The Intracellular Delivery of a Recombinant Peptide Derived from the Acidic Domain of PIAS3 Inhibits STAT3 Transactivation and Induces Tumor Cell Death

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Abstract

Signaling components, which confer an “addiction” phenotype on cancer cells, represent promising drug targets. The transcription factor signal transducers and activators of transcription 3 (STAT3) is constitutively activated in many different types of tumor cells and its activity is indispensible in a large fraction. We found that the expression of the endogenous inhibitor of STAT3, protein inhibitor of activated STAT3 (PIAS3), positively correlates with STAT3 activation in normal cells. This suggests that PIAS3 controls the extent and the duration of STAT3 activity in normal cells and thus prevents its oncogenic function. In cancer cells, however, the expression of PIAS3 is posttranscriptionally suppressed, possibly enhancing the oncogenic effects of activated STAT3. We delimited the interacting domains of STAT3 and PIAS3 and identified a short fragment of the COOH-terminal acidic region of PIAS3, which binds strongly to the coiled-coil domain of STAT3. This PIAS3 fragment was used to derive the recombinant STAT3-specific inhibitor rPP-C8. The addition of a protein transduction domain allowed the efficient internalization of rPP-C8 into cancer cells. This resulted in the suppression of STAT3 target gene expression, in the inhibition of migration and proliferation, and in the induction of apoptosis at low concentrations [half maximal effective concentration (EC₅₀), <3 μmol/L]. rPP-C8 did not affect normal fibroblasts and represents an interesting lead for the development of novel cancer drugs targeting the coiled-coil domain of STAT3.

Introduction

The signal transducer and activator of transcription 3, STAT3, is an essential mediator of cytokine and growth factor signaling. The activation of latent STAT3 is dependent on its phosphorylation by tyrosine kinases. Phosphorylation induces dimerization and translocation into the nucleus, in which P-STAT3 binds to response elements present in the promoters of various target genes (1, 2). Several counter-acting proteins tightly regulate the extent and the duration of this signal. They include the suppressors of cytokine signaling, the Src-homology 2 domain-containing phosphatases, the translocation inhibitor Grim-19, and the protein inhibitor of activated STAT3 (PIAS3; refs. 3, 4).

In contrast to the transient STAT3 activation in normal cells, STAT3 is persistently activated in many types of cancer cells including leukemia, melanoma, and cancers of the breast, head and neck, brain, and pancreas (reviewed in refs. 5, 6). P-STAT3 promotes tumor development by the induction of genes encoding apoptosis inhibitors (e.g., Bcl-xl, Survivin, and Mcl-1), cell cycle regulators (e.g., cyclin D1/D2 and c-Myc), regulators of angiogenesis (e.g., vascular endothelial growth factor and HIF1α), and genes promoting migration and invasion (e.g., matrix metalloproteinases).

The role of the negative STAT3 regulator, PIAS3, has not been extensively studied in tumor cells harboring constitutively activated STAT3 molecules. PIAS3 belongs to the PIAS protein family also comprising PIAS1, PIAS-xα and PIAS-xβ, and PIASγ (7, 8). These molecules positively or negatively regulate the activity of over 60 proteins, many of which are transcription factors e.g., STAT1, STAT5, p53, NFκB, TIF2, Gfi-1, Smad, and AR (9). Modulation of transcription factor activity by PIAS proteins most likely involves multiple molecular mechanisms, including the recruitment of histone deacetylases (HDAC), the promotion of the sumoylation of transcription factors, the induction of the dissociation of dimers, or the sequestration of transcription factors to subnuclear structures (9, 10).

PIAS3 is comprised of distinct functional domains. The NH₂-terminal is highly conserved among members of the PIAS protein family and involved in binding, e.g., to the p65 subunit of NFκB (11). The central domain, amino acid positions 327 to 369, is predicted to form a structure, which is similar to the RING domain in E3 ligases. It
displays SUMO-1 ligase activity toward p53, AR, LEF1, Septin, and IFN regulatory factor-1 (reviewed in ref. 12). The COOH-terminal regions of the PIAS proteins are highly diverse. The COOH-terminal domain of PIAS1 was found to be involved in STAT1 binding (13).

A few studies have addressed the effects of exogenous PIAS3 overexpression in cancer cells: (a) about 89% of glioblastoma samples were found to be PIAS3 negative and P-STAT3 positive and the ectopic expression of PIAS3 in a glioblastoma cell line caused the inhibition of the transcriptional activity of STAT3 (14); (b) overexpression of PIAS3 in melanoma and lung cancer cell lines inhibited cell growth and suppressed STAT3 activity (15, 16); (c) overexpression of PIAS3 in v-Src-overexpressing tumor cells suppressed STAT3 target gene expression (17); (d) exogenous expression, mediated by infection with an adenovirus encoding kChaP/PIAS3β in prostate cancer cells, induced apoptosis and reduced growth of prostate tumor xenografts in nude mice (18). These studies indicate that PIAS3 can counteract the function of constitutively active STAT3.

The downregulation of STAT3 by small interfering RNA, dominant-negative variants (STAT3-B), oligonucleotide decoys, and chemical compounds showed that STAT3 signaling is required to maintain a distinct subset of tumor cells in vitro and in vivo (reviewed in refs. 19, 20). These experiments have served to validate STAT3 as a promising drug target. However, the design of a potent and specific STAT3 inhibitor is not trivial because STAT3 is an intracellular protein and displays no enzymatic activity. The development of an inhibitor based on a peptide sequence known to bind to STAT3 with high affinity and specificity seems to be an attractive option. Insights into the structural and functional domain organization of PIAS3 could advance this purpose. Here, we analyzed the expression of PIAS3 in normal and tumor tissues, defined the interacting domains of PIAS3 and STAT3, and identified a short subfragment of PIAS3, which autonomously binds to STAT3. This peptide was linked to a protein transduction domain (PTD), recombantly expressed, purified, and introduced into tumor cells. This transducible peptide efficiently inhibits STAT3 signaling and subsequently migration, proliferation, and the survival of tumor cells.

Materials and Methods

Reagents
Recombinant interleukin-6, mitomycin C, anti-actin and anti-tubulin were obtained from Sigma. P-STAT3, CyclinD1, His-tag, and cMyC antibodies were purchased form Cell Signaling. Anti survivin and rabbit anti PIAS3 were from Acris Antibodies; mouse-derived anti PIAS3 and anti-Lamin B1 were obtained from Abcam. STAT3 and STAT5 antibodies were from Santa Cruz. MG-132 was purchased from Calbiochem.

