Regulation of PSMB5 Protein and β Subunits of Mammalian Proteasome by Constitutively Activated Signal Transducer and Activator of Transcription 3 (STAT3)

Potential Role in Bortezomib-Mediated Anticancer Therapy*

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Background: Malignancies are frequently associated with altered abundance of proteasome and elevated proteasome activity.

Results: EGF-induced PSMB5 expression requires STAT3 activation.

Conclusion: The catalytic subunits and activities of the mammalian proteasome are regulated by STAT3.

Significance: Proteasome function is regulated by oncogenic signaling.

The ubiquitin–proteasome system facilitates the degradation of ubiquitin-tagged proteins and performs a regulatory role in cells. Elevated proteasome activity and subunit expression are found in several cancers. However, the inherent molecular mechanisms responsible for increased proteasome function in cancers remain unclear despite the well investigated and defined role of the mammalian proteasome. This study was initiated to elucidate the mechanisms involved in the regulation of β subunits of the mammalian proteasome. Suppression of STAT3 tyrosine phosphorylation coordinately decreased the mRNA and protein levels of the β subunits of the 20 S core complex in DU145 cells. Notably, PSMB5, a molecular target of bortezomib, was shown to be a target of STAT3. Knockdown of STAT3 decreased PSMB5 protein. Inhibition of phospho-STAT3 substantially reduced PSMB5 protein levels in cells expressing constitutively active-STAT3. Accumulation of activated STAT3 resulted in the induction of PSMB5 promoter and protein levels. In addition, a direct correlation was observed between the endogenous levels of PSMB5 and constitutively active STAT3. PSMB5 and STAT3 protein levels remained unaltered following the inhibition of proteasome activity. The EGF-induced concerted increase of β subunits was blocked by inhibition of the EGF receptor or STAT3 but not by the PI3K/AKT or MEK/ERK pathways. Decreased proteasome activities were due to reduced protein levels of catalytic subunits of the proteasome in STAT3-inhibited cells. Combined treatments with bortezomib and inhibitor of STAT3 abrogated proteasome activity and enhanced cellular apoptosis. Overall, we demonstrate that aberrant activation of STAT3 regulates the expression of β subunits, in particular PSMB5, and the catalytic activity of the proteasome.

The ubiquitin–proteasome system facilitates the degradation of regulatory and abnormal proteins. Polyubiquitinated proteins are targeted for degradation by the 26 S proteasome. The 19 S regulatory complex recognizes polyubiquitinated proteins, whereas proteolytic activity resides within the 20 S catalytic core of the 26 S proteasome. The 20 S proteasome is comprised of two outer α and two inner β rings (αββα). Each of the α and β rings consists of seven subunits, termed α1-α7 and β1-β7. The α subunits maintain the structure, whereas the core β rings contains proteolytically active subunits such as PSMB5 (β5, chymotrypsin-like), PSMB6 (β1, caspase-like), and PSMB7 (β2, trypsin-like). The proteasome activity is controlled stringently and attuned to cellular requirements. Aberrations of this pathway lead to pleiotropic defects in all aspects of cell function. Because of their indispensable role, inhibitors of the proteasome are routinely used as antineoplastic agents in clinics (1–4). Most of the proteasome inhibitors target the chymotrypsin-like activity of PSMB5, although they coinhibit the caspase-like and/or trypsin-like activity at higher concentrations. Bortezomib, a peptide boronic acid congener, is used clinically as a frontline therapy for multiple myeloma as a single agent or in combination with standard therapies. Bortezomib binds and inhibits the chymotrypsin-like activity of the PSMB5 subunit. However, resistance to bortezomib develops in a majority of patients, thereby limiting its clinical efficacy (5–7). Hence, elucidation of the mechanism of regulation of PSMB5 is imperative to identify the molecular basis of bortezomib resistance.

Suppression of the proteasome activity or autophagy in mammalian cells by pharmacological inhibitors results in the induction of PSMB5 and other proteasome genes in a concerted manner (8, 9). Nrf1 and Nrf2 are key transcription factors involved in the up-regulation of proteasome genes in response to pharmacological inhibition of proteasome activities. The catalytic subunits and activities of the mammalian proteasome are regulated by STAT3.

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to proteasome inhibition. Under normal conditions, Nrf1 undergoes endoplasmic reticulum-mediated degradation, whereas Keap1-bound Nrf2 is degraded by the ubiquitin-proteasome system. In response to proteasome inhibition, these transcription factors translocate to the nucleus and form heterodimers with Maf proteins. The Nrf1/Maf or Nrf2/Maf heterodimers transactivate proteasome genes through the antioxidant response elements in the promoters (10–12). Brain-specific, conditional knockout mice of Nrf1 exhibit a coordinated down-regulation of the basal levels of various proteasomal genes (13). Conversely, endogenous levels of proteasome subunits remain unaltered in mouse embryonic fibroblasts of nrf1- and nrf2-disrupted mice (11). Therefore, on the basis of these observations, Nrf1 or Nrf2 largely regulate the feedback response to proteasome inhibition and contribute minimally to the basal expression of proteasome genes. However, the transcription factors that regulate the basal level expression of proteasome subunits are largely unknown (14).

