Novel Roles of Unphosphorylated STAT3 in Oncogenesis and Transcriptional Regulation

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Abstract

Signal transducer and activator of transcription 3 (STAT3) is phosphorylated on tyrosine residue 705 in response to growth factors or cytokines to form activated homodimers that drive gene expression. Because the *stat3* promoter has a binding site for STAT3 dimers, the amount of STAT3 protein increases when STAT3 is activated (e.g., in response to interleukin 6). Unphosphorylated STAT1 is known to drive the expression of certain genes. To explore the possibility of a similar role for the induced expression of unphosphorylated STAT3, we overexpressed either Y705F STAT3, which can not be phosphorylated on residue 705, or wild-type STAT3 in normal human mammary epithelial cells or STAT3-null mouse cells. The levels of many mRNAs were affected strongly by high levels of either form of STAT3. Some genes whose expression was increased by overexpressed STAT3, but not by activated STAT3 dimers, encode well-known oncoproteins (e.g., *MRAS* and *MET*). In many tumors, STAT3 is activated constitutively, and thus the unphosphorylated form is likely to be expressed highly, driving oncogene expression by a novel mechanism. In addition, expression of the *stat3* gene is increased strongly in response to interleukin 6, and the high levels of unphosphorylated STAT3 that result drive a substantial late phase of gene expression in response to this cytokine. Thus, unphosphorylated STAT3, which activates gene expression by a novel mechanism distinct from that used by STAT3 dimers, is very likely to be an important transcription factor both in cancer and in responses to cytokines. (Cancer Res 2005; 65(3): 939-47)

Introduction

Signal transducers and activators of transcription (STAT) are phosphorylated on a single tyrosine residue by Janus-activated kinase family kinases in response to cytokines, followed by dimerization and transport to the nucleus, where they activate the transcription of many cytokine-responsive genes (1). STAT3 was first described as a DNA-binding protein activated in interleukin 6 (IL-6)–stimulated hepatocytes and capable of interacting selectively with an enhancer element in the promoters of acute phase genes (2). Cytokines and growth factors activate STAT3, including growth hormone and the IL-6 family of cytokines, which use the common receptor gp130 (2). The importance of STAT3 is underscored by the failure of mice lacking this protein to progress beyond day 4 of embryogenesis. STAT3 plays important roles in myeloid development, based on results with dominant-negative mutants (3, 4).

It has been reported widely that the activation of STAT3 is likely to contribute importantly to the development of cancer. A constitutively active (DNA binding) variant of STAT3 induces oncogenic transformation and tumorigenesis (5). Constitutively tyrosine phosphorylated, active STAT3 has been observed in transformation by v-Src, v-Eyk, v-Ros, and v-Fps (6, 7), and STAT3 activation has been seen at a high frequency in many human tumors, including breast and head and neck carcinomas, lymphomas, leukemias, melanomas, and pancreatic, lung, ovarian, and brain tumors (7, 8). In addition, STAT3 can be activated by several different oncogenic kinases, such as v-SRC, c-SRC, v-ABL, and v-FPS (9, 10). Abrogation of constitutive STAT3 activity by dominant-negative STAT3 mutant proteins induces apoptosis and growth arrest in breast cancer cell lines (11), suggesting an important role for constitutively active STAT3 in breast cancer development, possibly through an aberrant epidermal growth factor receptor pathway or activation of SRC. SRC_mediated activation of STAT3 also correlates with the development of prostate and ovarian carcinomas (12), and autocrine stimulation by IL-6 induces prostate cancer cell growth that is accompanied by the activation of STAT3 (13). Furthermore, IL-6 is required for glioma development in a mouse model (14).

From our previous work (15), we know that STAT1 can drive the constitutive expression of several genes in the complete absence of tyrosine phosphorylation, a function quite distinct from its role in inducible, phosphorylation-dependent gene expression in response to IFNs and other cytokines. In the work reported here, we show that unphosphorylated STAT3 also drives gene expression and that the increase in STAT3 levels that follow its IL-6–dependent activation by tyrosine phosphorylation drives a distinct subset of genes. Furthermore, mRNAs or proteins whose expression is driven by overexpression of unphosphorylated STAT3 (e.g., MET and MRAS) are present at high levels in many cancers.

Materials and Methods

Cells and Constructs. STAT3-null cells were derived by standard methods from embryos of mice carrying a conditional floxed allele of STAT3 (16), following retroviral gene transduction of Cre recombinase. Mutant cells were reconstituted with wild-type or Y705F mouse STAT3 by retroviral gene transduction of recombinant retroviruses or of an empty control virus. STAT3-expressing retroviruses and control viruses also expressed green fluorescent protein (GFP) from a bicistronic mRNA, and reconstituted cells were sorted for high expression of GFP. Single cell clones derived by fluorescence-activated cell sorting, based on GFP expression, were expanded and assayed for STAT3 expression by the Western method.

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STAT3-null (1L4), LoSTAT3wt (2L1), HiSTAT3wt (2L6), and HiY705F (3M9) mouse embryonic fibroblasts (MEF) were grown in DMEM at 37°C under 10% CO_2. hTERT-HME1 cells were grown in MCDB 170 medium with supplements of bovine pituitary extract, hydrocortisone, insulin, gentamicin, human epidermal growth factor, and amphotericin-B, all from Clonetics, Palo Alto, CA. All media were supplemented with 10% FCS and 100 μg/mL penicillin-streptomycin mix (Life Technologies, Carlsbad, CA). Cells were left untreated or treated with IL-6 (200 ng/mL), together with IL-6 soluble receptor (250 ng/mL). Antibodies against STAT3 (C-20), Cyclin B1, CDC2, Ran GAP1, PLK-1, and CDC25C were from Santa Cruz Biotechnologies, Santa Cruz, CA and antibody against tyrosine 705-phosphorylated STAT3 (pTyr-STAT3) was from Cell Signaling Technology, Beverly, MA. The pLEGFP-N1 retroviral expression vector was from Clontech. The pLEGFP-STAT3-WT or pLEGFP-STAT3-Y705F vectors were generated by inserting wild-type or Y705F human STAT3 cDNA into the *Hin*dIII site of the pLEGFP-N1 vector. To obtain infectious retrovirus, each construct was transfected into BOSC23 packaging cells (Clontech) and supernatant media, collected after 48 hours, were used to infect hTERT-HME1 cells on three consecutive days. hTERT-HME1 cells were transiently transfected with the ecotropic receptor 2 days before virus infection. Stably transduced hTERT-HME1 cell pools were selected in 300 g/mL neomycin and shown to express STAT3 proteins of the expected size by Western analysis.