Cell Lines
Human low-grade (Hs-683), high-grade (MZ-54, MZ-18, U-87, and U-373), and murine high-grade glioblastoma cell lines (Tu-9648, Tu-2449) were kindly provided by Donat Kögel and Jakob Weißenberger (Neuroscience Center, Frankfurt, Germany; ref. 21). These cell lines were not further tested nor authenticated. Human breast cancer cell lines (SK-BR-3, MDA-MB-468, and MCF-7) as well as the murine melanoma cell line B16 were purchased from the American Type Culture Collection. The human breast cancer cells (MDA-MB-231, MDA-MB-453) and human prostate cancer cells (PC3) were kindly provided by Cord Hartman and Gert Carra (Georg Speyer Haus, Institute for Biomedical Research, Frankfurt, Germany). The murine breast cancer cell line 4T1 was kindly provided by Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland). The MDA-MB and 4T1 cells were verified by morphology, and epidermal growth factor receptor and ErbB2 receptor expression pattern. Primary human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection at passage 2 and were kindly provided by Carmen Dübel (Institute of Cardiovascular Regeneration, Frankfurt, Germany). B16 and PC3 cells were grown in RPMI; normal mouse HC11 breast cells were cultivated in RPMI containing 10 ng/mL insulin and 5 μg/mL epidermal growth factor; and all other cancer cell lines were grown in DMEM.

Preparation of Tissue Lysates
Mouse organs or tissues were dissected and dissociated in 5 mL standard radioimmunoprecipitation assay buffer per gram tissue. Samples were incubated on ice for 20 min and sonified. After centrifugation (15,000 rpm, 20 min, 4°C) supernatants were stored at −20°C. For Western blot analysis, 100 μg of each sample were loaded on a polyacrylamide gel. Western blots were done according to standard procedures.

Plasmid Construction
The hPIAS3L gene was amplified from cDNA. mPIAS3 was amplified from plasmids p513-flag-mPIAS3-wild-type and p513-flag-mPIAS3L-wild-type (22) kindly gifted by Hélène Boeuf (Université Bordeaux, Bordeaux, France) using specific primers. To generate the NH2- and COOH-terminal fragments primers, 5′-aaacataggtagtagttccc-3′ and 5′-aactcctctagctcggacacccggag-3′, or 5′-aatctagggtcgccgtaaagtt-3′ and 5′-aatcctctctccggagcgttc-3′ were used, respectively. The products were cloned into vector pGAD-T7 (Clontech). Additionally, smaller fragments of hPIAS3 were amplified and cloned in pGAD-T7 as well as in pET-30a(+) (Merck) for bacterial expression. To construct the bait plasmids, the different STAT genes were amplified from cDNA samples. The fragments were cloned into pGBK-T7 (Clontech). To prevent the autoactivation of the bait constructs, the COOH-terminal transactivation domain of the STATs is not included in any of the bait constructs.

Coimmunoprecipitation Experiments
Cell lysates were prepared with NP40 lysis buffer and 100 μg protein were mixed with 50 μg purified PIAS3 peptides. The mixtures were rotated for 4 h at 4°C. Specific
antibodies (1–2 μg) bound to 5 μL protein A–coated magnetic beads (Invitrogen) were added overnight. The beads were washed thrice with phosphate buffer and the bound complexes were released by boiling. Immunocomplexes were analyzed in a Western blot according to standard procedures.

Messenger RNA Analysis
Total RNA was isolated from cell lysates using the RNeasy Mini kit (Qiagen) and the SuperScript II Reverse Transcriptase kit (Invitrogen) was used for synthesis of cDNA. Q-PCR amplification of PIAS3 and 18S transcripts was done using forward primer 5′-gccacagtcatgcccgtc-3′ and reverse primer 5′-ttggccatgaactagctgg-3′, or primers 5′-gggaggtagtgagcaaaaaaatc-3′ and 5′-ttgccccctgaatctc-3′, respectively. Q-PCR was done using SYBR Green I (Abgene) for labeling. The reactions were done in triplicate. To calculate the relative mRNA levels, the cycle threshold value obtained by amplification of 18S mRNA was used for normalization. To amplify transcripts of STAT3 target genes in human (h) and murine (m) cell lysates, primers used were as follows: hCyclinD1, 5′-tgccatgaacgctgttcgc-3′ and 5′-gtagtcgctggagctgagc-3′; hActin, 5′-tagcagctgcgtgctggagg-3′; mCyclinD1, 5′-tggacagccgtgcctggtc-3′ and 5′-tgaggctgctggagctgagc-3′; mActin, 5′-agtttggatgcgcggag-3′; hStat3, 5′-gccagctgccctgcag-3′ and 5′-ctcgatggcacggcgcac-3′; mStat3, 5′-tgtggccttgccagccgtc-3′ and 5′-acaggactgaggccccag-3′; hPIAS3, 5′-atggccactgccgcatcc-3′; mPIAS3, 5′-tcccccctggactgggctgtact-3′, or reverse primer 5′-gccacagtcatgcccgtc-3′; h18S, 5′-gtggccttgtggagaggttg-3′; m18S, 5′-gggggtcgacg-3′; mBclXL, 5′-cctcccctggactgggctgtact-3′; hStat1, 5′-tggcagctgtacctcaag-3′ and 5′-tggcagctgtacctcaag-3′; hStat5a, 5′-tggcagctgtacctcaag-3′ and 5′-tggcagctgtacctcaag-3′; hStat5b, 5′-tggcagctgtacctcaag-3′ and 5′-tggcagctgtacctcaag-3′; hmitomycin c (10 μg/mL) was added to prevent further proliferation of the cells. The cells were incubated for 16 h and the scratches were photographed again. The number of cells that moved into the scratch was determined. For the Transwell assays, 1 × 10^6 cells/mL pretreated for 2 d with peptides were seeded into a Transwell insert (Greiner bio-one) without serum. Transwell inserts were placed in a 12-well plate containing 1 mL DMEM (with 0.2% bovine serum albumin) and plates were incubated overnight at 37°C. The next day, the number of cells at the bottom of the Transwell was determined by counting or staining with crystal violet.

