.5 μg of pSVβ-gal using Fugene transfection reagent according to the manufacturer's instructions. Transfected DNA was left on cells overnight. The next day, each well was divided into two 10-cm plates and allowed to recover overnight. The next morning, media were changed to control (SFM) or 10 ng/ml IL-4 for 48 hours.

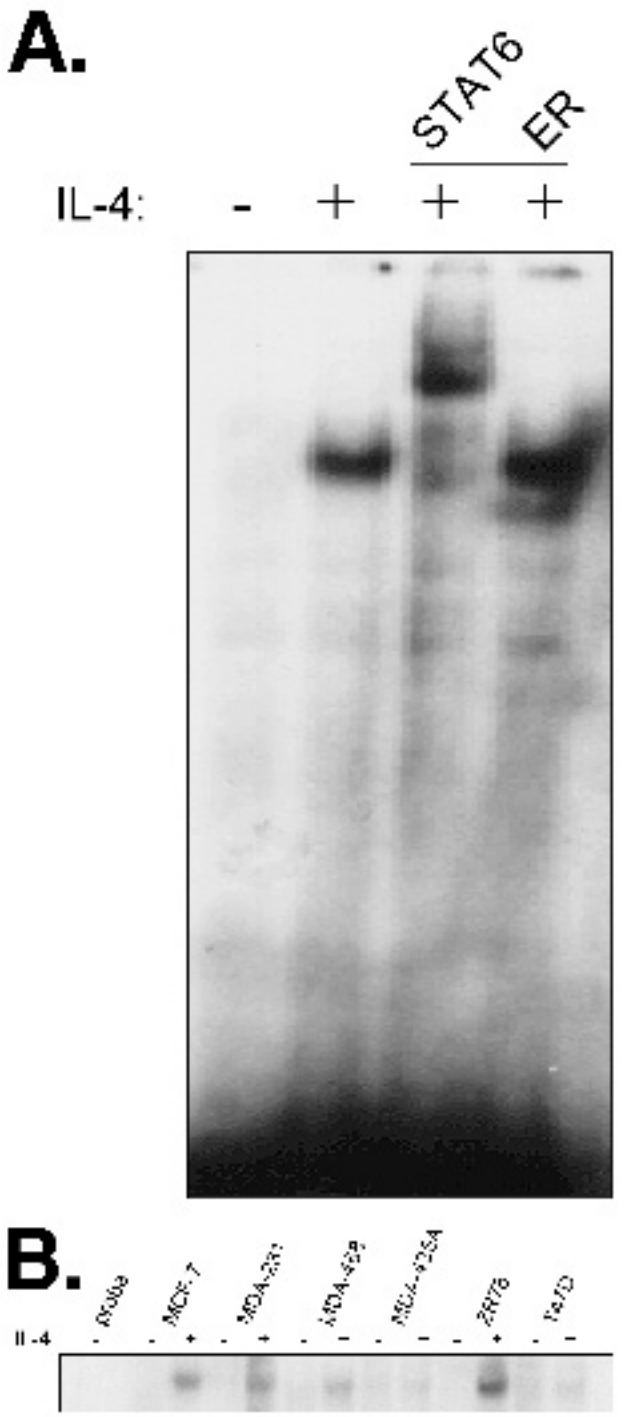
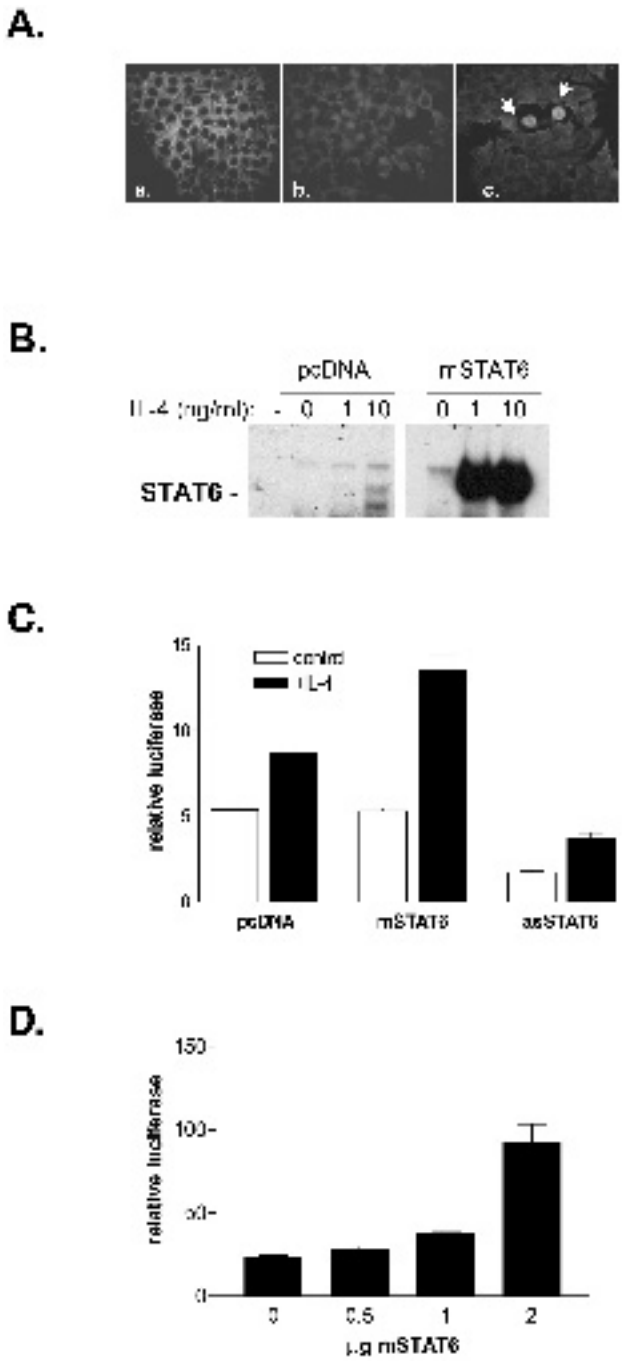


