Selective inhibition of the function of tyrosine-phosphorylated STAT3 with a phosphorylation site-specific intrabody

Mi Young Koo[a], Jiyoung Park[b], Jung Mi Lim[a], Sei Yoon Joo[a], Seung-Pil Shin[c], Hyun Bo Shim[a], Junho Chung[d], Dongmin Kang[a], Hyun Ae Woo[b,e], and Sue Goo Rhee[f,1]

[a]Division of Life and Pharmaceutical Sciences, [b]Graduate School of Pharmaceutical Sciences, and [e]Global Top 5 Research Program, Ewha Womans University, 52 Ewhayeodae-gil, Seodaemun-gu, Seoul 120-750, Korea; [c]Genitourinary Cancer Branch, Research Institute National Cancer Center, 323 Ilsan-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-769, Korea; [d]Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, 28 Yeongun-dong, Jongro-gu, Seoul 110-799, Korea; and [f]Yonsei Biomedical Research Institute, Yonsei University College of Medicine, 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, Korea

Edited* by Joseph Schlessinger, Yale University School of Medicine, New Haven, CT, and approved March 19, 2014 (received for review September 9, 2013)

Signal transducer and activator of transcription 3 (STAT3) is a multifunctional protein that participates in signaling pathways initiated by various growth factors and cytokines. It exists in multiple forms including those phosphorylated on Tyr^{705} (pYSTAT3) or Ser^{727} (pSSTAT3) as well as the unphosphorylated protein (USTAT3). In addition to the canonical transcriptional regulatory role of pYSTAT3, both USTAT3 and pSSTAT3 function as transcriptional regulators by binding to distinct promoter sites and play signaling roles in the cytosol or mitochondria. The roles of each STAT3 species in different biological processes have not been readily amenable to investigation, however. We have now prepared an intrabody that binds specifically and with high affinity to the tyrosine-phosphorylated site of pYSTAT3. Adenovirus-mediated expression of the intrabody in HepG2 cells as well as mouse liver blocked both the accumulation of pYSTAT3 in the nucleus and the production of acute phase response proteins induced by interleukin-6. Intrabody expression did not affect the overall accumulation of pSSTAT3 induced by interleukin-6 or phorbol 12-myristate 13-acetate (PMA), the PMA-induced expression of the c-Fos gene, or the PMA-induced accumulation of pSSTAT3 specifically in mitochondria. In addition, it had no effect on interleukin-6–induced expression of the gene for IFN regulatory factor 1, a downstream target of STAT1. Our results suggest that the engineered intrabody is able to block specifically the downstream effects of pYSTAT3 without influencing those of pSSTAT3, demonstrating the potential of intrabodies as tools to dissect the cellular functions of specific modified forms of proteins that exist as multiple species.

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family of transcription factors (STAT1 to STAT6) and was originally identified as an acute phase response (APR) factor that is activated by interleukin (IL)-6. STAT3 regulates the expression of a variety of genes in response to its activation by IL-6 family cytokines, peptide growth factors, interferons (IFNs), and oncoproteins. As with other STAT proteins, the transactivation function of STAT3 is activated when a critical tyrosine residue (Tyr^{705} in STAT3) is phosphorylated, which results in dimerization of the protein through reciprocal interactions between the phosphotyrosine and a Src homology 2 (SH2) domain. The tyrosine-phosphorylated form of STAT3 (pYSTAT3) translocates from the cytosol to the nucleus, where it binds to the IFN-γ–activated sequence (GAS) in target promoters and thereby activates transcription (1–4).

Most STAT proteins also contain a serine phosphorylation site (Ser^{727} in STAT3). Although the serine-phosphorylated form of STAT3 (pSSTAT3) also participates in transcriptional regulation, such phosphorylation can have a positive or negative effect on transactivation activity (5). The ultimate biological outcome of pSSTAT3 signaling appears to depend on the extracellular stimulus, gene promoter, cell type, and activation status of the cell (5). Whereas all of the cell surface receptors known to increase pYSTAT3 abundance also increase the amount of pSSTAT3, pSSTAT3 is also generated in the absence of pYSTAT3 in response to several stimuli (5). Moreover, pSSTAT3 is also found in mitochondria and regulates mitochondrial respiration (6–8). STAT3 is also acetylated on a lysine residue, with this modification being essential for the formation of stable dimers (3). A feature of STAT3 that distinguishes it from other STAT proteins is its prominent nuclear localization in the absence of its tyrosine phosphorylation. Unphosphorylated STAT3 (USTAT3) thus shuttles between the cytoplasmic and nuclear compartments, binds to DNA, and functions as a transcriptional activator and a chromatin or genomic organizer (9, 10).