Results
PIAS3 Is Differentially Expressed in Normal Mouse Tissues
The expression pattern of STAT3 in different tissues has been analyzed in multiple studies, but only limited data are available on the expression of its endogenous inhibitor PIAS3. Northern blot analysis, reverse transcription-PCR analysis, and immunohistochemical analyses have been done (7, 24-25). We extended these studies and carried out Western blot analyses of freshly prepared mouse tissue protein extracts. These studies were complemented by the analysis of latent STAT3 and activated P-STAT3 levels, and immunohistochemical analyses have been done (7, 24-25). We extended these studies and carried out Western blot analyses of freshly prepared mouse tissue protein extracts. These studies were complemented by the analysis of latent STAT3 and activated P-STAT3 levels. Previous studies showed that PIAS3 mRNA can be detected in nearly all tissues, but our experiments indicate that the PIAS3 protein is absent in the kidney, liver, heart, and brain (Fig. 1A). We found high PIAS3 expression in the muscles and in involuted breast tissue, and low expression in the spleen and the lungs (Fig. 1A). We detected a band of 68 kDa, which corresponds to the expected size of PIAS3. In addition, we found a band of higher molecular weight in spleen and lung extracts. This band represents the 85-kDa sumoylated form of PIAS3 (25, 26). Interestingly, the expression of PIAS3 largely corresponds to the activation pattern STAT3 in the same tissues. These data...
FIGURE 1. Expression of STAT3, P-STAT3 (Y705), and PIAS3 in normal mouse tissues, breast and brain tumor cell lines, and transplanted tumor tissues. A, Western blot analysis with antibodies specific for STAT3, P-STAT3 (activated STAT3 phosphorylated at Y705), and PIAS3 of protein extracts obtained from normal mouse tissues. Arrows, antibodies recognize the respective human and murine proteins. The PIAS3 antibody detects PIAS3 (68 kDa) as well as its sumoylated form (85 kDa). B, Western blot analysis of protein extracts obtained from mammary gland tissue at different developmental stages were analyzed with antibodies specific for STAT3, P-STAT3, and PIAS3. Tissues were obtained at virgin (puberty), pregnant, early lactation, late lactation, early involution, and late involution stages. C, Western blot analysis of protein extracts obtained from human breast cancer cells (MCF-7, SK-BR-3, MDA-MB-231, MDA-MB-435, MDA-MB-468, and T47D) and murine mammary cancer cells (4T1). D, Western blot analysis of protein extracts obtained from human glioblastoma cell lines (MZ-54, MZ-18, U-87, Tu-2449, Tu-9648, Hs-883). STAT3, P-STAT3, and PIAS3 expression was determined with specific antibodies. The WHO-classifications of the glioblastomas from which the cell lines were derived are indicated below the blot. E, comparison of the expression levels of P-STAT3 in protein extracts obtained from tumor cell lysates (lane1, PC3 cells; lane 3, B16 cells; lane 5, U-87 cells; lane 7, Tu-2449 cells; lane 9, SK-BR-3 cells) or protein extracts obtained from tumors obtained upon transplantation of these tumor cell lines into NMRI-nu/nu mice (lane 2, PC3; lane 6, U-87; lane 10, SK-BR-3, BALB/c mice (lane 8, 4T1), or C57Bl/6 mice (lane 4, B16). Extracts were prepared from the cancer cell lines (CL) grown in culture and from tumors (T) grown for 2 wk after s.c. transplantation of the cells into the recipient mice. F, Western blot analysis of PIAS3 expression in the tumor extracts (T) obtained as described in E. Protein extracts obtained from HepG2 lysates were included as a positive control. In all blots, actin and β-tubulin antibodies were used to control the loading of the gels.
could indicate a coordinate control of PIAS3 expression and P-STAT3 activation in normal tissues. We found high levels of PIAS3 in cells of the involuting mammary gland. We extended our studies and analyzed the expression of STAT3, activated P-STAT3, and PIAS3 during different stages of the developmental cycle of the mammary gland (Fig. 1B). STAT3 is rather uniformly expressed during all stages of development, but it becomes activated mainly in developing glands during puberty and pregnancy. P-STAT3 levels decrease during lactation and increase again during involution. The involution phase, is induced by cessation of suckling, and is accompanied by massive cellular apoptosis and the activation of STAT3 (27). PIAS3 is expressed in glandular cells of virgin mice, these levels decrease during pregnancy and lactation, but again increase during involution (Fig. 1B). The PIAS3 protein levels thus correlate with the P-STAT3 levels. These data confirm the coordinate regulation of STAT3 activation and PIAS3 expression in the mammary gland and suggest that STAT3 activity is dynamically counteracted by the upregulation of PIAS3-expression particularly at the end of the involution process.

**PIAS3 Protein Expression Is Lost in Cancer Cells**

*In vitro and In vivo*

We found a correlation between PIAS3 expression and P-STAT3 levels in normal tissues. Because STAT3 is highly activated in, e.g., breast cancer and glioblastoma cells, we investigated PIAS3 expression in these cancer cells by Western blotting. Five of seven breast cancer cell lines are both positive for P-STAT3 and negative for PIAS3 expression (Fig. 1C). In accordance with data reported earlier, all of the glioblastoma cell lines express PIAS3, in contrast to most primary glioblastoma tissues (14). Brantley et al. (14) suggested that there is a difference in the *in vitro* and *in vivo* systems. However, we found an inverse correlation for P-STAT3 and PIAS3 expression. We found very high levels of P-STAT3 in Tu-2449 and Tu-9648 cells and relatively low levels in the low-grade glioma cell line HS-683. The high-grade murine glioblastoma cells expressed very low levels of PIAS3, whereas the HS-683 cells showed the highest PIAS3 expression (Fig. 1D). Thus, in both cancer types, the levels of PIAS3 expression are inversely correlated with P-STAT3 levels.

We investigated, if the PIAS3 expression levels are influenced by the *in vivo* growth conditions. PC3, B16, U-87, 4T1, and SK-BR-3 cancer cells, expressing constitutively activated STAT3, were injected s.c. in mice. The tumors were allowed to grow until they reached a volume of 100 to 200 mm³ before the mice were sacrificed. Protein extracts were prepared and the P-STAT3 levels in the tumor tissues were compared with those found when the cells were grown on culture plates (Fig. 1E). P-STAT3 was detectable in all tumor samples derived from these cell lines. A higher STAT3 activation was generally found in the cells derived from tumor tissue. We also investigated the PIAS3 levels in the tumor tissue–derived cells and found that they do not express PIAS3 (Fig. 1F). Only in PC3 cell–derived tumors, a weak band of 85 kDa, corresponding
to sumoylated PIAS3, was detected. Thus, differently from HepG2 cells and prostate cancer cells (28), PIAS3 is absent in most breast cancer and glioblastoma cell lines grown in cell culture or as xenografts in mice. The protection afforded by PIAS3 to normal tissues against detrimental effects of P-STAT3 seems to be lost in these cancer cells.