Neoplastic growth is frequently associated with increased proteasome activity and subunit expression to maintain protein homeostasis (15–19). Because of their stringent mode of activation, Nrf1 and Nrf2 are unlikely to promote the increased proteasome subunit expression in malignancies unless they are deregulated. Accordingly, colon tumors showed a gain of proteasome activity because of increased nuclear localization of Nrf2 (20). Thus, to cope with increased proteasome function in malignancies, it is plausible that the proteasome genes are regulated coordinately by oncogenic transcription factor(s). In this study, we investigated the mechanisms underlying increased proteasome subunit expression and activity in cell lines harboring oncogenic STAT3 protein. Importantly, PSMB5 has been shown to be a STAT3 target.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—All cell lines were purchased from the ATCC and maintained at 37 °C in a humidified incubator with 5% CO2. DU145, A549, HeLa, MDA-MB-231, and LNCaP cells were cultured in DMEM with 10% FBS. HEK293T and PC-3 cells were grown in minimal essential medium and F12, respectively, supplemented with 10% FBS. Transfections were carried out using Lipofectamine 2000 reagent (Invitrogen) according to the instructions of the manufacturer. All plasmids for transfection were prepared using Qiagen columns (Hilden, Germany). DU145 cells were washed three times with medium without serum following 24 h of serum starvation. Human EGF (catalog no. 324831, Calbiochem, Darmstadt, Germany) was added whenever required at a final concentration of 100 ng/ml.

Chemicals, Expression Vectors, and Antibodies—Stattic (catalog no. 573099), WP1066 (catalog no. 573097), EGFR inhibitor 324674 (EI)3 (catalog no. D00067055), PI3K/asket inhibitor LY294002 (catalog no. 440202), and MEK/ERK inhibitor PD98059 (catalog no. 513000) were obtained from Calbiochem. Bortezomib was from Natco Pharma, India. The pRC/CMV/STAT3-FLAG expression construct was a gift from Dr. Vijay Gupta (Scripps Research Institute). Resveratrol (catalog no. R5010); epoxomicin (catalog no. E3652); MG-132 (catalog no. C2211); a purified fraction of 20 S proteasome (catalog no. P3988); and antibodies for PSMB5 (catalog no. SAB2101895), GFP (catalog no. G1546), ubiquitin (catalog no. U5379), and tubulin (catalog no. T5168) were from Sigma-Aldrich. Bcl-2 (catalog no. sc-492) antibody was from Santa Cruz Biotechnology. AKT (catalog no. 4691), phospho-AKT-Ser-473 (catalog no. 4060), ERK1/2 (catalog no. 4695), phospho-ERK1/2 (catalog no. 4370), EGFR (catalog no. 4267), phospho-EGFR-Tyr-1068 (catalog no. 3777), STAT1 (catalog no. 9175), phospho-STAT1-Tyr-701 (catalog no. 9167), STAT3 (catalog no. 9132), and phospho-STAT3-Tyr-705 (catalog no. 9145) were from Cell Signaling Technology (Danvers, MA). The antibodies for the β subunits and 20 S proteasome core (catalog no. PW 8905) were from Enzo Life Sciences. The secondary antibodies anti-mouse IgG HRP (catalog no. NA9310) and anti-rabbit IgG HRP (catalog no. NA9340) were from Amersham Biosciences Pharmacia.

Construction of shRNA—The shRNAs targeting STAT3 were constructed using a U6 promoter-based vector as described previously (21). The desired synthetic oligonucleotides targeting the nucleotide sequences of STAT3 (NM_139276.2) were annealed and cloned into the BbsI-XbaI-digested mU6 pro vector. The two sequences of oligonucleotides cloned were STAT3sh1 (5′-tttgCAATTGGAGTACGTGCAGAAGttttGTTGAATTC-3′); and STAT3sh2 (5′-ttggAGAGGAGGCAATTTGGAA AGTTcttagaAGCTTTCATgGtCCTTCCCTTtttt-3′). This vector expressed the sense, 9 bp of hairpin, and antisense sequence. The mU6pro vector expressing shRNA of an unrelated sequence of the same length was used as a control (21).