Figure 2. *STAT6 DNA binding activity is induced by IL-4 in breast cancer cell lines. (A) MCF-7 cell nuclear extracts were incubated with labeled probe corresponding to the STAT6 binding site in the Fcγ1 gene promoter. Anti-STAT6 or irrelevant antibodies (anti-ER) were added to the same reactions to produce a supershifted complex. (B) Breast cancer cells were treated with IL-4 (10 ng/ml) for 30 minutes. Nuclear extracts were isolated and 10 μg of protein were incubated with labeled probe. Data shown are representative of three independent experiments.*

Cells were then washed with 1× PBS, fixed in 3.7% formaldehyde for 15 minutes at room temperature, and then incubated in X-gal buffer overnight. Buffer was removed and cells were washed three times with 1× PBS and then cells were examined by microscopy.

Focus Formation Assay

MCF-7 cells were plated at 1.0×10^6 cells per 10-cm dish and transfected the next day. Ten micrograms total of DNA (pcDNA or mSTAT6) was transfected. The next day, cells were transferred to 150-cm dishes and allowed to adhere overnight. The following day, selection was begun using 450 ng/ml G418. Selection was continued for 3 weeks and then resulting foci were stained with 0.1% crystal violet.



Results

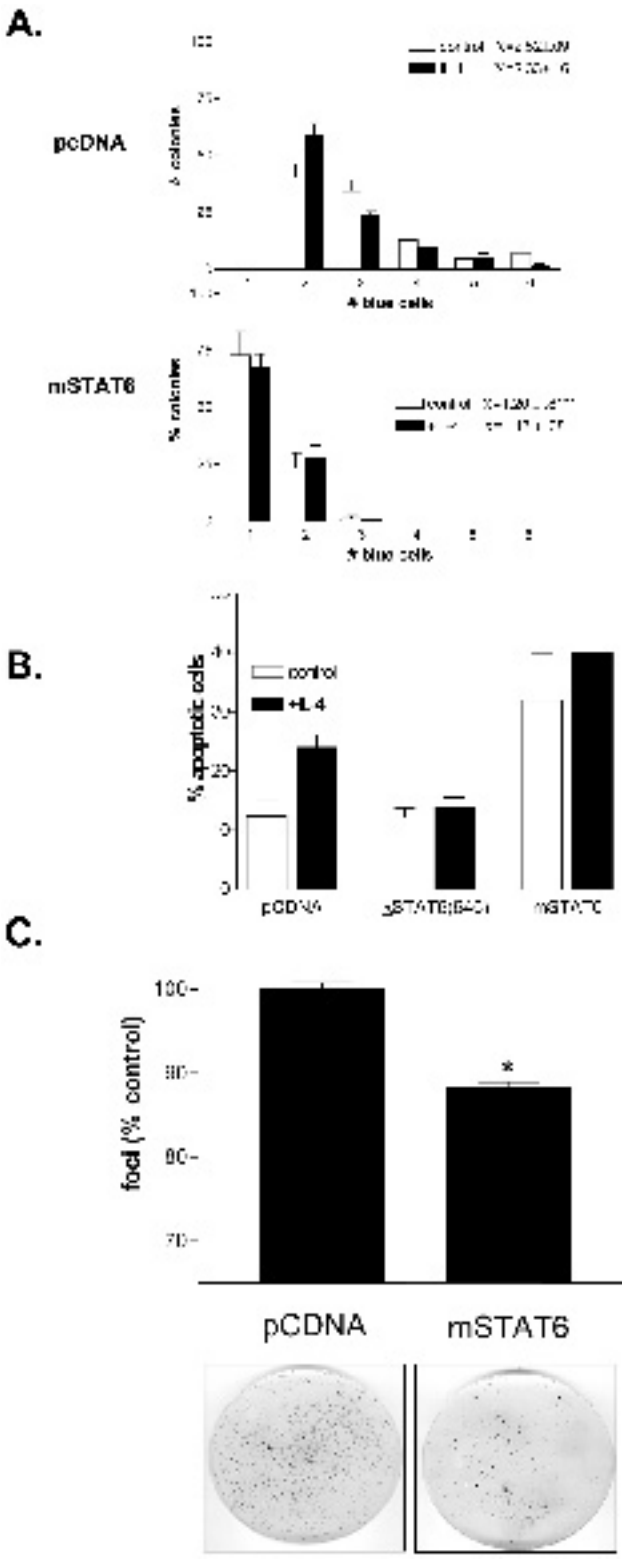
Activation of Signaling Molecules by IL-4 in Human Breast Cancer Cells

We first examined which signaling molecules were activated by IL-4 in human breast cancer cells. Treatment of MCF-7 cells with IL-4 resulted in detection of an approximately 180-kDa tyrosine phosphorylated protein in total cell lysates (Figure 1*A*.). As this band is similar