PIAS3 mRNA Is Constantly Expressed in Breast Tissues and Breast Cancer Cell Lines

PIAS3 protein expression seems largely absent in cancer cells. We analyzed the molecular level at which the down-regulation of PIAS3 gene expression occurs and measured the PIAS3 mRNA in cancer cells by quantitative real-time PCR (Q-PCR; Fig. 2A). The results show that PIAS3 mRNA is present and that the levels of PIAS3 mRNA do not vary greatly (0.9- to 1.4-fold) when breast cancer cell lines expressing PIAS3 (e.g., MDA-MB-453) and lines expressing no detectable PIAS3 protein were compared. Similar results have previously been obtained in the analysis of glioblastoma cells (14).

We observed that the expression of PIAS3 protein in the mammary gland depends on the differentiation stage of the epithelial cells and is dependent on the stage of the cycle of pregnancy and lactation (Fig. 1B). We also analyzed PIAS3...
mRNA levels during these stages and found that PIAS3 mRNA remains relatively constant (between 0.7 and 1.0; Fig. 2B). These data suggest that PIAS3 gene expression is posttranscriptionally regulated in normal cells as well as in cancer cells.

A possible mechanism for the absence of PIAS3 protein in tumor cells, even in the presence of normal mRNA levels, could be found in an enhanced degradation rate of the protein. We investigated this option and incubated HepG2, 4T1, and Tu-9648 cells in the presence or absence of the proteasome inhibitor MG-132 (Fig. 2C). In PIAS3-expressing HepG2 cells, the PIAS3 mRNA is translated into PIAS3 protein and the cellular concentration is not influenced by MG-132. In 4T1 cells and in Tu-9648 cells, only very little PIAS3 is found in the absence of MG-132 (Fig. 2C). The addition of MG-132 resulted in a slight increase of PIAS3 in 4T1 cells and an appreciable increase in PIAS3 in Tu-9648 cells. These data indicate that increased proteasomal degradation constitutes at least one mechanism by which tumor cells downregulate the expression of PIAS3.

**Delimitation of the PIAS3 and STAT3 Interaction Domains**

The absence of PIAS3 in most cancer cells suggests that the loss of STAT3 inhibition might be a crucial mechanism to maintain the tumor cells. This implies that the development of a STAT3-inhibiting peptide derived from PIAS3 could be a promising approach. To define such an inhibitor, we first analyzed the molecular interactions of STAT3 and PIAS3 with the help of the yeast two-hybrid system. Sequences of STAT3 (bp 1-2004) or STAT1 (bp 1-2085) were fused to the GAL4-DNA binding domain, and STAT3 and STAT1 bait constructs were derived. The sequences of human hPIAS3, murine mPIAS3 (variant 1), and its shorter variant 2 lacking the L-insert (mPIAS3L) were fused to the GAL4-transactivation domain (GAD) and the hPIAS3, mPIAS3L, and mPIAS3 prey constructs were obtained. Yeast cells were transformed with the bait and prey constructs, and double transformants were grown on plates lacking leucine, tryptophan, and histidine, or on leucine, tryptophan, and histidine plates with 30 mmol/L 3-AT. This selective growth medium is used to select for protein interactions under stringent conditions. The growth of the yeast cells indicated strong interactions between STAT3 and hPIAS3 or mPIAS3L, and a weaker interaction between STAT3 and mPIAS3 (Fig. 3A). hPIAS3 does not interact with the highly homologous STAT1 protein in which 52% of the amino acids are identical to those found in STAT3.

Similar to STAT1 and STAT3, PIAS1 and PIAS3 exhibit a high sequence homology. It has been shown previously that STAT1 interacts with the COOH-terminal of hPIAS1, comprising amino acid positions 392 to 541. It was therefore reasonable to postulate that PIAS3 and STAT3 might interact through similar domains. However, a previous report showed that an NH$_2$-terminal fragment of mPIAS3, comprising amino acid positions 82 to 132, interacts with STAT3 in murine cell lines (15, 29). We performed yeast two-hybrid analyses with the NH$_2$ terminal (amino acid positions 1-319) and the COOH terminal (amino acid positions 320-619) fragments of hPIAS3. These fragments were integrated in prey constructs and STAT3 served in a bait construct (Fig. 3A-C). The yeast growth analysis

![FIGURE 4](image-url)

**FIGURE 4.** The COOH terminal fragments C5 and C8 of hPIAS3 interact with STAT3 in coimmunoprecipitation (IP) experiments. A, the amino terminal fragment of hPIAS3 (amino acid positions 9-326; N) and the COOH terminal fragment of hPIAS3 (amino acid positions 329-628; C) were expressed as His-tagged recombinant proteins and purified by FPLC. The two purified fragments are shown on the left in a Coomassie blue-stained gel. The recombinant proteins were incubated with 100 μg of protein extracts obtained from Tu-9648 cell lysates for 4 h at 4°C. STAT3 was isolated from the protein mixture with STAT3-specific antibodies and protein A–coated beads. The immunoabsorbed proteins were analyzed by Western blotting (WB) with a His-tag specific antibody. The COOH-terminal, but not the NH$_2$-terminal PIAS3 fragment, interacts with STAT3 (right). B, the COOH terminal hPIAS3 fragments C5 (amino acid positions 474-543) and C8 (amino acid positions 400-523) were expressed as His-tagged recombinant proteins and purified by FPLC. The two purified fragments are shown on the left in a Coomassie blue–stained gel. The purified fragments were incubated with protein extracts from Tu-9648 cell lysates; STAT3 antibodies were added; and the immune complexes were isolated on protein A–coated beads. The immunoabsorbed proteins were analyzed by Western blotting with a His-tag specific antibody. Both hPIAS3 fragments, C5 and C8, interact with STAT3 (right). C, yeast two-hybrid interaction analysis of a STAT3 bait construct with the C5 and C8 prey constructs. Serial dilutions of transformed yeast cells transformed with both constructs were grown on plates containing selection medium. The interaction of C8 with STAT3 allows the growth of the yeast cells under slightly more stringent conditions when compared with C5. The analysis of a p53 bait and a SV40 Large T-antigen prey construct served as a positive control.
indicated that STAT3 is not able to interact with the NH2-terminal fragment neither of hPIAS3 nor of mPIAS3. Instead, the results clearly show that STAT3 interacts with the COOH-terminal fragment of hPIAS3 (Fig. 3C). We conclude that a strong STAT3-interacting motif is present in the COOH-terminal fragment of hPIAS3.