Expression Array Experiments. Total RNAs were extracted from cells with Trizol (Life Technologies). Approximately 15 μg of RNA were used to obtain cRNA according to standard Affymetrix protocols (17). The cRNAs were hybridized with human (U133A) or mouse (U74Av2) arrays and data were analyzed using the Affymetrix gene chip software. Expression was normalized against the Affymetrix spike RNA levels as well as against glyceraldehyde phosphate dehydrogenase (GAPDH) and actin mRNA levels in the all samples. The expression of genes in HiSTAT3wt or HiY705F MEFs was compared with STAT3-null MEFs as the baseline. The expression of genes in hTERT-HME1 cells overexpressing wild-type or Y705F STAT3 was compared with hTERT-HME1 cells as the baseline.

Expression Analysis Using a Tumor Tissue Array. Cancer Profiling Array I (Clontech) consists of 241 cDNAs, synthesized from human tumors and normal tissues (http://www.bdbiosciences.com/clontech/techinfo/manuals/PDF/PT3578-1.pdf and http://www.bdbiosciences.com/clontech/techinfo/manuals/PDF/7841-1.pdf). Each pair was independently normalized, based on the expression of three housekeeping genes. Human *stat3*, *met*, and *mras* cDNAs were radiolabeled using a [α-^{32}P]-dCTP and hybridized overnight at 68°C using ExpressHyb Hybridization Solution (Clontech), washed, and exposed to Phosphor Screen (Molecular Dynamics, Eugene, OR). Signal intensities were calculated for individual spots using a STORM-840 phosphoimager (Molecular Dynamics).

Western Analysis. Cells at 80% confluence in 100-mm dishes were washed once with PBS and the cell pellets were lysed for 20 minutes at 4°C in 100 μL lysis buffer [50 mmol/L HEPES (pH 7.0), 250 mmol/L NaCl, 0.1% NP40, 10% glycerol, 1 mmol/L phenylmethanesulfonyl fluoride, 2 μg/mL aprotinin, 25 μg/mL leupeptin, 5 μg/mL pepstatin A, and 1 mmol/L DTT]. Extracts containing equal quantities of proteins, determined by the Bradford method (Bio-Rad, Hercules, CA), were separated by SDS-PAGE (10% acrylamide) and transferred to polyvinylidenedifluoride membranes (Millipore, Bedford, MA). Membranes were probed with antibodies specific for STAT3, Cyclin B1, CDC2, PLK-1, or Ran GAP1. Horseradish peroxidase–coupled goat anti-rabbit or anti-mouse immunoglobulins were used for visualization, using the enhanced chemiluminescence Western detection system (Perkin-Elmer Life Sciences, Boston, MA).

Northern Analysis. Twenty micrograms of total RNA were fractionated in a 1% agarose/formaldehyde gel and transferred to a Hybond-XL membrane (Amersham Biosciences UK Limited, Buckinghamshire, United Kingdom). The RNAs were cross-linked to the membrane by using a UV cross-linker before hybridization. [α-^{32}P]-dCTP (6,000 Ci/mmol, NEN, Boston, MA) was used to generate ^{32}P-labeled cDNA probes by random priming (Roche Molecular Biochemicals, Laval, Quebec, Canada). The membrane was prehybridized for 6 hours with 5× saline-sodium phosphate-EDTA (75 mmol/L NaCl, 50 mmol/L NaH_2P0_4, and 4 mmol/L EDTA) plus 2% SDS and 100 μg/mL ssDNA at 65°C. After the addition of specific probes, hybridization took place for 36 hours at 65°C. The membranes were washed thrice with 2× saline-sodium phosphate-EDTA plus 0.1% SDS. Selected genes from the array were also examined by the Northern procedure. Mouse and human cDNA probes for these genes were cut from I.M.A.G.E. clones, obtained from Invitrogen (Carlsbad, CA) or the American Type Culture Collection (Manassas, VA). Templates for the mouse and human *gapdh* cDNAs were obtained by reverse transcription-PCR. Transfers were normalized for loading by comparing the intensities of the 18 S and 28 S rRNA bands or the *gapdh* mRNA levels on the same membranes.

Electrophoretic Mobility Shift Analysis. hTERT-HME1 cells, untreated or treated with IL-6, were lysed in 10 mmol/L HEPES (pH 7.9), 60 mmol/L KCl, 1 mmol/L EDTA, 0.1 mmol/L EGTA, 0.075% NP40, 1 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L DTT, 1 mmol/L phenylmethanesulfonylfluoride, 2 μg/mL aprotinin, 25 μg/mL leupeptin, and 5 μg/mL pepstatin. After incubation for 15 minutes on ice, debris was removed by centrifugation at 16,000 × *g* at 4C for 30 minutes. Protein concentrations were determined by the Bradford method. Binding reactions contained 1 L of lysate (5-10 g of protein), 1 μL of sonicated salmon sperm DNA (0.5 mg/mL), 1.4 μL of 5 shift buffer [100 mmol/L HEPES (pH 7.9), 200 mmol/L KCl, 30 mmol/L $MgCl_2$, 0.5% NP40, 5 mmol/L DTT, and 5 mmol/L phenylmethylsulfonyl fluoride], 1.5 μL of bovine serum albumin (1 mg/mL), 3.8 μL of H_2O, and 0.3 μL of probe, end-labeled with T4 polynucleotide kinase and [-^{32}P]-ATP. The probe was the SIE consensus sequence (top strand 5′-TCGACATTT-CCCGTAAATC-3′) from the *c-fos* gene promoter (18); 10^4 dpm of labeled probe was used in each binding reaction. Protein-DNA complexes were separated by electrophoresis in gels composed of 4% acrylamide-bisacrylamide (29:1), 0.5× TBE (45 mmol/L Tris-HCl, 44 mmol/L boric acid, and 1 mmol/L EDTA), and 5% glycerol. For supershift analyses, whole cell extracts were preincubated for 20 minutes at room temperature with polyclonal antibodies specific for STAT3 (C-20) before adding radiolabeled probe.