The biological effects of STAT3 are diverse, likely reflecting its activation by a wide range of cytokines, growth factors, and oncoproteins as well as the actions of variously modified STAT3 species in the nucleus, cytosol, and mitochondria. The deciphering of such diverse STAT3 functions will require dissection of the role of each covalently modified form of STAT3. Intrabodies, which are intracellular, recombinant, single-chain antibody fragments that comprise the heavy (V_H) and light (V_L) antigen binding domains connected by a linker, are an attractive option for neutralization of the function of a protein posttranslationally modified at a specific site, given the possibility of developing high-affinity binders to the modified site and their intrinsic specificity.

We have now generated an intrabody that binds specifically to the tyrosine-phosphorylated sequence of pYSTAT3 and have studied the effects of its expression on downstream signaling in

Significance

In response to a variety of extracellular stimuli, signal transducer and activator of transcription 3 (STAT3) is phosphorylated on Tyr705 (pYSTAT3) or Ser727 (pSSTAT3), and signal transmission by those stimuli depend on pYSTAT3 as well as pSSTAT3 and unphosphorylated protein (USTAT3). Here we prepared an intrabody (an antibody that is expressed within the cell) that binds specifically to the tyrosine-phosphorylated site of pYSTAT3 and demonstrated that the engineered intrabody is able to block selectively the downstream effects of pYSTAT3 without influencing those of pSSTAT3 and USTAT3 in cultured cells as well as mouse liver. Our results demonstrate the potential of intrabodies as tools to dissect the cellular functions of specific modified forms of proteins that exist as multiple species.

Author contributions: M.Y.K., H.B.S., D.K., H.A.W., and S.G.R. designed research; M.Y.K., J.P., J.M.L., and S.Y.J. performed research; S.-P.S. and J.C. contributed new reagents/analytic tools; M.Y.K. and S.G.R. analyzed data; and S.G.R. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]To whom correspondence should be addressed. E-mail: RHEESG@yuhs.ac.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1316815111/-/DCSupplemental.

BIOCHEMISTRY

HepG2 human hepatoma cells stimulated with IL-6 and in mouse liver stimulated with IL-6 or lipopolysaccharides (LPS). Adenovirus-mediated expression of the intrabody blocked signaling downstream of pYSTAT3 but not that downstream of pYSTAT1. Intrabody expression affected neither the abundance of nor signaling by pSSTAT3. Comparison of the effects of intrabody expression with those of STAT3 knockdown by RNA interference (RNAi) or STAT3 inhibition with a chemical agent revealed the effectiveness and benefits of intrabodies for characterization of a protein like STAT3 that has multiple functions dependent on different types of covalent modification.

Results

Generation of an Intrabody That Binds Specifically to the Tyrosine-Phosphorylation Site of pYSTAT3. A rabbit–human chimeric antigen-binding fragment (Fab) phagemid library was prepared with mRNA isolated from the bone marrow and spleen of rabbits immunized with an 11-amino acid peptide (PGSAAPpYLKTK, designated the pYSTAT3 peptide) that corresponds to the sequence surrounding the phosphorylated Tyr^{705} residue of STAT3. The presence of pYSTAT3-reactive antibodies in the serum of immunized rabbits was detected by immunoblot analysis of IL-6–stimulated HepG2 cells (Fig. S1*A*). To select for specific binders of pYSTAT3, we subjected phage to four rounds of panning with the pYSTAT3 peptide conjugated to BSA and immobilized on magnetic beads. Fab fragments (Fig. S1*B*) expressed in the *Escherichia coli* system from 10 randomly selected clones were tested for their reactivity with the pYSTAT3 peptide with the use of an ELISA. All of the selected Fabs were found to bind to the BSA-conjugated pYSTAT3 peptide but not to the corresponding unphosphorylated STAT3 peptide (Fig. S1*C*). Sequencing analysis revealed that 4 of the 10 clones were highly related to each other, differing by only seven amino acid residues in their light-chain complementarity-determining regions (LCDRs), with all amino acid residues being identical in their heavy-chain complementarity-determining regions (HCDRs) (Fig. S1*D*). The remaining 6 clones differed from each other in all CDRs including HCDR3 (Fig. S1*D*), a key region for antigen binding.