PIAS1 and PIAS3 are highly homologous in their NH2-terminal domains but differ strongly in their COOH-terminal sequences. These differences might determine the specificities of their interactions with different STATs. The shortest fragment of PIAS1, previously shown to interact with STAT1, is located between amino acid positions 392 and 541 (13). In the corresponding region of PIAS3, we found two short sequences of 15 amino acids with low homologies to PIAS1 (L1: amino acid position 418-439 and L2: amino acid position 461-494) flanking an acidic domain (Fig. 3B). This region of hPIAS3 was further investigated. A prey construct, comprising amino acids 400 to 523 and spanning the L1, A, and L2 domains (named C8), was derived. In a second prey construct, the GAD was fused to a fragment comprising the L2 domain, amino acids 474 to 543 (named C5). Figure 3C and D show that the hPIAS3-C8 fragment and the overlapping hPIAS3-C5 constructs both mediate strong interactions with STAT3.

We also defined the domain within STAT3, which is being recognized by PIAS3. For this purpose, four functional domains of STAT3 were fused to the GAL4-DNA binding domain and used as bait constructs (Fig. 3D), the NH2-terminal domain, the coiled-coil domain (CC), the DNA binding domain, and the dimerization domain (SH2). The STAT3 bait and the PIAS3 prey constructs were introduced into yeast cells and analyzed in two hybrid experiments. All fragments derived from the COOH-terminal region of PIAS3 (PIAS3-C, PIAS3-C5, and PIAS3-C8), but not the fragments derived from the NH2 terminus, strongly interact with the CC of STAT3. There is no or only

**FIGURE 5.** Cellular uptake of the purified, recombinant COOH-terminal hPIAS3 fragment, rPP-C8, through protein transduction and inhibition of STAT3 target gene expression. A, schematic representation of the transducible rPP-C8 peptide. rPP-C8 is fused to a PTD comprising nine arginines residues (9R) and two terminal His tags. In the Flag-rPP-C8 construct, the NH2-terminal His-tag has been replaced by a Flag-tag. This epitope binds a Flag-specific antibody used to detect the protein by immunofluorescence. The cysteine residues present in rPP-C8 are indicated. Numbers above the constructs refer to the amino acid positions in hPIAS3. B, purified His-tagged rPP-C8 peptides were added to the medium (1 μmol/L) of growing 4T1 cells. Cytoplasmic and nuclear fractions of the cells were prepared at different times after rPP-C8 addition and the presence of rPP-C8 in these fractions was analyzed by Western blotting. A His-tag–specific antibody was used. The quality of the subcellular fractionations was confirmed by showing the presence of lamin B1 in the nuclear fraction and β-tubulin in the cytoplasmic fractions. These proteins also served as controls for the equal loading of the lanes. C, immunofluorescence analysis of the intracellular localization of Flag-rPP-C8 in SK-BR-3 cells 4 h after its addition to the medium. Before fixation of the cells, they were washed with 0.1 mol/L acetic acid in PBS to remove peptides attached to the cell surface. Flag-rPP-C8 was detected with an antibody directed against the Flag epitope and an Alexa 488–coupled secondary antibody. All cells show an even distribution of the peptide in the cytoplasm. In the nucleus, the peptides accumulate in dotted structures. Nuclei were stained with 4′,6-diamidino-2-phenylindole. D, confocal laser scanning microscopy of SK-BR-3 cells stained with anti-Flag antibodies to visualize rPP-C8 in nuclear bodies 4 h after the addition of Flag-rPP-C8 to the cells. Nuclei were stained with TO-PRO-3-iodide (Molecular Probes). C8. The levels of P-STAT3 and STAT3 were determined in nuclear and cytoplasmic extracts by Western blotting of protein extracts with specific antibodies. Expression of Actin was determined to verify equal loading of the gel.
significantly less interaction with the other STAT3 domains (Fig. 3D). This result is not necessarily expected. It has previously been suggested that PIAS3 might inhibit STAT3 through interference with its DNA-binding function (7). Our results show that PIAS3 does not recognize the DNA binding domain of STAT3.

The experiments were verified by biochemical interaction studies with recombinant proteins. The NH2 terminal (amino acids 9-326) and COOH-terminal (amino acids 329-628) PIAS3 fragments were fused to a His tag and cloned into bacterial expression vectors. The proteins were purified from bacterial lysates by FPLC (Fig. 4A). They were added to Tu-9648 cell lysates and coimmunoprecipitation experiments were done with a STAT3-specific antibody. The COOH-terminal fragment, but not the NH2-terminal fragment of PIAS3 is able to bind to STAT3 (Fig. 4A). These results confirm the yeast two-hybrid analyses. Similar experiments were carried out with recombinantly expressed and purified PIAS3-C5 and PIAS3-C8 peptides, named rPP-C5 and rPP-C8. We found that both rPP-C5 and rPP-C8 are able to bind to STAT3, with rPP-C8 slightly better than rPP-C5 (Fig. 4B). These differences in binding strengths were confirmed in additional yeast two-hybrid analyses. The ability of the yeast cells expressing the PIAS3-C8 fragment to grow under more stringent selection conditions, i.e., on -LTA plates, shows that the PIAS3-C8 fragment binds with higher affinity to STAT3 when compared with the PIAS3-C5 fragment (Fig. 4C).

Expression, Purification, and Cellular Delivery of rPP-C8

The expression of the PIAS3 gene in tumor cells was shown to cause the suppression of STAT3 function. These experiments were based on gene transfer methods. We investigated if we could use the rPP-C8 peptide to elicit a similar effect upon introduction into cancer cells. Because peptides do not readily enter cells, rPP-C8 was fused to a PTD. This domain is comprised of a homopolymeric stretch of nine arginines and promotes the uptake of its cargo (23, 30). Two His-tags were added for the affinity purification of the recombinant products (Fig. 5A). The purified peptides were found to be rather stable when incubated for prolonged periods of up to 40 hours in PBS, DMEM, or cell lysates at 37° (data not shown). This indicates that the peptides might be suited for cellular transduction experiments.