Measurement of Caspase 3 Activity—Following treatment, cell pellets were washed twice with phosphate-buffered saline and suspended in lysis buffer (50 mM HEPEs (pH 7.4) containing 5 mM CHAPS and 5 mM DTT) for 15 min on ice. Subsequently, cells were centrifuged for 15 min at 14,000 × g under cold conditions, and clear supernatant was collected. The supernatant was added to assay buffer (20 mM HEPEs (pH 7.4), 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) containing caspase 3 substrate (Ac-DEVD-AMC, 40 μM) in a final volume of 100 μl as described earlier (22). The incubation was performed for 1 h at 37 °C with readings recorded at 5-min intervals. Fluorescence released by AMC was measured at 360-nm and 460-nm excitation and emission wavelengths, respectively. Values were normalized to protein concentration and expressed as fold change of activity relative to DMSO control.

Construction of Adenoviral Vectors—Adenoviral vectors were generated using the AdEasyTM adenoviral vector system from Stratagene. The cDNA was isolated from the pRC-CMV-FLAG-mSTAT3 plasmid by PCR and cloned into the NotiI/XhoI site of pAdtrack-CMV plasmid coexpressing green fluorescent protein as a marker to monitor infection efficiency. The cDNA was under the control of the CMV promoter terminated by the simian virus 40 (SV40) polyadenylation signal, resulting in pAdSTAT3. The pAdtrack-CMV plasmid was utilized as a control vector. The adenoviruses were generated as described previously (23).
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Cloning of the PSMB5 Promoter and Luciferase Assays—Genomatix software predicted a 965-bp PSMB5 promoter. The sequence was amplified by PCR employing human genomic DNA with sequence-specific primers (PSMB5, 5'-GGGTTAC-CTGTTGACATATTATGCACTTC AACC-3' (forward) and 5'-CCGGCCTG GAAGACAGATCTCGAC-CTAGATCCAG-3' (reverse)). Subsequently, the PSMB5 promoter was cloned into the pGL3-basic luciferase vector and sequenced to ensure the absence of mutations. The sequence was matched with Homo sapiens chromosome 14 with sequence identification NC_000014.8 from nucleotides 23,503,983–23,504,947. Putative transcription factor binding sites were determined by using MatInspector from Genomatix software. To determine the promoter activity, PC-3 cells plated in 12-well plates were transfected with 100 ng of pWT-PSMB5 promoter-reporter construct and required concentrations of STAT3 plasmids. The plasmid concentrations were kept constant at 1 µg by the addition of control plasmids. 30 h post-transfection, cellular lysates were prepared for luciferase activity assays according to protocols described by Promega. Relative luciferase activities were calculated after normalizing the values with β-galactosidase enzyme activities (24).

Measurement of Proteasome Activities—Peptidase activities of the proteasome were measured in cellular lysates by the addition of 50 µM fluorogenic peptide substrates, Suc-Leu-Leu-Val-Tyr-AMC (for chymotrypsin-like) and Z-Leu-Leu-Glu-AMC (for caspase-like activity) in assay buffer (50 mM Tris-HCl (pH 7.8), 20 mM KCl, 5 mM MgCl₂, and 1 mM dithiothreitol). The reaction mixture was incubated for 20 min at 37 °C. Later, the reaction was stopped by the addition of 125 mM sodium borate buffer (pH 9.0) containing 7.5% ethanol, as described previously (12). Fluorescence released by AMC was measured at 360-nm and 460-nm excitation and emission wavelengths, respectively, in an EnSpire multimode plate reader (PerkinElmer Life Sciences). Enzymatic activities were normalized to protein concentration and expressed as a percentage of activities relative to the DMSO control.

RT-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen). Semi-quantitative RT-PCR was carried out as described previously (21). RNA was reverse-transcribed using reagents from the first-strand cDNA synthesis kit (Invitrogen). The PCR conditions for PSMB1–PSMB7 were one cycle of 3 min at 95 °C, 1 min at 95 °C, 1 min at 56 °C, and 1 min at 72 °C and one cycle of 7 min final extension at 72 °C. The primers employed for the amplification of individual subunits were as follows: PSMB1, 5’-TTTGCCTGATACTTTAC-3’ (forward) and 5’-TACGCCCCCTTTCCTTCTTT-3’ (reverse); PSMB2, 5’-AAGGCCCGCCTAATTTCCTTCTT-3’ (forward) and 5’-AGGTTTGCAGATTTGAAGTTG-3’ (reverse); PSMB3, 5’-GAAGGGGAAAGAAGCTTGTTG-3’ (forward) and 5’-CCTGTTGCTGATTTGCTC-3’ (reverse); PSMB4, 5’-TACGCTCTGGGGATGTTAGT-3’ (forward) and 5’-TCTAGCGACTCTGCTGTCT-3’ (reverse); PSMB5, 5’-CCATACCTGCTAGCCACCAT-3’ (forward) and 5’-GCACCTCTGAGTACCATC-3’ (reverse); PSMB6, 5’-CCAAGCTTAGTACCATC-3’ (forward) and 5’-GCCGAAGCTGATGTTG-3’ (forward) and 5’-AAACT-AGGGTCAAGTGGAC-3’ (reverse); 18 S, 5’-CCTGGGCTGATTCATTTTAC-3’ (forward) and 5’-TGCCCCAGATCTGGTTCT-3’ (reverse); and Bcl-2, 5’-CTACGAGTTGATCCGAGATGTG-3’ (forward) and 5’-GGTCCGGCTGATATTCCAGT-3’ (reverse). PSMB1, 3, and 4 were amplified for 27 cycles. PSMB2 and 5 were amplified for 25 cycles. PSMB6 and 7 were amplified for 22 cycles. Bcl-2 and 18 S RNA were amplified for 28 and 18 cycles, respectively, in 25-µl volumes of PCR mixture (Fermentas) in an Eppendorf Master Cycler®.