Results

Overexpression of Wild-Type or Y705F STAT3 in Human or Mouse Cells. hTERT-HME1 cells, primary human mammary epithelial cells immortalized by the hTERT subunit of telomerase, have relatively low levels of STAT3, comparable with the levels in normal human fibroblasts (data not shown). These cells were infected with retroviral vectors capable of expressing wild-type or Y705F STAT3. In stable pools of cells expressing wild-type STAT3, we saw no evidence of STAT3 tyrosine phosphorylation or in the ability of STAT3 to bind to a GAS probe, showing that the level of activated STAT3 dimers was undetectable in these assays (data not shown). Lysates of the pooled cells were analyzed by the Western method. Both wild-type and Y705F STAT3 were overexpressed by ca. 10-fold, as judged by densitometric quantitation of the bands shown in Fig. 1*A*. The pooled cells grew at rates very similar to those of parental hTERT-HME1 cells.

We also used STAT3-null MEFs to study the effect of STAT3 expression against a null background. Clones were obtained from cells expressing wild-type or Y705F STAT3. Western analysis indicated that clones 2L6 (HiSTAT3wt) and 3M9 (HiY705F) expressed high levels of wild-type or Y705F STAT3, respectively, that clone 1L4 has no STAT3, and that that clone 2L1 (LoSTAT3wt) has an approximately normal level of STAT3 (Fig. 1*B*). Clones 2L6 and 1L4 grew most rapidly (doubling time about 16 hours) and 3M9 and 2L1 grew most slowly (doubling time about 24 hours). Because clonal differences and differences in growth rates are likely to contribute to differences in gene expression, we used the data from the MEFs primarily to confirm and extend to mouse cells differences in expression observed with the human cells.

Overexpressed Wild-Type and Y705F STAT3 Drive Gene Expression. An array-based analysis of gene expression revealed that the relative levels of more than a thousand mRNAs changed in response to the overexpression of wild-type or Y705F STAT3. In hTERT-HME1 cells, 1,420 mRNAs changed, positively or negatively, by ≥2-fold in response to overexpressed wild-type STAT3 and 1,191 mRNAs changed by the same amount in response to overexpressed Y705F STAT3 (data not shown). Furthermore, 869 mRNAs changed in common by ≥2-fold in both cell populations, and 84 of these changed by ≥4-fold (examples in Table 1*A*; full list available upon request). These mRNAs include several that are induced to very high levels (10-to 40-fold). Analysis of the mouse cell lines provided complementary data. In addition to the obvious cell-type differences in the two experiments, mouse cells overexpressing wild-type or Y705F STAT3 are compared with STAT3-null cells, rather than to cells with low level expression of STAT3, which was the baseline in the human system. In MEFs, 1,054 mRNAs changed by ≥2-fold in cells overexpressing wild-type STAT3 and 1,268 mRNAs changed by the same amount in cells overexpressing Y705F STAT3. Furthermore, 413 mRNAs changed in common by ≥2-fold in both cell types, and 115 of these changed by ≥4-fold (Table 1*B*, full list available upon request). Some of the genes that respond to unphosphorylated STAT3 are known to be regulated also by phosphorylated STAT3 homodimers [e.g., *socs-2* (19), *c-myc*, *dp1* (20), *c-fos*, *c-jun* (21), and *bcl-x* (22)]. (22)]. In the case of STAT1, the *lmp2* gene is known to respond both to homodimers of phosphorylated STAT1 and to unphosphorylated STAT1-IRF1 heterodimers through distinct but overlapping DNA elements (15). On the other hand, several genes not shown in Table 1*B* were induced in MEFs only in response to overexpressed Y705F and not wild-type STAT3 [e.g., *small inducible cytokine a5* (*scyA5*), *tiap*, *meiosis-specific nuclear structural protein 1* (*mns1*), *the p70/p85 S6 kinase* gene, *formin*, *P21-activated kinase-3* (*mpak-3*), *heat shock protein 86*, *thymopoietin*, and *TFIIH*]. Several genes were induced in MEFs only in response to overexpressed wild-type STAT3 but not Y705F (e.g., *Survivin*, *BUB1*, and *TACC3*). Some of the gene chip data have been confirmed in both cell systems Fig. 2. Most of the genes identified in the gene chip analyses were confirmed by Northern, Western, or reverse transcription-PCR analyses. Those genes confirmed by Northern or Western analyses are shown in Fig. 2; for example, genes confirmed in hTERT-HME1 cells by Northern analysis include *bcl2-related protein a1* (*bcl2a1*), *Ran GTPase activating protein 1*(*Ran GAP1*), *tis11d*, *met*, and *rantes* (Fig. 2*A*); genes confirmed by Western analysis in MEFs include *cyclin B1*, *polo-like kinase 1* (*plk-1*), *Ran GAP1*, *cdc2*, and *cdc25c* (Fig. 2*B*) and by Northern analysis *survivin*, *tacc3*, *bub1*, *serine/threonine kinase 6* (*s/t kinase 6*), *Ran GAP1*, *met*, *tis11d*, and *plk-1* (Fig. 2*C*). Others, which were confirmed by reverse transcription-PCR are not shown.

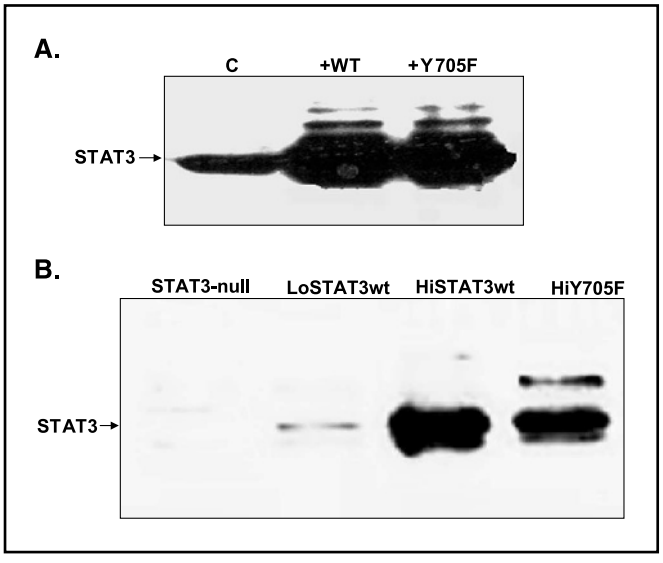

Figure 1. Western analyses of STAT3 expression. The cells were infected with retroviral constructs and stable pools were selected with 300 μg/mL G418. *A,* expression of wild-type or Y705F human STAT3 in hTERT-HME1 cells. *C,* control; *+WT,* overexpression of wild-type STAT3; *+Y705F,* overexpression of Y705F STAT3. *B,* expression of wild-type or Y705F mouse STAT3 in STAT3-null MEFs. LoSTAT3wt, MEFs reconstituted with a low level of wild-type STAT3; HiSTAT3wt, MEFs reconstituted with a high level of wild-type STAT3; HiY705F, MEFs reconstituted with a high level of Y705F STAT3.