Tumor cell lines were exposed to purified rPP-C8 for increasing periods of time and the uptake into cells was monitored. 4T1 cells were treated by addition of 1 μmol/L rPP-C8 to the growth medium; cellular subfractions were prepared; and the presence of rPP-C8 was analyzed in cytoplasmic and nuclear protein extracts by Western blotting with specific antibodies. Expression of Actin was determined to verify equal loading of the gel.
The uptake of rPP-C8 was also visualized by immunofluorescence microscopy. SK-BR-3 cells were treated for 4 hours with Flag-rPP-C8. This variant contains a Flag epitope and can thus be detected by immunofluorescence (Fig. 5A). We observed uptake of the peptide into the cytoplasm and nucleus of all cells (Fig. 5C). Confocal laser scanning microscopy provided a higher resolution and we found that the peptides accumulate in subnuclear dotted structures (Fig. 5D). This observation coincides with previous reports that showed that PIAS3 accumulates in dot structures, also called nuclear granules (30, 31). The experiments show that the recombinantly expressed and purified rPP-C8 peptides quickly enter cells upon addition to the medium. Because the intracellular levels peaked at 2 to 4 hours, rPP-C8 was regularly replenished in experiments in which cells were treated for prolonged time periods.

**Transduction of rPP-c8 Inhibits STAT3 Target Gene Expression**

STAT3 regulates the expression of target genes involved in proliferation and survival, migration, and angiogenesis. If the transduced peptides are able to bind to STAT3 and possibly sequester it to subnuclear sites, they also have the potential to inhibit STAT3 target gene expression. We analyzed the immediate effects of the peptides on the expression level of two antiapoptotic target genes of STAT3, *BclXL* and *Survivin*, and of two genes regulating proliferation, *Cyclin D1* and *c-Myc*. MZ-54, Tu-9648, and 4T1 tumor cells were treated for 24 hours with rPP-C8. After this time period, fresh peptides were added to the medium. RNA was prepared from the cells at different time points after the second addition of rPP-C8. Transcript levels of the selected STAT3 target genes were determined by reverse transcription-PCR (Fig. 5E). The downregulation of the Survivin, *BclXL*, and *Cyclin D1* transcripts was observed within 4 hours in MZ-54 and Tu-9648 cells. We also monitored the levels of protein expression of these targets as a function of rPP-C8 exposure. 4T1 cells were treated for 48 hours with the peptides. After that time, the rPP-C8 was replenished and protein extracts were prepared at different times after addition (Fig. 5F). Treatment of 4T1 cells with rPP-C8 caused a strong reduction in the levels of *BclXL*, *Survivin*, and *Cyclin D1* within 40 to 240 minutes. Similar decreases were found in Tu-9648 cells (data not shown). Exposure of the cells to rPP-C8 did not cause a reduction in the level of *c-Myc* within the 4-hours observation period.

The inhibition of *BclXL*, *Survivin*, and *Cyclin D1* gene expression shows that the uptake of rPP-C8 regulates relevant proliferation and cell cycle functions in cancer cells. We investigated if these effects are mediated through the regulation of STAT3 or P-STAT3 levels in the transduced cells. Increasing concentrations of rPP-C8 were added to the medium of Tu-9648 cells and nuclear and cytoplasmic protein extracts were prepared. Figure 5G shows that the levels of STAT3 and P-STAT3 remain rather stable and are not affected by the exposure of the cells to rPP-C8. We conclude that the effects of rPP-C8 on target gene expression are caused by the functional inhibition of P-STAT3, possibly by subnuclear sequestration into dotted structures and not by interference with tyrosine phosphorylation.

**Transduction of rPP-C8 Inhibits Migration of Glioblastoma Cells**

We investigated the phenotypic consequences for cancer cells, treated for 2 days with the PIAS3-derived peptides, and initially focused on cell motility. One major characteristic of glioblastomas is their propensity to infiltrate surrounding normal tissues; this is a major obstacle for their surgical removal. The interference with the migratory potential of glioblastoma cells might therefore be of clinical significance. The STAT3 target genes *MMP-2* and *MMP-9* are suspected to be involved in the regulation of migration and invasion. We analyzed the effects of rPP-C8 on glioblastoma cell migration. Tu-2449, Tu-9648, and MZ-54 cells were grown for 2 days in the presence of the peptide. On the third day, the monolayer was scratched with a pipette tip and photographed. The extent to which the cells
were able to migrate into the scratch was examined 16 hours later. The control cells were able to fill the scratch within this time period. Seventy percent less cells were found in the gap area after rPP-C8 treatment (Fig. 6A). Additional migration assays were done by seeding 5 x 10^5 treated or nontreated Tu-2449, Tu9648, and MZ-54 cells in a Transwell insert. MCF-7 cells, which are not able to migrate, were used as a negative control. After 16 hours, the number of cells that migrated to the bottom surface of the membrane was counted (Fig. 6B). The rPP-C8 peptide clearly inhibited (2- to 4-fold) the migration of the three different glioblastoma cell lines.

**Transduction of rPP-C8, but not rPP-C8Δcys, Affects Cancer Cell Growth**

The migration assays described above were carried out after treatment of the cells for 2 to 3 days. We prolonged the exposure time to 3 to 5 days and observed additional cellular phenotypes. The Tu-9648, Tu-2449, MZ-54, and 4T1 cancer cell lines were seeded at low density and treated daily with 2 μmol/L of rPP-C8. After 5 days, they were photographed to analyze their morphology and stained with crystal violet to visualize the overall cell density in the wells (Fig. 7A). The PBS-treated control cells were confluent after 5 days. Treatment of the glioblastoma and breast cancer cells with the peptides strongly reduced the cell density. The remaining cells displayed a rounded morphology or were detached from the plate. In contrast, the growth of normal primary cells (murine mammary epithelial cells and HUVEC) and of normal immortalized cells (NIH-3T3, fibroblasts and HC11, mammary epithelial cells) was largely unaffected (Fig. 7A). These cells are not P-STAT3 dependent. T47D cells that express PIAS3 were also not affected. The differential sensitivity of the cell lines indicates that rPP-C8 is not generally cytotoxic but selectively affects the growth and survival of cells, which constitutively express activated STAT3 in the absence of endogenous PIAS3.