Western Blot Analysis—Following treatments, cells were washed twice with PBS and lysed in 1× SDS sample buffer. Proteins were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were washed twice with Tween Tris-buffered saline before blocking nonspecific binding with 5% nonfat dry milk (Blotto, Santa Cruz Biotechnology) or 3% bovine serum albumin. The primary antibodies were used at 1:1000 dilutions, and the membrane was incubated for 2 h at room temperature. The phosphoproteins were detected using primary antibodies at 1:500 dilution and incubation of 2 h at room temperature in 0.3% BSA containing Tween-Tris-buffered saline. The membranes were washed three times, and the detection was performed by using horseradish peroxidase-conjugated secondary antibody as described previously (21).

Statistical Analysis—Statistical differences were calculated using Student’s t test. When significant differences were observed, p values for pairwise comparisons were calculated by employing a two-tailed t test. p < 0.01 was considered significant.

RESULTS

Suppression of Activated STAT3 Down-regulated PSMB5 Expression—Bortezomib treatment of head and neck squamous cell carcinoma cell lines lead to a significant up-regulation of total and phosphorylated STAT3 (25). In another study, bortezomib-resistant clones of human monocytes (THP1 cell line) manifested a marked increase in PSMB5 protein levels (26). These observations suggest a possible role of STAT3 in the regulation of PSMB5 expression. To explore this, we opted for DU145 cells, which harbor aberrantly high levels of activated STAT3 (27). DU145 cells were treated with Stattic, an inhibitor of STAT3, at 10 µM concentrations for various time periods (28). Stattic treatment resulted in the down-regulation of STAT3 tyrosine phosphorylation (p-STAT3) from 3 h onwards, whereas total STAT3 protein levels remained unaffected. The protein levels of Bcl-2, a target of STAT3, also decreased significantly by 3 h of treatment. Similar to p-STAT3 and Bcl-2, PSMB5 protein levels also decreased correspondingly. Moreover, PSMB5 protein levels were undetectable from 12 h of treatment (Fig. 1A). To validate the observations with Stattic, we employed another STAT3 inhibitor, WP1066 (29).

Treatment with WP1066 resulted in a significant reduction of PSMB5 protein levels by 18 h. p-STAT3 levels also concomitantly decreased from 18 h of treatment. Similar to Stattic, STAT3 protein levels remained unchanged in WP1066-treated cells (Fig. 1B). The difference in the inhibition of p-STAT3 protein levels in Stattic- and WP1066-treated cells was probably due to differences in the efficacy of the inhibitors. Previous
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FIGURE 1. STAT3 inhibitors reduce PSMB5 expression in DU145 cells. A and B, DU145 cells were treated with Stattic (10 μM) or WP1066 (10 μM) for different time periods. DMSO was used as a vehicle control (C). Following termination of treatment, cells were harvested and subjected to Western blot analysis employing specific antibodies for PSMB5, p-STAT3, STAT3, and Bcl-2. The p-STAT3/STAT3 antibodies detect the α and β isomers of STAT3. Tubulin was used as a loading control. C, DU145 cells were treated with different concentrations of resveratrol (25–100 μM) for 24 h, and an immunoblot analysis was performed for proteins. D, DU145 cells were treated with Stattic (10 μM) for 3–24 h. Following treatment, total RNA was isolated. PSMB5 and Bcl-2 mRNA levels were examined by semiquantitative RT-PCR. 18 S RNA was used as a loading control. E, DU145 cells were transfected with 2 μg of STAT3 shRNA constructs for 30 h. Scrambled shRNA was employed as control. GFP and tubulin were used as transfection and loading controls. Cells were harvested 30 h after transfection and probed for PSMB5, p-STAT3, STAT3, and Bcl-2 proteins. F, p-STAT1 and STAT1 protein levels were analyzed in DU145 cells treated with Stattic (10 μM) for the indicated time intervals. RT-PCR analysis and Western blot images were from pooled samples of triplicate cultures, and data are representative of three independent experiments.