Induction of STAT3-Dependent Genes in Response to IL-6. Because the STAT3 promoter contains a GAS site and is known to be activated by phosphorylated STAT 3 homodimers, we conducted an analysis of the response of hTERT-HME1 cells to IL-6 over a long period of time (32 hours) to distinguish between genes that respond quickly to phosphorylated STAT3 and those that respond more slowly to the secondary increase in the levels of unphosphorylated STAT3 Fig. 3. The level of STAT3 homodimers increased rapidly in IL-6–treated cells, peaking at 4 to 8 hours and returning to the low levels characteristic of untreated cells by 32 hours (Fig. 3*A*). Consistent with this result, the level of tyrosine-phosphorylated STAT3 followed a similar time course (Fig. 3*B*). In contrast, the total amount of STAT3 continued to increase throughout the experiment (Fig. 3*B*), reaching a maximum at 16 to 32 hours of ca. 24 times the level in untreated cells, as analyzed by densitometry. Three genes known to respond to phosphorylated STAT3 dimers through GAS elements in their promoters are induced rapidly in response to IL-6 and the levels of their mRNAs decline in parallel with the decline of STAT3 homodimers (Fig. 3*C*). Of course, it must be true that these mRNAs have short half-lives. In striking contrast, the expression of four genes that are induced specifically by the increased expression of unphosphorylated STAT3 and not by phosphorylated STAT3 dimers is increased little or not at all 2 hours after treatment with IL-6 (Fig. 3*D*). These genes were identified in an array experiment in which hTERT-HME1 cells were treated with IL-6 for 4 hours (data not shown, full list available upon request). However, their expression continues to increase throughout the course of the experiment, in parallel with the increased levels of total STAT3 (Fig. 3*D*). An analysis of their stability in cells treated with actinomycin D showed that *c-myc* and *mras* mRNAs have very similar half-lives of about 30 minutes (data not shown). Therefore, the high level of *mras* mRNA in IL-6–treated cells (Fig. 3*D*) must be sustained by continued transcriptional activation in response to increased amounts of unphosphorylated STAT3. This experiment shows clearly that the full response of STAT3-dependent genes to IL-6 occurs in two phases, an initial induction of genes with GAS sites, including the *stat3* gene itself, followed by a secondary induction of genes whose expression depends, directly or indirectly, on unphosphorylated STAT3, and not STAT3 homodimers. The activation of this latter set of genes is driven by the induced expression of STAT3. This situation is very likely to hold

Table 1. Differential gene expression in cells overexpressing wild-type or Y705F STAT3

Accession no.	WT	Y705F	
	Fold change		Gene name
A, gene expression profiles in hTERT-HME1 cells			
1405	28	42	Human T cell–specific protein (RANTES)*
210615	20	18	Neuropilin-1 soluble isoform 11 (NRP1)
209783	17	12	Human Tis11d primary response gene*
219514	17	11	Angiopoietin-like 2 (ANGPTL2)
33814	17	2.1	Serine/threonine kinase 6
206538	13	62	Muscle RAS oncogene (MRAS)*
204760	14	6.5	Nuclear receptor subfamily D1 (NR1D1)
202861	11	15	Period (PER1)
203765	11	8.6	Grancalcin (GCL)
212125	11	10	Ran GTPase activating protein 1 (Ran GAP1)*
211607	9.2	7.5	Epidermal growth factor receptor (EGFR)
201641	8.6	5.7	Bone marrow stromal cell antigen 2 (BST2)
205681	8.6	6.1	BCL2-related protein A1 (BCL2A1)*
200784	5.3	4.3	Low density lipoprotein-related protein 1
203710	4.3	4.0	Inositol 1,4,5-triphosphate receptor 1 (ITPR1)
207186	4.3	4.3	Fetal Alzheimer antigen (FALZ)
220370	4.0	4.3	KIAA1453 protein (KIAA1453)
210163	3.5	3.7	Interferon stimulated T-cell α chemoattractant (I-TAC)
209956	3.3	4.6	Calciumcalmodulin-dependent protein kinase II β
213807	2.7	6.9	Met proto-oncogene*
206167	2.5	2.3	Rho GTPase activating protein 6 (Rho GAP6) *
202508	2.3	−2.5	Synaptosomal-associated protein, 25kD (SNAP25)
202237	−4.9	−4.3	Nicotinamide *N*-methyltransferase (NNMT)
202036	−5.7	−4.0	Secreted apoptosis related protein 2 (SARP2)
B, gene expression profiles in MEFs			
93099	39	2.0	GM-CSF
93099	21	14	Polo-like kinase 1*
101059	20	37	Necdin
100309	18	16	Met proto-oncogene*
100885	15	2.1	Interleukin 6
99435	12	8.6	Fibroblast growth factor 7
93943	12	17	Tis11d primary response gene
101019	11	7.5	Cathepsin C
93940	11	7.5	Paraoxonase 3
98603	9.2	10.4	RAN GTPase activating protein 1*
102934	8.3	2.0	Cell division cycle control protein 25C*
104527	8.1	4.0	Rad51
93881	7.0	2.3	TGN38B
97182	6.7	3.3	Cyclin E2
99186	6.5	3.5	Cyclin A2
101937	6.5	7.9	Cdc2*
100885	5.9	2.0	Serine/threonine kinase (nek2)
99632	5.0	3.3	Mitotic checkpoint component Mad2
160092	4.6	3.7	Interferon β
92639	4.5	2.1	Serine/threonine kinase 6*
160501	4.4	5.7	Rabkinesin-6
102963	4.3	2.0	E2F-1
160159	3.5	2.5	Cyclin B1*
100156	3.3	4.0	CDC46
94761	3.3	3.7	Small inducible cytokine A7
100368	3.3	2.8	Caspase9S
102292	3.0	−2.5	GADD45 protein (*gadd45*) gene
98349	2.6	3.0	IL-6 signal transducer (gp130)
95785	2.6	2.8	RAB7 (member RAS oncogene family)
97991	2.5	2.5	Ki-ras cellular oncogene
93870	2.5	2.8	Braf transforming gene
100302	2.5	3.7	mafF
93613	2.1	2.8	mSmad3
93456	−4.0	3.25	BMP-4
98924	−8.6	−16	ART3
98406	−294	−22	Cathepsin Z (*ctsZ* gene)