in size to the IRS proteins, we immunoprecipitated both IRS-1 and IRS-2 from whole-cell lysates and then used anti-phosphotyrosine immunoblotting to detect phosphorylation. We found that IRS-1 is heavily phosphorylated whereas IRS-2 is only minimally phosphorylated after IL-4 treatment (Figure 1*A*). This is in contrast to hematopoietic cells where IL-4 treatment predominantly activates IRS-2 [41], but consistent with the previous finding that IRS-1 is the predominant substrate for IGF-I, insulin, and IL-4 in ERα positive breast cancer cells [15]. In addition to IRS-1 and IRS-2, IL-4 has been reported to activate the transcription factor, STAT6. Therefore, we examined activation of STAT6 by immunoprecipitation followed by anti-phosphotyrosine immunoblotting. We found that IL-4 treatment resulted in activation of STAT6 in MCF-7 cells (Figure 1*A*). Treatment of MCF-7 cells with IL-4 did not cause changes in the level of total IRS-1, IRS-2, or STAT6 (Figure 1*A*).

As both IRS-1 and STAT6 have been reported to interact with the IL-4Rα chain [29], we next determined if activation of IRS-1 and STAT6 by IL-4 was independent of one another. We took advantage of a previous observation in our laboratory that IGF-I treatment can induce degradation of IRS-1 within 24 hours [19]. To reduce levels of IRS-1, we pretreated MCF-7 cells in serum-free media (SFM) alone or SFM plus IGF-I for 24 hours and then exposed the cells to IL-4 for 10 minutes. IRS-1 protein levels were analyzed by immunoblotting and found to be decreased to an undetectable level by IGF-I treatment (Figure 1*B*). STAT6 phosphorylation by IL-4 in the