studies report that resveratrol inhibits Src tyrosine kinase activity and, thereby, blocks STAT3 phosphorylation in DU145 cells (30). To further confirm STAT3-dependent PSMB5 subunit expression, we employed resveratrol. Treatment of DU145 cells with resveratrol suppressed p-STAT3 levels in a dose-dependent manner. The reduction in p-STAT3 levels was associated with decreased PSMB5 protein levels in resveratrol-treated cells (Fig. 1C). Further, STAT3 inhibition drastically reduced the mRNA levels of PSMB5 (Fig. 1D). The decrease in protein levels was preceded by reduced mRNA levels of PSMB5 in Stat3-treated cells. To provide further evidence for the requirement of STAT3 in the regulation of PSMB5, we employed a STAT3-directed shRNA. Both constructs of STAT3 shRNA significantly knocked down total STAT3 and p-STAT3 protein levels. In accordance with our earlier observations, knockdown of STAT3 protein resulted in lower PSMB5 protein levels (Fig. 1F). To validate the specificity of STAT3 inhibitors, Stattic-treated DU145 cells were analyzed for p-STAT1 levels. Notably, the tyrosine-phosphorylated STAT1 levels were undetectable in DU145 cells, and total STAT1 levels also remained unchanged (Fig. 1F). Overall, pharmacological inhibition and shRNA-mediated down-regulation of STAT3 levels decreased PSMB5 protein in DU145 cells. Thus, STAT3 activation is essential for PSMB5 expression.

Coordinated Decrease of β Subunits of Proteasome because of STAT3 Inhibition—Previous reports demonstrated that proteasome genes are regulated coordinately through a common transcription factor (10–12). Because the expression of PSMB5 is regulated by STAT3, we analyzed whether the other β subunits are also regulated coordinately by STAT3. To test this assumption, we treated DU145 cells dose-dependently with Stattic for 24 h. It was observed that, like PSMB5, protein levels of PSMB6, PSMB7, and other β subunits decreased significantly in a concerted manner in STAT3-inhibited cells (Fig. 2A). Stattic also decreased the levels of p-STAT3 and Bcl-2 protein levels. Next, under similar treatment conditions, we examined the mRNA levels of other β subunits. Similar to protein levels, the mRNA levels of β subunits decreased substantially in Stat3-treated cells (Fig. 2B). Thus, blockade of activated STAT3 coordinately reduced the mRNA and protein levels of the β subunits of the proteasome in DU145 cells.

Stattic Down-regulated PSMB5 Protein Levels and Decreased Proteasome Activities—In addition to DU145, other cancer cell types, such as the A549, HeLa, and MDA-MB-231 cell lines, display constitutively active STAT3 (31, 32). Hence, we analyzed whether suppression of p-STAT3 affects PSMB5 protein levels.
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levels as observed in DU145 cells. Consequently, these cell lines were treated in a dose-dependent manner with Static in 24 h. Concomitant with a decrease of p-STAT3 levels in Static-treated A549 cells, PSMB5 and Bcl-2 protein levels were also reduced (Fig. 3A). PSMB5 protein levels also decreased in the HeLa and MDA-MB-231 cell lines because of compromised activities in cells that harbor constitutively active STAT3. Consequently, we monitored the proteasome activities in cells that harbor constitutively active STAT3. Chymotrypsin-like activity significantly decreased to 90% in A549 cells. In comparison, the same activity was reduced to 78 and 85% at 10 μM concentrations of Static in the HeLa and MDA-MB-231 cell lines, respectively (Fig. 3A). Chymotrypsin-like activity was reduced significantly to 92% in DU145 cells (Fig. 3G). In addition, trypsin-like and caspase-like activities were also reduced significantly in STAT3-inhibited cells (Fig. 3, D–G). Taken together, suppression of p-STAT3 levels decreased PSMB5 protein and reduced proteasome activities in cell lines possessing constitutively activated STAT3.