NOTE: The expression of 22,000 genes in human Affymetrix U133A arrays and 12,000 genes in mouse Affymetrix U74Av2 arrays was analyzed. Some of the induced genes were chosen to be shown here because of their roles in regulating the cell cycle or in tumorigenesis. Full list available upon request. In human Affymetrix arrays, gene expression in hTERT-HME1 cells infected with empty vector was taken as the baseline. In mouse Affymetrix arrays, gene expression in STAT3-null cells was taken as baseline.
*Expression of genes has been confirmed by the Northern or Western methods.

also for responses to other cytokines that activate STAT3, especially cytokines in the IL-6 family that use the common gp130 receptor.

Expression of STAT3 in Tumors. In order to obtain more information about STAT3 expression in tumors, we interrogated commercially available arrays (Clontech) of cDNAs made from total pools of mRNAs derived from individual tumors or normal tissues. The data are presented in Fig. 4 and are summarized in Table 2. It is clear that high levels of STAT3 mRNA are present in many, but not all, tumors and also in some "normal" tissues. However, the tumor samples include unknown fractions of

normal cells, for example, infiltrating lymphocytes, which can obscure the situation in the tumors themselves, and the "normal" cells, if derived from tissues near the tumor, may be influenced by, for example, factors secreted from the tumor. Although the relative levels of expression of STAT3 in normal versus tumor cells could not be reliably quantified, it is clear that STAT3 is readily detectable in many tumors and may regulate downstream gene expression important for the behavior of the neoplastic cells.

Expression of STAT3-Regulated Genes in Human Tumors. Expression of the *cdc2*, *cyclin B1*, *e2f-1* (data not shown), and *mras* genes (Fig. 3*D*) is induced by overexpressed unphosphorylated STAT3 and not by STAT3 homodimers, because the induced expression of these mRNAs is not seen at early time points. CDC2, Cyclin B1, and E2F-1 have already been reported to be expressed at high levels in lung, head and neck carcinomas (23, 24), but their expression has not been linked to STAT3 levels. To investigate whether these proteins are overexpressed in the

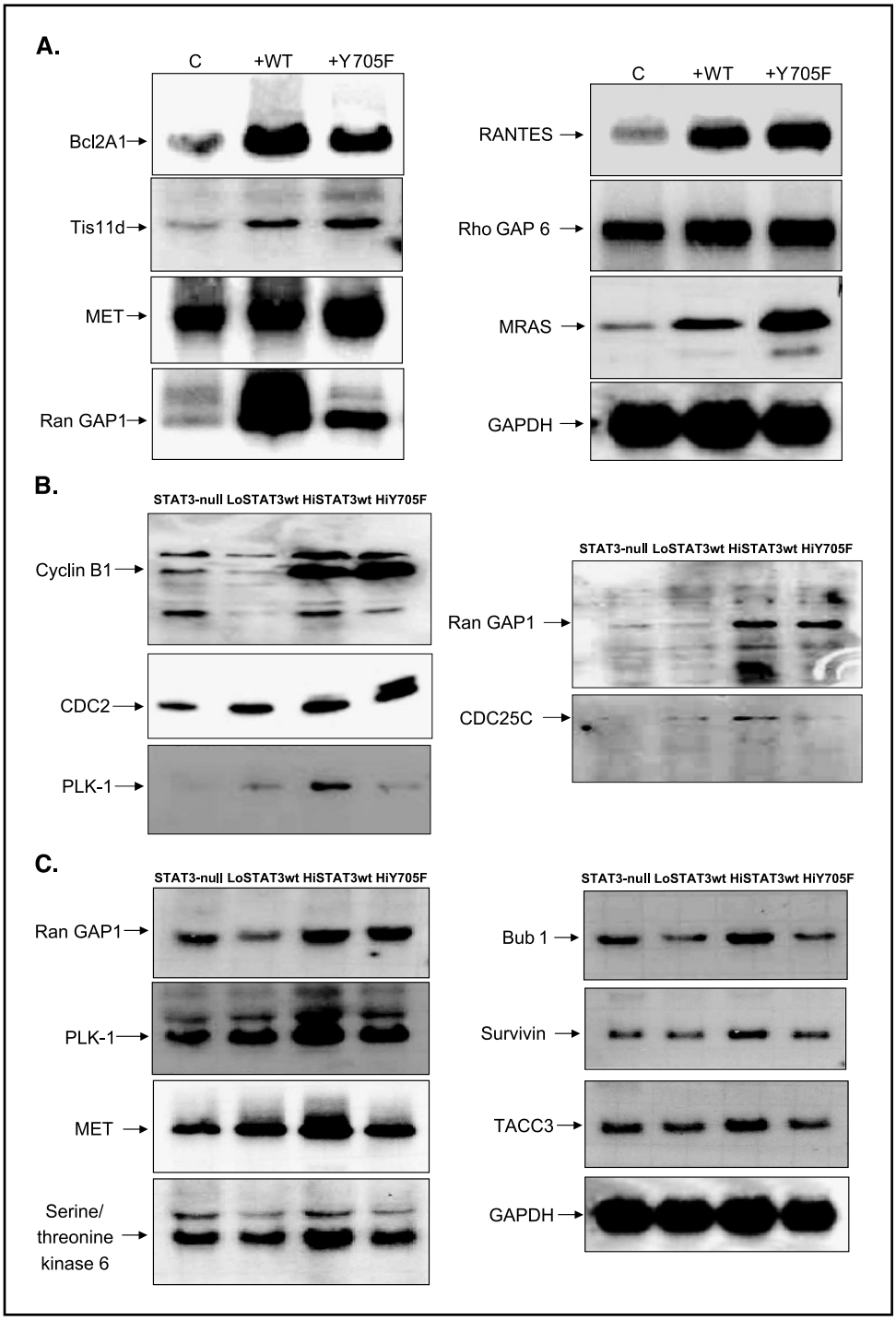

Figure 2. *A,* Northern and Western analyses of gene expression driven by overexpression of wild-type or Y705F STAT3. Northern profiles in hTERT-HME1 cells. *B,* Western profiles in MEFs. *C*, Northern profiles in MEFs.