We reported earlier that aggregation of recombinant proteins attached to a PTD strongly affects their cellular uptake and impedes the intracellular inhibition by peptide aptamers (23). Aggregation can be caused by the intermolecular cross-linking and the formation of disulfide bridges. rPP-C8 contains three cysteine residues and we observed partial aggregation of rPP-C8 during its purification. We investigated if its efficacy could be further improved by replacing the cysteine residues by serines through site-directed mutagenesis. The mutated variant, rPP-C8Δcys, was found to be highly soluble and could be purified in a monomeric form (Fig. 7B). However, when rPP-C8Δcys was added to 4T1 cells or Tu-9648 cells, no inhibition of cell growth was observed (Fig. 7C). Although the peptides devoid of cysteines entered the cells more efficiently than the parental peptides, they were not able to affect growth of the cells. This indicates that at least one of the cysteines in rPP-C8 is required for the binding to STAT3. This conclusion was confirmed by coimmunoprecipitation experiments (Fig. 7D).
Transduction of rPP-C8 Inhibits Proliferation and Induces Apoptosis in Cancer Cells

rPP-C8 suppresses the expression of STAT3 target genes involved in cellular growth (Fig. 5E-F) and consequently reduces cell density (Fig. 7A). We measured the proliferation of the peptide-treated cancer cells in 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt assays as a function of time. SK-BR-3, Tu-2449, Tu-9648, MZ-54, and NIH-3T3 cells, 2,000 cells each, were seeded into 96-well plates and grown for 3 d. One, 2, or 4 μmol/L of rPP-C8 were added to the medium and the medium was changed every 24 h. Addition of PBS was used as a control. The proliferation of the cells was determined after 72 h by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt proliferation assays. The assays were done in duplicate and repeated thrice (**, P < 0.05). B, 5,000 Tu-9648 cells were seeded in 16-well E-plates (Roche) and grown for 24 h at 37°C. Then, the cell index in all wells (determined in a cell analyzer, Xcelligence, Roche) was normalized and set to 0. Different concentrations of rPP-C8 were added to the medium and proliferation of the cells was recorded as a function of the rPP-C8 concentrations by growing the cells for an additional 24 h in the cell analyzer. The EC50 for the two peptides was calculated using the GraphPad Prism program (P = 0.0038). C, morphologic changes induced in Tu-2449 and Tu-9648 cells upon exposure to rPP-C8. Cells were treated for 72 h with rPP-C8 and photographed at a ×40 magnification. Early and late apoptotic cells were observed. D, apoptosis induction by rPP-C8 in Tu-9648, Tu-2449 cells, and NIH-3T3 cells was measured by the detection of histone-complexed DNA fragments (mononucleosomes and oligonucleosomes) in the cytoplasm of rPP-C8 treated cells. The enrichment factor indicates the increase in cytoplasmic nucleosomes in peptide-treated Tu-9648 and Tu-2449 cells compared with PBS-treated cells and NIH-3T3 cells; the control cell value is considered 1. Assays were done with the Cell Death Detection ELISA* kit (Roche) according to the instructions of the manufacturer (*, P > 0.05; **, P < 0.05).

Transduction of rPP-C8 inhibits proliferation and causes the induction of apoptosis of breast and glioma tumor cells, but does not affect normal fibroblasts. A, SK-BR-3, Tu-2449, Tu-9648, MZ-54, and NIH-3T3 cells, 2,000 cells each, were seeded into 96-well plates and grown for 3 d. One, 2, or 4 μmol/L of rPP-C8 were added to the medium and the medium was changed every 24 h. Addition of PBS was used as a control. The proliferation of the cells was determined after 72 h by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt proliferation assays. The assays were done in duplicate and repeated thrice (**, P < 0.05). B, 5,000 Tu-9648 cells were seeded in 16-well E-plates (Roche) and grown for 24 h at 37°C. Then, the cell index in all wells (determined in a cell analyzer, Xcelligence, Roche) was normalized and set to 0. Different concentrations of rPP-C8 were added to the medium and proliferation of the cells was recorded as a function of the rPP-C8 concentrations by growing the cells for an additional 24 h in the cell analyzer. The EC50 for the two peptides was calculated using the GraphPad Prism program (P = 0.0038). C, morphologic changes induced in Tu-2449 and Tu-9648 cells upon exposure to rPP-C8. Cells were treated for 72 h with rPP-C8 and photographed at a ×40 magnification. Early and late apoptotic cells were observed. D, apoptosis induction by rPP-C8 in Tu-9648, Tu-2449 cells, and NIH-3T3 cells was measured by the detection of histone-complexed DNA fragments (mononucleosomes and oligonucleosomes) in the cytoplasm of rPP-C8 treated cells. The enrichment factor indicates the increase in cytoplasmic nucleosomes in peptide-treated Tu-9648 and Tu-2449 cells compared with PBS-treated cells and NIH-3T3 cells; the control cell value is considered 1. Assays were done with the Cell Death Detection ELISA* kit (Roche) according to the instructions of the manufacturer (*, P > 0.05; **, P < 0.05).
shown by the sigmoid shape of the response curve. We obtained an EC50 value of 2.7 μmol/L for rPP-C8 in Tu-9648 glioblastoma cells. 

Addition of rPP-C8 in the medium of STAT3-dependent cancer cells reduced the expression of the antiapoptotic target genes Survivin and BclxL. Treatment of the cells also affected their morphology and signs of apoptosis were observed (Fig. 8C). Therefore, we quantitated the induction of apoptosis as a function of rPP-C8 exposure. The apoptotic process is characterized by nuclear DNA fragmentation resulting in the release of nucleosomes into the cytoplasm. We determined the presence of nucleosomes in the cytoplasm of Tu-9648, Tu-2449, and NIH-3T3 cells after 24 or 48 hours of peptide treatment by an ELISA assay. There is a clear increase (2- to 8-fold) in the number of apoptotic Tu-9648 and Tu-2449 cells when compared with PBS-treated cells or the NIH-3T3 cells used as controls (Fig. 8D).

Our experiments show that rPP-C8, the fragment of PIAS3 identified in the yeast two-hybrid assay, can bind STAT3 specifically and efficiently in vitro. Fused to a PTD, rPP-C8 can be expressed as a recombinant protein. This protein is efficiently taken up by cells, binds intracellularly to STAT3, and inhibits transactivation by P-STAT3. The peptide derived from the COOH-terminal acidic region of PIAS3 acts as potent STAT3 inhibitor.