PSMB5 Expression Is Induced by STAT3—Compromised STAT3 activation leads to decreased PSMB5 protein. Therefore, we examined whether increased levels of activated STAT3 induce PSMB5 expression. To investigate this possibility, we used a human prostate cancer cell line, PC-3, that does not express endogenous STAT3 (34). PC-3 cells were infected with adenovirus expressing wild-type STAT3 protein for 48 h. Endogenous or overexpressed STAT3 protein levels were absent in uninfected and control-infected cells. Accumulation of p-STAT3 protein levels in PC-3 cells resulted in a significant increase of PSMB5 protein levels to 1.8-fold relative to control-infected cells (Fig. 4A). Next, we analyzed whether STAT3 enhanced expression of PSMB5 through promoter activation. A bioinformatics analysis predicted a 965-bp promoter for PSMB5, with a TATA box harboring 294 bp of the 5′ untranslated region and 108 bp of exon 1. Consequently, the cloned promoter in the pGL3-basic vector was analyzed for basal and
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EGF-induced Up-regulation of PSMB5 Is Mediated by STAT3 Activation—The elevated levels of epidermal growth factor receptor (EGFR) in association with loss of androgen regulation results in androgen-independent growth of DU145 cells (36). In addition, aberrant EGFR levels are found to be frequently associated with activated STAT3 (37). Hence, we investigated whether EGF regulates PSMB5 protein in DU145 cells. To examine this possibility, cells were serum-starved for 24 h and later treated with EGF (100 ng/ml) for various time points until 24 h. PSMB5 protein levels were increased by 3 h and gradually reached a maximum by 24 h. The maximal levels of PSMB5 protein were associated with higher p-STAT3 levels (Fig. 5A). Next, we analyzed whether EGF-induced PSMB5 expression requires STAT3. To elucidate this, serum-starved DU145 cells were pretreated with inhibitors of EGFR (324674) and STAT3 (Stattic and WP1066) for 2 h. Subsequently, cells were treated with EGF for 24 h. EGF-induced STAT3 activation and PSMB5 protein levels were decreased significantly either by E1 or STAT3 inhibitors (Fig. 5B). EGF/EGFR can activate the PI3K and MEK/ERK signaling pathways (38). Next, we investigated whether these downstream pathways were also responsible for PSMB5 induction. However, treatment with inhibitors of PI3K/AKT (LY294002) and MEK/ERK (PD98059) did not prevent EGF-induced PSMB5 expression (Fig. 5C). The efficacy of all inhibitors was substantiated by suppression of their target kinases. Because β subunits are coordinately regulated by STAT3, we determined whether EGF also induced the expression of β subunits. EGF was added to cells pretreated with or without EGFR inhibitor for 24 h. EGF-induced concerted increases in the protein levels of β subunits were reduced in EGFR inhibitor-treated cells (Fig. 5D). Taken together, these results suggest that EGF induced the expression of β subunits and, in particular, that the induction of the PSMB5 subunit was through STAT3 activation.

PSMB5 Protein Levels Are Unresponsive to EGF in STAT3-negative PC-3 Cells—PC-3 cells display overexpressed EGFR and are responsive to EGF (39). Hence, to validate that EGF-induced PSMB5 activation requires STAT3, serum-starved PC-3 cells were pretreated with EGFR pathway inhibitors for 2 h. Subsequently, cells were treated with EGF for 24 h. EGF induced the phosphorylation of EGFR (Fig. 6A). In contrast, p-STAT3 and STAT3 were undetectable in PC-3 cells. Moreover, phosphorylation of AKT and ERK proteins was also observed with EGF treatment. Notably, PSMB5 protein levels remained unaffected by EGF or pharmacological inhibition of EGFR, ERK, and AKT proteins (Fig. 6, A and B). Blockade of p-STAT3 decreased PSMB5 protein levels and proteasome activities in STAT3-activated cells (Fig. 3, A–G). We analyzed whether PC-3 cells respond similarly to STAT3 inhibition. Treatments of cells with STAT3 inhibitors did not alter the protein levels of PSMB5 in PC-3 cells that lack endogenous STAT3 (Fig. 6, C and D). Correspondingly, chymotrypsin-like, trypsin-like, and caspase-like activities of the proteasome remained unaltered in Stattic-treated cells (Fig. 6E). Taken
together, sustained proteasome activities in STAT3 inhibitor-treated cells and loss of EGF-induced PSMB5 expression was due to the absence of STAT3 in PC-3 cells.

**PSMB5 and STAT3 Protein Levels Are Not Altered by Proteasome Inhibitors**—Proteasome inhibition elicits a feedback-dependent induction of PSMB5 gene expression (8, 10–12). In addition, bortezomib also induced the activation of STAT3 in head and neck squamous cell carcinoma cell lines (25). Hence, we analyzed whether proteasome inhibitors activate STAT3 and PSMB5. To test this possibility, DU145 cells were treated with bortezomib, epoxomicin, and MG-132 for 24 h. In response to proteasome inhibition, p-STAT3 and PSMB5 protein levels were unaffected and remained similar to control levels (Fig. 7A). Because the protein levels of PSMB5 and STAT3 were unaltered, we analyzed whether cells exhibit resistance to proteasome inhibitors by examining the chymotrypsin-like and caspase 3 enzyme activities. Chymotrypsin-like activity was inhibited significantly to 90% in cells treated with bortezomib when compared with the control. In addition, chymotrypsin-like activity in epoxomicin- and MG-132-treated cells was reduced to 86 and 58%, respectively (Fig. 7B). Nevertheless, bortezomib induced a robust caspase 3 response compared with the other two inhibitors (Fig. 7C). Thus, DU145 cells were susceptible to proteasome inhibitors, although the protein levels of PSMB5 or p-STAT3 remain unaltered.