same cancers in which STAT3 is expressed, expression of MRAS and MET mRNAs was assessed in Clontech cDNA arrays (Fig. 4; Table 2). In tumors of the breast, uterus and thyroid, *met* and *mras* mRNAs are overexpressed in almost all samples analyzed, regardless of the expression levels of *stat3* mRNA. However, in tumors of the colon, stomach, ovary, lung, kidney, and rectum, there is a strong correlation between overexpression of the mRNAs for *met* and *mras* with overexpression of *stat3* mRNA. When the data for these six tumor types are considered together, *met* mRNA is overexpressed in 93% of the tumors in which *stat3* mRNA is overexpressed but in only 29% of the tumors where it is not, and *mras* is overexpressed in 74% of the tumors in which *stat3* mRNA is overexpressed but in only 16% of the tumors where it is not. The coincidence of *stat3* mRNA expression with the expression of *met* and *mras* mRNAs in these tumors is interesting, especially when account is taken of the limitations concerning the unknown fraction of normal cells in the tumor preparations.

Discussion

Unphosphorylated STAT3 and Gene Expression. The results presented above make the case that unphosphorylated STAT3 has an important role in transcription. Similarly to STAT1 (15), unphosphorylated STAT3 is required for the constitutive expression of certain genes. These genes were identified in an array experiment in which untreated STAT3-null and LoSTAT3wt cells were compared (data not shown; full list available upon request). The *stat3* promoter has a GAS element that allows it to respond to STAT3 dimers, but not to STAT1 dimers (25). However, this promoter has not been studied in detail, and it may well be that other stimuli can increase the expression of STAT3 mRNA and protein. We have shown that the activation of STAT3 in response to IL-6 greatly stimulates *stat3* gene expression, thus indirectly driving the primary inducible expression of those genes that use unphosphorylated STAT3 as a transcription factor. It is to be expected that other cytokines that activate STAT3 through the common gp130 receptor subunit, or stimuli that activate STAT3 by other means, will cause a similar indirect response. Therefore, STAT3 serves two quite distinct roles in cytokine-dependent transcription: as a part of the primary response through the action of STAT3 dimers and as a secondary part of the complete response through the action of increased amounts of unphosphorylated STAT3. It is very interesting that STAT1 dimers drive STAT1 expression but not STAT3 expression, and vice versa, so that stimuli that cause the formation of one or the other dimer are specifically reinforced (26).

Mechanism of Transcriptional Activation by Unphosphorylated STAT3. Changes in gene expression in response to unphosphorylated STAT3 may be due either to the direct effect of STAT3 binding to a responsive promoter or to an indirect effect (e.g., if a gene regulated directly by STAT3 encodes a transcription factor that activates additional genes). In Table 1, it can be seen that