Figure 3. *STAT6 overexpression increases IL-4–mediated DNA binding and promoter activity. (A) STAT6 expression was detected by immunofluorescence with anti-STAT6 antibody followed by fluorescein-conjugated anti-mouse secondary antibody in untransfected MCF-7 cells (a) and cells transfected with pcDNA (b) or mSTAT6 (c). Cells overexpressing mSTAT6 are indicated with arrows. Data shown are representative of three independent experiments. Equal magnification (40×) is shown in each panel. (B) MCF-7 cells transiently transfected with 10 μg pcDNA or mSTAT6 were treated with increasing amounts of IL-4. Nuclear extracts were prepared and then 10 μg of each nuclear extract was incubated with labeled probe. Data shown are representative of two independent experiments. (C) COS-7 cells were transfected in triplicate with pKB350luc, β-gal expression construct, and vector alone (pcDNA), wild-type mSTAT6, or antisense mSTAT6 (asSTAT6). After 12 hours, media was changed and cells were incubated in the absence (−) or presence (+) of IL-4 (50 ng/ml). Luciferase and β-gal activities were determined 24 hours later. Results are expressed as relative luciferase activity, which is defined as luciferase divided by β-gal activity in the same sample. Error bars represent the mean of triplicate samples ± SEM. (D) Increasing amounts of mSTAT6 were transfected in triplicate into COS-7 cells treated with IL-4 (50 ng/ml). Luciferase was measured and normalized to β-gal activity in the same samples. Error bars represent the mean of triplicate samples ± SEM. Data shown are representative of three independent experiments.*

absence of IRS-1 was then determined by immunoprecipitation of STAT6 followed by anti-phosphotyrosine immunoblotting. Phosphorylated STAT6 protein was detected following IL-4 treatment either in the presence or absence of IRS-1 (Figure 1*B*). In addition, phosphorylated STAT6 protein was detected in MDA-MB-231 and MDA-MB-435A cells, neither of which phosphorylate IRS-1 in response to IL-4 (data not shown).



Characterization of STAT6 Expression and Activation in Breast Cancer Cells

We have previously shown that inhibition of IRS-1 protein by antisense expression reduced IGF-I–mediated proliferation and protection from apoptosis but had no effect on IL-4–mediated growth inhibition [13]. Because IL-4 treatment induced phosphorylation of STAT6 in addition to IRS-1, we speculated that STAT6 might function to mediate IL-4–induced growth effects in breast cancer cells. To test this hypothesis, we first examined IL-4–mediated STAT6 DNA binding activity in breast cancer cell lines. We found that IL-4 treatment of MCF-7 cells resulted in the formation of a protein–DNA complex, which was supershifted by STAT6 antibody, but not by an unrelated antibody to the ERα (Figure 2*A*). As shown in Figure 2*B*, we then examined STAT6 DNA binding in a panel of breast cancer cell lines and found that IL-4 activated STAT6 binding in all cells tested (although at a very low level in MDA-MB-435A cells) and that the level of activation was consistent with our previously published result of IL-4R mRNA expression in the same cell lines [12].

To examine the role of STAT6 in IL-4–mediated growth effects, we transiently overexpressed a full-length murine STAT6 cDNA (mSTAT6) in MCF-7 cells. STAT6 expression was detected by immunofluorescence using anti-STAT6 antibody followed by a fluorescein-conjugated anti-mouse secondary antibody. Cells either untransfected or transfected with vector alone showed uniform staining, which was primarily cytoplasmic (Figure 3*A, panels* (*a*) and (*b*), respectively). Following transient transfection with mSTAT6 some cells showed intense staining in both the cytoplasm and nucleus consistent with over expression of mSTAT6 (Figure 3*A, panel* (*c*)). Next, we determined if transient overexpression of mSTAT6 affected IL-4–mediated STAT6 DNA binding in MCF-7 cells. Figure 3*B* shows that MCF-7 cells transiently transfected with vector alone (pcDNA) respond to increasing amounts of IL-4 with increased STAT6 DNA binding and overexpression of mSTAT6 dramatically increased the amount of IL-4–mediated STAT6 DNA binding.

To determine if overexpression of mSTAT6 resulted in increased transactivation of an IL-4–responsive promoter we examined transcriptional activation of murine γ1