**Combination Treatment of Bortezomib and STAT3 Inhibitors Exacerbates Proteasome Dysfunction and Cellular Apoptosis**—Abrogation of p-STAT3 was found to decrease proteasome subunit expression and activity in DU145 cells (Figs. 2A and 3G). In comparison, bortezomib treatment reduced proteasome activity. However, PSMB5 protein levels remained unchanged (Fig. 7, A and B). Furthermore, synergistic activation of cell death in response to combined treatment of bortezomib and STAT3 inhibitors was observed in head and neck squamous cell carcinoma cell lines (25). Hence, we reasoned that cotreatment would further reduce proteasome function and potentiate cell death. To examine this, DU145 cells were treated with Static, WP1066, bortezomib, or a combination of STAT3 inhibitors with bortezomib for 18 h. Chymotrypsin-like activity was decreased to 90% in response to bortezomib. In comparison, the same activity was reduced to 70% in WP1066-treated and 98% in Static-treated cells (Fig. 8A). However, cotreatment with STAT3 inhibitors and bortezomib completely abolished chymotrypsin-like activity. In addition, trypsin-like and caspase-like activities of the proteasome were inhibited significantly in cotreatment (Fig. 8A). We also exam-
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In this study, we report that PSMB5 is a target of STAT3. Suppression of STAT3 tyrosine phosphorylation by either chemical inhibitors or shRNA-mediated knockdown of STAT3 reduced PSMB5 protein levels. Blockade of p-STAT3 resulted in the concerted down-regulation of mRNA and protein levels of β subunits, leading to reduced proteasome activities. PSMB5 promoter and protein levels were induced by p-STAT3. EGF-induced expression of PSMB5 requires STAT3 activation. PSMB5 and STAT3 protein levels were unresponsive to proteasome inhibition. Bortezomib-induced loss of chymotrypsin-like activity and cell viability was potentiated by STAT3 inhibitors.

STAT3 is activated persistently in various malignancies and induces oncogenic processes through expression of various prosurvival genes (37). Hence, small-molecule inhibitors of tyrosine-phosphorylated STAT3 are explored actively for tumor therapy. The inhibition of constitutively active STAT3 induced apoptosis in DU145 cells (27). In our study, STAT3 inhibitors reduced PSMB5 expression and induced apoptosis. Similarly, proteasome inhibitors also triggered apoptosis, corroborated by increased caspase 3 activity. However, under similar experimental conditions, PSMB5 or phospho-STAT3-Tyr-705 levels remained unaffected, suggesting that the decreased levels of PSMB5 were not a general feature of apoptosis. Thus, lower levels of transcriptionally active STAT3 resulted in decreased levels of PSMB5 protein. The possibility of STAT1-dependent regulation of PSMB5 was ruled out because Stat3 selectively binds the SH2 domain of STAT3 over STAT1 (28). In addition, phospho-STAT1 levels were undetectable in DU145 cells, which is in accordance with an earlier report (41). A reduction in the mRNA levels of PSMB5 by STAT3 inhibitors further substantiates STAT3-dependent regulation of PSMB5 (Fig. 1D). Moreover, the mRNA and protein levels of Bcl-2, a known STAT3 target, concomitantly decreased with PSMB5, suggesting a transcriptional role of STAT3. Furthermore, a promoter analysis of PSMB5 revealed putative STAT3 binding sites with the canonical TT (N)₄₋₆ AA at −439 bp, +87 bp, and +230 bp relative to TSS (42). Accordingly, the PSMB5 promoter was activated in a dose-dependent manner by STAT3. In addition, exogenous expression of STAT3 in PC-3 cells resulted in the induction of PSMB5 protein. Hence, it is likely that STAT3 transcriptionally activates the gene expression and promoter activity of PSMB5 through the STAT3-responsive sites. Intriguingly, LNCaP and PC-3 cells expressed basal levels of PSMB5 protein when compared with DU145 cells. An abundance of proteasome is important for normal cell survival and indispensable for malignancies (6). Other oncogenic transcription factors may regulate the expression of PSMB5 in cell types lacking constitutive STAT3 levels.