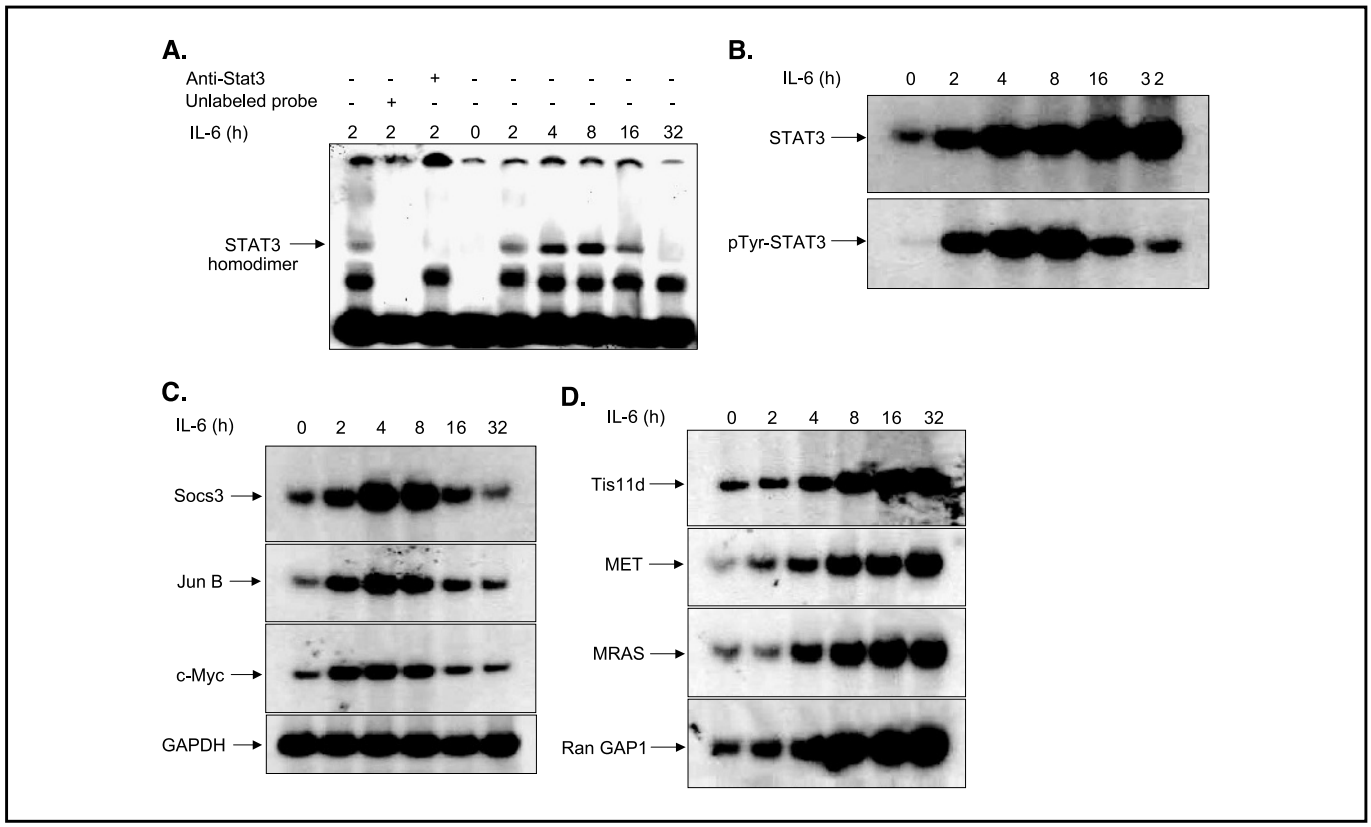

Figure 3. Kinetics of STAT3 activation and downstream gene expression in response to IL-6. *A,* electrophoretic mobility shift assays. Whole cell extracts were made from hTERT-HME1 cells, untreated or treated with IL-6 for different times. An hSIE element derived from the *c-fos* promoter was used as the probe. *Lane 2,* a 100-fold molar excess of unlabeled probe was added to the binding reaction. *Lane 3,* 1 μL of anti-STAT3 antibody was added to the binding reaction. *B,* Western analysis. Cell extracts (100 μg of protein per lane) from hTERT-HME1 cells treated with IL-6 were analyzed. Expression profiles of genes activated by phosphorylated (*C*) and unphosphorylated (*D*) STAT3. Samples (20 μg total RNA per lane) from hTERT-HME1 cells treated with IL-6 were analyzed by the Northern method.

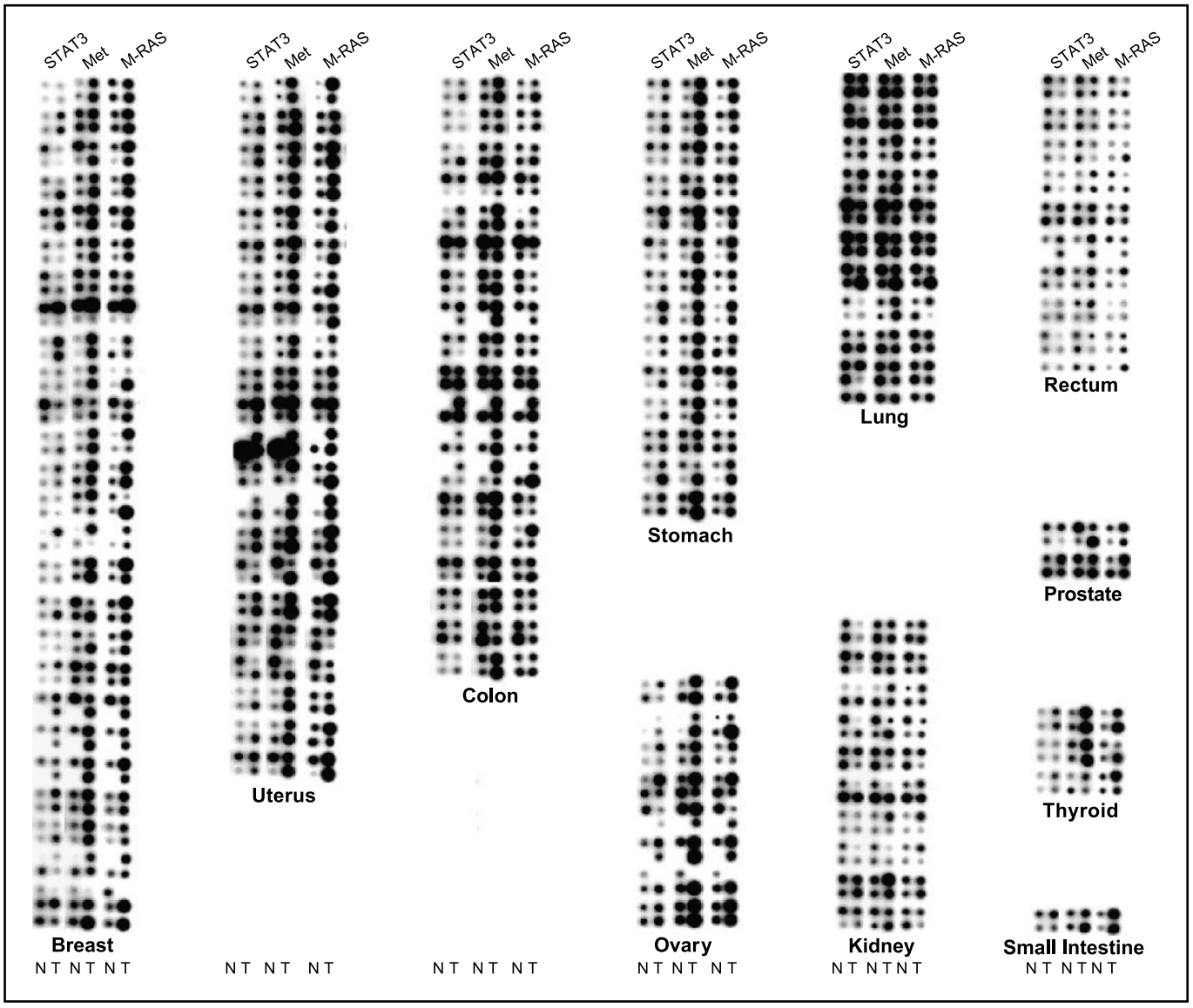

Figure 4. Correlation of human *stat3, met*, and *mras* expression in cDNA arrays. The Cancer Profiling Array (Clontech) includes normalized cDNAs from 241 tumor and corresponding normal tissues from individual patients. It was hybridized separately with radioactive probes for ubiquitin (as an internal control, data not shown), *stat3*, *met*, and *mras*. Signals were detected by phosphorimaging, followed by scanning and manipulation with Adobe Photoshop. *N,* normal; *T,* tumor.

overexpression of wild-type and Y705F STAT3 have similar but distinct effects on gene expression. It is unlikely that the differences are due to the formation of dimers of unphosphorylated STAT3 in the case of the wild-type protein because no stimulus has been given to the cells, there was no detectable tyrosine phosphorylation (data not shown) and the pattern of gene expression is quite distinct from the pattern induced by IL-6, which does cause the formation of dimers. By analogy with the limited information currently available on how unphosphorylated STAT1 drives gene expression (15), it seems possible that STAT3 forms heterodimers with other transcription factors (yet to be identified) and that such heterodimers might recognize half of a GAS element plus a DNA element to which the non-STAT partner can bind. The substitution of tyrosine by phenylalanine may affect the structure or affinity for DNA of such heterodimers and thus may affect gene expression independently of the ability of residue 705 to become phosphorylated.