Figure 4. *STAT6 mediates growth inhibition and apoptosis in MCF-7 cells. (A) MCF-7 cells were transfected with pcDNA or mSTAT6 along with β-gal expression construct as an indicator of transfection in a 10:1 ratio. Cells were treated with IL-4 for 48 hours, fixed with 3.7% formaldehyde, and then stained with X-gal to identify transfected cells. Colonies of blue cells were counted as a measure of proliferation of transfected cells. Error bars represent the mean of three independent experiments ± SEM. **P < 0.001 (two-way ANOVA) for mSTAT6 control versus pcDNA control. (B) Cells were transfected with pcDNA, mSTAT6, or ΔSTAT6(645) as described in (A) and the percentage of blue cells that had apoptotic morphology was measured. Error bars represent the mean of three independent experiments ± SEM. (C) MCF-7 cells were transfected with pcDNA, mSTAT6, and then cultured in the presence of neomycin for 3 weeks. Resulting foci were stained with 0.1% crystal violet and then counted in triplicate. Error bars represent the mean of triplicate counts ± SEM. Data shown are representative of three independent experiments. *P < 0.05 (Student's t-test).*

immunoglobulin heavy-chain promoter cloned upstream of firefly luciferase (pKB350luc). Figure 3*C* shows that addition of IL-4 resulted in an approximate 1.6-fold induction of relative luciferase activity when cells were transfected with vector alone (pBL2 basic), presumably through activation of endogenous STAT6. Transient overexpression of mSTAT6 enhanced IL-4–mediated induction and, conversely, transient transfection of antisense mSTAT6 (asSTAT6) resulted in inhibition of both IL-4–induced and basal promoter activity. Finally, in Figure 3*D* we show a dose-responsive increase in activation corresponding to increased expression of mSTAT6 in the presence of IL-4.

The Role of STAT6 in IL-4–Mediated Growth Inhibition and Apoptosis

We next determined the role of STAT6 in IL-4–mediated growth inhibition and apoptosis. Because transient transfection of MCF-7 cells results in gene expression in a relatively small percentage of transfected cells, we used a single-cell proliferation assay that has been previously described [16,21]. MCF-7 cells cotransfected with a β-galactosidase expression vector and pcDNA alone (pcDNA) or the mSTAT6 construct. After treatment with IL-4 for 48 hours, the cells were stained with X-gal, and colonies of blue cells were examined for cell number. Cells transfected with vector alone had a mean of 2.52±0.09 cells per colony, representing roughly two doublings in the absence of IL-4 (control) (Figure 4*A*). IL-4 treatment significantly reduced the mean number of blue cells per colony to 2.33±0.16 ($P<0.05$). Interestingly, expression of mSTAT6 alone dramatically reduced the number of blue cells per colony compared to pcDNA (1.20 vs. 2.52, $P<0.001$), and a small additive effect due to IL-4 was observed.

Using the single-cell proliferation assay, we also examined the effect of overexpression of mSTAT6 on IL-4–induced apoptosis. To do this, we determined the percentage of blue cells that were apoptotic based on morphologic characteristics including reduced cytoplasm and chromatin condensation. Figure 4*B* shows that cells transfected with pcDNA responded to IL-4 with a two-fold increase in the number of cells undergoing apoptosis. In contrast, transfection of wild-type mSTAT6 resulted in a large increase in the basal rate of apoptosis, which was only slightly further increased by IL-4 treatment. To determine if inhibition of STAT6 function would block IL-4 induced apoptosis, we transfected cells with a dominant negative STAT6 construct that lacks the transactivation domain (ΔSTAT6(645)). This construct inhibited transactivation of the murine γ1 immunoglobulin heavy-chain promoter and STAT6 DNA binding induced by IL-4 (data not shown). Introduction of this ΔSTAT6(645) dominant negative construct into these cells eliminated IL-4–induced apoptosis.

Taken together, these results suggested that overexpression of STAT6 was responsible for IL-4 effects in MCF-7 cells. Furthermore, overexpression of mSTAT6 was capable of inducing apoptosis in the absence of additional IL-4. Therefore, the ability of mSTAT6 to inhibit the growth of MCF-7 cells in a colony-forming assay was examined. MCF-7 cells were transfected with pcDNA or mSTAT6 and then foci were allowed to form for 3 weeks under G418 selection (Figure 4*C*). Transfection of mSTAT6 significantly inhibited the number of foci ($P<0.05$) compared to transfection of vector alone. In contrast, ΔSTAT6(645) did not influence colony number in this assay.