Malignant conditions are often associated with high rates of protein synthesis. Hence, a wide range of cancers exhibit either higher proteasome activity or subunit expression or both to overcome this predicament (15–19). However, the molecular

Regulation of PSMB5 by STAT3
determinants responsible for the regulation of proteasome activity remain unclear. The results of this study demonstrate, for the first time, that activated STAT3 modulates the proteasome activities through the regulation of PSMB5 and other subunits. Hence, decreased protein levels of PSMB5 resulted in reduced proteasome activities in STAT3-suppressed cells. However, proteasome inhibitors reduced proteasome activity in DU145 cells without affecting the endogenous levels of PSMB5. This was probably due to the presence of copious amounts of constitutive STAT3 regulating the basal expression of PSMB5. Thus, STAT3 inhibitors impair cellular proteasome activities, leading to accumulation of polyubiquitinated proteins similar to proteasome inhibitors. However, STAT3 inhibitors do not interact with mammalian proteasome subunits because the molecular structures of STAT3 inhibitors differ markedly from known structural classes of proteasome inhibitors. The lack of inhibition by STAT3 inhibitors on chymotrypsin-like activity of the purified 20 S proteasome clearly indicates that there is no cross-reactivity between the STAT3 inhibitors and the proteasome per se. Overall, persistently activated STAT3 levels function as an important determinant of proteasome activity in cell lines harboring constitutive STAT3.

Proteasome genes are regulated coordinately by Nrf1 and Nrf2 in response to proteasome inhibition (8, 10–12). Similarly, the expression levels of other β subunits were reduced concertedly because of EGFR or STAT3 inhibition. It is likely that promoters of β subunits may contain STAT3-responsive sites. STAT3 has been shown as one of the targets of EGF signaling in FIGURE 8. The synergistic effect of bortezomib and STAT3 inhibitors on proteasome dysfunction and apoptosis. A, DU145 cells were treated with bortezomib (BTZ, 100 nM), Stattic (10 μM), or WP1066 (10 μM) or a combination of bortezomib and STAT3 inhibitors for 18 h. DMSO was used as a vehicle control. Chymotrypsin-like (CT-like), trypsin-like (T-like), and caspase-like (C-like) activities of the proteasome were measured. B, the chymotrypsin-like activity of the purified 20 S proteasome incubated with different concentrations (0.1, 5, and 10 μM) of bortezomib, Stattic, or WP1066. C, control. C, Under similar treatment conditions as in A, cell lysates were analyzed for PSMB5, p-STAT3, STAT3, 20 S core, and tubulin by Western blotting. D, levels of polyubiquitinated (Poly-Ub) proteins were determined by immunoblot analysis, and tubulin was employed as loading control. The bottom panel demonstrates the densitometric analysis of polyubiquitinated proteins. E, caspase 3 activity of cells treated with Stattic (10 μM) or WP1066 (10 μM) in the presence or absence of bortezomib for 18 h (n = 3). *, p < 0.01 compared with controls; #, p < 0.01 compared with the BTZ-treated group. Western blot images are the pooled samples from triplicate cultures. Data are representative of three independent experiments.
addition to the PI3K and ERK pathways (38). However, EGF-induced activation of the PI3K or ERK pathway did not affect PSMB5 expression in DU145 and PC-3 cells. Further, PSMB5 protein levels remained unchanged in response to EGF in STAT3-negative PC-3 cells. In contrast, EGF-dependent activation of p-STAT3 induced PSMB5 protein in DU145 cells. Hence, EGF-activated STAT3 possibly regulates the expression of β subunits of the 20 S proteasome and may contribute to the optimal growth of DU145 cells through efficient protein homeostasis. In support of these observations, recent studies in Caenorhabditis elegans also demonstrated that EGF signaling activates the ubiquitin proteasome system to modulate the life span of the worm (43).

PSMB5 is the molecular target of bortezomib, a Food and Drug Administration-approved proteasome inhibitor used clinically to ameliorate relapsed multiple myeloma and mantle cell lymphoma (44, 45). However, bortezomib resistance mechanisms pose a major obstacle in clinical therapy (7). The status of PSMB5 was elucidated in bortezomib-resistant cell line models. These studies suggested that bortezomib resistance was either due to mutations in or overexpression of the PSMB5 subunit (46, 47). However, observations from these cell line models remain unverified in clinical samples (48–50). In addition, the feedback regulation of proteasome gene expression also contributes to bortezomib resistance (14). Hence, constitutively activated STAT3 levels in cancers may presumably circumvent the effect of bortezomib regimen through up-regulation of PSMB5 protein. Therefore, cotreatment with bortezomib and an inhibitor of STAT3 may suppress bortezomib resistance. Because STAT3 regulates the basal expression of β subunits, blockade of STAT3 activation may, in part, alleviate the feedback response to proteasome inhibition. Moreover, lower expression of proteasome subunits reduces the number of active sites required for proteasome inhibition. In agreement with this hypothesis, knockdown of the 20 S core subunits of the proteasome sensitized HCT116 cells to bortezomib (51). Therefore, cotreatment involving bortezomib and STAT3 inhibitors might prove useful to inhibit proteasome function and STAT3 signaling in cancers (Fig. 8, A–E). Overall, the present findings on STAT3 dependent PSMB5 regulation underscore an alternative mechanism for bortezomib insensitivity. Finally, the oncogenic properties of STAT3 regulate the expression of PSMB5, β subunits, and activities of the mammalian proteasome to support malignant progression.

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