Discussion

The multitude of molecules involved in the formation and progression of cancer represent new opportunities for drug discovery and development. The most promising drug targets are molecules that are functionally indispensable for the survival of cancer cells, whereas their temporary inhibition does not cause cell death in normal cells. Such components have been identified and include signaling molecules or transduction pathways, which impose an “addiction phenotype” on cancer cells (32). The transcription factor STAT3 represents such a promising drug target and its central role in cellular transformation is well documented. Nevertheless, the development of therapeutic strategies targeting STAT3 has been difficult; it displays no substrate-binding pockets inhabitable by small molecules, but functions through protein-protein and protein-DNA interactions. Our strategy is based on the premise that peptides and proteins efficiently binding to the functional domains of STAT3 have the potential to block such essential interactions.

PIAS3 is a natural binding partner of STAT3 and a regulator of STAT3 signaling. The binding affinity and specificity of their interacting domains has probably been optimized in an evolutionary process. We used PIAS3 as a starting point for the development of a specific STAT3 inhibitor. Our observation that the PIAS3 protein is present in normal cells, which are capable of expressing high levels of P-STAT3 upon cytokine induction (Fig. 1A), emphasizes its essential function in the temporal regulation of STAT3 function. Furthermore, we found that sumoylated PIAS3 appeared during late stages of involution in the mammary gland (Fig. 1B). The induction of involution in the mammary gland is accompanied by a strong P-STAT3 activation and results in the massive apoptosis of alveoli. Sumoylation results in an increased protein stability and possibly points at a role for PIAS3 to limit P-STAT3 activity at the end of the involution process (33). From these data, it is tempting to speculate that PIAS3 protects normal cells from P-STAT3 addiction by fine tuning P-STAT3 activity and preventing its oncogenic effects. This protection mechanism seems lost in tumor cells. Tumor cells seem to down-regulate PIAS3 protein expression and thus allow the persistent action of P-STAT3. This then results in uncontrolled proliferation (Fig. 1C and D). Similar observations were recently described for primary brain tumor samples and ALK-“ALK” lymphoblastic lymphomas (14, 34), assigning a role as a tumor suppressor to PIAS3.

Because the PIAS3 gene is constitutively transcribed (Fig. 2), the downregulation of the PIAS3 protein might involve factors regulating its stability. Phosphoinositide 3-kinase/AKT has been linked to PIAS3 activity (16) and nitric oxide has been found to destabilize PIAS3 and regulate its sumoylation (35). A cross-talk between hSiah2 (seven in absentia) and PIAS3 regulates PIAS3-dependent activation. hSiah2 is involved in the proteasome-dependent degradation of PIAS3 (36). We found that treatment of 4T1 and Tu-9648 cells with a proteasome inhibitor led to an increase in PIAS3 protein levels. This also confirms previous data (14). In addition to these regulatory mechanisms, other processes affecting translation, e.g., miRNA-mediated translational inhibition, might be involved (37). The definition of the mechanisms that prevent PIAS3 protein expression, in the absence of P-STAT3 in normal tissues and in the presence of P-STAT3 in tumor cells, will be of great interest.

Our observations, and previously published data, indicate that tumor cells might be sensitive to the restoration of PIAS3 function. They encouraged us to derive a STAT3-specific inhibitor from PIAS3, which can be delivered into tumor cells, suppress STAT3-dependent transcription, and the associated cellular phenotypes. Because the data published with respect to the interactions of hPIAS1 and mPIAS3 with STAT1 and STAT3 were not entirely consistent, we performed detailed in vitro interaction studies of STAT3 and PIAS3 using the yeast two-hybrid system and in vitro with coimmunoprecipitation experiments. We mapped the interaction domains of STAT3 and hPIAS3 and found that the NH2-terminal CC of STAT3 interacts strongest with the COOH-terminal acidic domain of PIAS3. This does not confirm previously published studies of the group of Razin. These investigators described the NH2 terminus of mPIAS3 as the major STAT3 interaction domain (29, 38). The discrepancies between the data might be due to different methods used in the interaction analyses, the use of different cell types, and different PIAS3 variants. Our findings are consistent with domains found to mediate the STAT1/IPAS1 interaction (13). STAT1 and STAT3 as well as the NH2 terminus of PIAS1 and PIAS3 are highly homologous in sequence (72% and 70%, respectively) and in structure. Because
PIAS3 is able to clearly discriminate between STAT1 and STAT3 binding, it is reasonable to assume that the less homologous acidic region (39%) mediates this function.

We clearly identified the CC of STAT3 as the binding region for PIAS3. This has interesting implications for the mechanism by which rPP-C8 inhibits STAT3 function. rPP-C8 does not seem to affect the tyrosine phosphorylation of STAT3; therefore, it probably acts after the activation step. In contrast to PIAS3, which is only found in the nucleus of cells, rPP-C8 is also localized in the cytoplasm of transduced cells. This might imply that the peptides bind to activated STAT3 molecules and possibly prevent the translocation of these complexes to the nucleus. Additionally, we found rPP-C8 in dot-like structures in the nucleus. This could support the previous suggestion that PIAS3 proteins sequester transcription factors away from the promoters of their target genes into subnuclear compartments (8).

The important role of activated STAT3 in cellular transformation has triggered the search for specific STAT3 inhibitors. Although considerable effort has been invested, only a few specific and selective STAT3 inhibitors have been described. Kinase inhibitors that interfere with the activation step, e.g., the Jak-kinase inhibitor AG490, Jak-Inhibitor I, and WP1066, or compounds, which affect multiple other targets in addition to STAT3, e.g., curcumin, resveratrol, and platinum compounds, have been investigated. Several other compounds might be rather STAT3 specific, among them are hpSx3ODN, Static, S3-M2001, curcubitacin, peptide P1a, and our previously identified peptide aptamer hTrx-3.8 that targets the STAT3-SH2 domain (23, 39-43).

It will be necessary in the future to further characterize these inhibitors, to understand their mechanisms of action, to compare their EC50 -values, to improve their efficacy, and to identify possible toxic side effects. Our study shows that STAT3 can be inhibited in a postactivation stage and that the CC of STAT3 can serve as a drug target site. The recombinant PIAS3 fragment, rPP-C8, specifically recognizes this domain of STAT3; it is not toxic in normal cells and offers new perspectives for structure based drug design.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Intracellular Delivery of a Recombinant Peptide Derived from the Acidic Domain of PIAS3 Inhibits STAT3 Transactivation and Induces Tumor Cell Death

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