Discussion

We have shown that IL-4 treatment results in growth inhibition and induction of apoptosis in human breast cancer cells [12]. These growth effects are associated with phosphorylation of two key components of distinct signaling pathways, IRS-1 and STAT6. We find activation of these two pathways occurs independently in breast cancer cells, consistent with findings made in hematopoietic cells [18,29]. Since we previously showed that inhibition of IRS-1 failed to abrogate IL-4–mediated growth effects [13], we hypothesized that STAT6 may be mediating these effects. Inhibition of STAT6 function by a dominant negative construct blocked the ability of IL-4 to induce apoptosis. Moreover, overexpression of mSTAT6 alone inhibited proliferation, increased the basal rate of apoptosis, and inhibited focus formation of MCF-7 cells. These findings show that STAT6 can induce apoptosis in breast cancer cells.

STAT6 is a cytoplasmic protein that dimerizes on phosphorylation, translocates to the nucleus, and activates transcription at specific promoter regions. There are several places in this signaling pathway where STAT6 may act to modulate cell survival. First, it is possible that STAT6 is interacting with other cytoplasmic proteins to impact growth or possibly cell death pathways. Although little is known about STAT proteins acting as cytoplasmic signal transducers, it has been reported that there is some cross talk between STAT proteins and the PI-3 kinase and Erk1/Erk2 MAPK pathways [1,9,30]. Unlike dimerization and transcriptional activation, which depend on tyrosine phosphorylation, interaction with these cytoplasmic pathways is associated with serine phosphorylation. Alternatively, the effects exerted by IL-4 in breast cancer cells may be due to transcriptional control of growth- and or death-regulating genes by STAT6. Consistent with this, Kaplan et al. [17] reported that STAT6 can modulate the transcription of the cyclin-dependent kinase inhibitor p27, thereby affecting cell growth. Finally, STAT6 action may be due to the specific nature of STAT6-mediated control of gene expression. Unlike other transcription factors, STAT6-mediated induction of transcription is associated with class-switch recombination [22,32]. This form of recombination involves introduction of double-strand breaks in the DNA followed by recombination of immunoglobulin receptor-coding sequences mediated by switch recombinases and potentially other proteins such as LR1 [4]. It is possible that components of this pathway are not expressed by breast cancer cells, resulting in STAT6-mediated transcription coupled to DNA double-strand breaks but without resolution of the recombination event, resulting in programmed cell death.

The data presented here suggest that STAT6 overexpression alone may be capable of inducing apoptosis, which is a novel result. In the single-cell proliferation assay, we observed a significant increase in the basal level of apoptosis when mSTAT6 was expressed, which was further increased in the presence of IL-4. It appears that STAT6 alone, in the absence of additional exogenous IL-4, is capable of inducing apoptosis. There are several possible explanations for this result. First, transient expression of mSTAT6 in MCF-7 cells resulted in a dramatic increase in IL-4–mediated STAT6 DNA binding (Figure 3*B*); compare 1 ng/ml IL-4 in the pcDNA-transfected lane with 1 ng/ml IL-4 in the mSTAT6-transfected lane). There was no detectable binding of STAT6 in the absence of IL-4, indicating that overexpression alone is not sufficient for activation of STAT6 DNA binding and that MCF-7 cells likely do not produce IL-4, consistent with previously published findings [36]. However, the dramatic increase in IL-4–mediated binding may explain the inhibition of focus formation in full serum by STAT6 overexpression observed in Figure 4*C* as full serum may contain IL-4 in addition to many other cytokines and growth factor.

In contrast to the previous findings by Chang et al. [5], we were unable to demonstrate IL-4–induced activation of STAT1 in breast cancer cells (data not shown). Whereas we have shown that interferon γ in breast cancer cells activates STAT1 and causes growth inhibition [11], IL-4 is unable to activate this pathway. As suggested by Chang et al., differences in STAT activation are likely responsible for the different biologic effects of IL-4 in cells. It appears that IL-4 causes growth inhibition of both breast and colon cancer cells despite activating different STAT pathways.

Due to toxicity observed in phase I and II clinical trials for IL-4 [6,39], IL-4 may never be a feasible therapeutic agent, despite its antitumor capabilities *in vitro* and in *in vivo* animal models. Therefore, the signaling pathway required for IL-4–mediated growth effects may be a source of novel therapeutic targets. As such, it will be very important to define what specific target genes are activated in response to STAT6. As with many important signaling molecules in breast cancer cells, including IGF-IR and IRS-1 [3,20], it will be crucial to determine if there is prognostic significance associated with STAT6 expression in human breast tumors. These are important areas of future investigation that may yield new therapeutic insights for regulating the growth of breast tumors and potentially other human cancers.

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