The crystal structure of STAT3 dimers bound to DNA indicates that, in addition to several well characterized conserved domains (linker, SRC homology 2, and transactivation), STAT3 also has a large NH_2-terminal coiled coil domain that projects laterally from the core structure (27, 28). This feature represents a large exposed surface that can bind to other proteins that interact with STAT3 or with other STATs (29, 30). There is limited coincidence between the sets of genes induced by unphosphorylated STAT3 in the human (Table 1*A*) and mouse (Table 1*B*) systems but, for example, *met*, *tis11d*, and *serine/threonine kinase 6* are induced in both situations. The lack of greater coincidence is perhaps not too surprising, given the many differences between the two experiments (species, cell type, comparison with normal levels of STAT3 in the human experiment and with STAT3-null cells in the mouse, analysis of clones of mouse cells versus a population of human cells, differences in growth rates of the cells studied in the mouse experiment).

Table 2. Expression of mRNAs in normal and tumor tissues

	Breast	Uterus	Colon	Stomach	Ovary	Lung	Kidney	Rectum	Thyroid	Prostate	Small intestine	Other
A, expression of *stat3* mRNA												
Total samples	50	44	39	27	16	21	20	18	6	4	2	2
Higher in tumor	28	33	8	18	10	4	2	4	4	1	1	0
Higher in normal	14	8	14	3	4	6	9	9	0	2	0	0
About equal	8	3	17	6	2	11	9	5	2	1	1	2
B, expression of human *stat3*, *met*, and *mras* mRNAs												
STAT3 overexpressed	28	33	8	18	10	4	2	4	4	1	1	0
MET overexpressed	22	26	8	16	10	4	2	3	3	0	1	0
M-RAS overexpressed	21	23	4	15	9	2	1	3	4	1	1	0
STAT3 is not overexpressed	22	11	31	9	6	17	18	14	2	3	1	2
MET overexpressed	17	8	13	3	2	4	4	2	2	1	1	0
M-RAS overexpressed	18	7	4	1	2	1	4	3	1	3	1	2

NOTE: The Cancer Profiling Array (Clontech) was hybridized with human *stat3*, *met*, and *mras* cDNAs-see Fig. 4. The intensity of signal in every cancer tissue spot was counted and compared with the counterpart normal tissue.

Functions of Induced Genes. Target genes of unphosphorylated STAT3 (*cdc2*, *cyclin B1 mras*, and *e2f-1*) are overexpressed in colon and breast carcinomas.[4] E2F-1 is a transcription factor that activates the synthesis of mRNAs encoding proteins needed during the cell cycle. Most E2F-1–regulated genes encode proteins involved in DNA replication or cell cycle progression. The products of some E2F-1–regulated genes, such as *cyclin D1* and *cyclin E* (31, 32), *cdc2* and *cyclin B1* (33, 34) contribute to the G_1-S and G_2-M transitions and inhibit apoptosis. Recently, clear evidence has been obtained that CDC2, Cyclin B1, and E2F-1 are overexpressed in mucosa-associated lymphoid tissues and head and neck carcinomas (23, 24). The product of another STAT3 target gene (*MRAS*) contributes to cellular transformation and differentiation (35, 36). Our findings reveal an important role for STAT3 in activating oncogene expression.

Expression and Location of STAT3 in Cancer Cells. We investigated STAT3 immunoreactivity in a variety of neoplastic tissue specimens obtained from different sources (National Cancer Institute TARP3, Zymed MaxArray, Cleveland Clinic Foundation Surgical Pathology specimens). Based on these limited data (not shown), we can say that STAT3 was detected in the majority of neoplasms examined (carcinomas of breast, prostate, colon, lung, brain and ovary, melanomas, lymphomas, and glioblastomas). Most of the STAT3 immunoreactivity was in the nucleus. This finding is consistent with observations in cell culture systems (37).

It is difficult to determine whether the level of a particular protein is altered in tumors compared with the corresponding normal tissues. Analysis of tissue extracts by the Northern, reverse transcription-PCR, or Western methods yields results that are readily quantified but lack specificity because of the cellular heterogeneity of tissues. For example, the most abundant cell type in normal colonic mucosa is the colonic epithelial cell, but some regions of the colon have a high frequency of lymphoid nodules and mucosal lymphocytes. Many genes of interest in cancer, such as regulators of cell cycle and apoptosis, are expressed by lymphocytes and contribute to the total signal in homogenates. Similarly, tumor-infiltrating lymphocytes, present in many neoplasms, will contribute to the expression seen in tumor tissues.

Immunocytochemistry and *in situ* hybridization enable one to distinguish the cells of interest from others, making them highly specific techniques. However, most *in situ* techniques used for human pathology specimens employ chromogenic detection and rely upon enzyme-linked and other amplification techniques to achieve the high sensitivity required for visualization. Large differences but not small differences in stain intensity are readily distinguished by the naked eye. Also, as noted above, the lability of specific proteins or phosphoproteins in tumors can only be addressed by rapid acquisition after surgery and rapid preservation of the specimens.

Upon long-term treatment of hTERT-HME1 cells with IL-6, the total amount of STAT3 was increased, whereas the level of phosphorylated STAT3 was similar to the very low level in untreated cells (Fig. 3*A* and *B*). Immunofluorescence analysis in these experiments showed that STAT3 was increased both in the cytoplasm and in the nucleus, but mostly in the nucleus (data not shown). Also, as noted above, the lability of specific proteins or phosphoproteins in tumors can only be addressed by rapid acquisition after surgery and rapid preservation of the specimens.

STAT3 and Cancer. The role of STAT3 in cancer has been assumed until now to be due solely to the activation of gene expression in response to STAT3 dimers, formed through tyrosine phosphorylation in response to oncogeneic tyrosine kinases such as SRC (11). It is now clear that the constitutive activation of STAT3 drives in turn the constitutive overexpression of STAT3, and it is possible that stimuli other than STAT3 dimers can also drive STAT3 expression. To analyze the impact of gene expression driven by unphosphorylated STAT3 in tumors, it will be helpful to determine the relative expression of

[4] J.B. Yang and G.R. Stark, unpublished observation.

proteins that are driven by the total amount of STAT3 or by STAT3 dimers, respectively, to help discriminate among different mechanisms in different tumors. For example, if genes that respond to unphosphorylated STAT3 are expressed in certain cancers but genes that respond to STAT3 dimers are not, it would indicate that the STAT3 overexpression in these cases is caused by a signal distinct from STAT3 dimers. Such information would be useful in seeking appropriate applications for drugs currently in development that target the formation or function of STAT3 dimers (38).

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