We converted the Fab phage clones to a single-chain variable fragment (scFv) form, in which V_H and V_L domains of each Fab are joined via a flexible polypeptide linker (GGSSRSSSSGGGGSGGGG), and we then fused scFv to the human fragment crystallizable (Fc) region to generate a scFv–Fc fusion protein. Each scFv–Fc fusion protein was also targeted for secretion by the addition of a mouse Ig kappa (Igκ) leader sequence to the NH_2 terminus, thus allowing the minibodies to be purified from culture supernatants. The fusion antibody derived from clone 17 (Fig. S2 *A* and *B*), one of the four closely related clones, was expressed at a much higher level compared with those derived from the other clones, and we therefore focused on the characterization of this fusion protein. Surface plasmon resonance analysis with various concentrations of scFv–Fc17 fusion protein (50–200 nM) and a fixed amount of BSA-conjugated pYSTAT3 peptide revealed that the fusion protein bound to the pYSTAT3 peptide with high affinity [dissociation constant (K_d) = 76 pM; association rate constant (k_{on}) = 1.37×10^4 $M^{-1}{\cdot}s^{-1}$; and dissociation rate constant (k_{off}) = $1.05 \times 10^{-5}{\cdot}s^{-1}$)] (Fig. S1*E*).

Immunoblot analysis indicated that STAT3 was recognized by scFv–Fc17 fusion protein in IL-6–stimulated HepG2 cells but not in unstimulated cells (Fig. S2*C*). HepG2 cell lysates were then subjected to immunoprecipitation with the fusion protein, and the resulting precipitates were subjected to immunoblot analysis with commercial antibodies to pYSTAT3, STAT3, and phosphotyrosine (Fig. S2*D*). A STAT3 band was detected by all three antibodies only in the immunoprecipitates prepared from IL-6–stimulated cells, and no other bands were detected by the antibodies to STAT3 or to phosphotyrosine. These results thus indicated that scFv–Fc17 fusion protein recognizes pYSTAT3 selectively over USTAT3 and that it precipitates only pYSTAT3 and not other tyrosine-phosphorylated proteins, despite the fact that multiple proteins are phosphorylated on tyrosine in response to stimulation with IL-6 (11).

To study the effects of intracellular expression of a pYSTAT3-specific antibody, we infected HepG2 cells with an adenovirus encoding the scFv region (V_L-linker-V_H) of scFv–Fc17 fusion protein fused to green fluorescent protein (GFP), with the fusion protein being designated intrabody 17 (Fig. 1*A*). The abundance of pYSTAT3 in HepG2 cells maintained in serum-free medium was increased in proportion to the amount of the infecting GFP–scFv17 virus, whereas it was unaffected by infection with an adenovirus encoding GFP alone (Fig. 1*B*). These results indicated that tyrosine phosphorylation of STAT3 occurs in unstimulated HepG2 cells and that tight binding of GFP–scFv17 to the nascent pYSTAT3 molecules and their consequent protection from protein tyrosine phosphatases results in the accumulation of pYSTAT3 in a manner dependent on GFP–scFv17 concentration. IL-6 stimulation also induced the accumulation of pYSTAT3 to much higher levels in cells expressing GFP–scFv17 than in those expressing GFP (Fig. 1*C*). In addition, IL-6 increased the abundance of STAT3 phosphorylated on Ser^{727} (pSSTAT3), but the expression of GFP–scFv17 had no effect on pSSTAT3 accumulation (Fig. 1*C*). This was confirmed after fractionation of IL-6–stimulated HepG2 cell lysates on longer SDS gels, which yielded two bands, the lower band containing STAT3 proteins phosphorylated only on Tyr^{705} and the upper

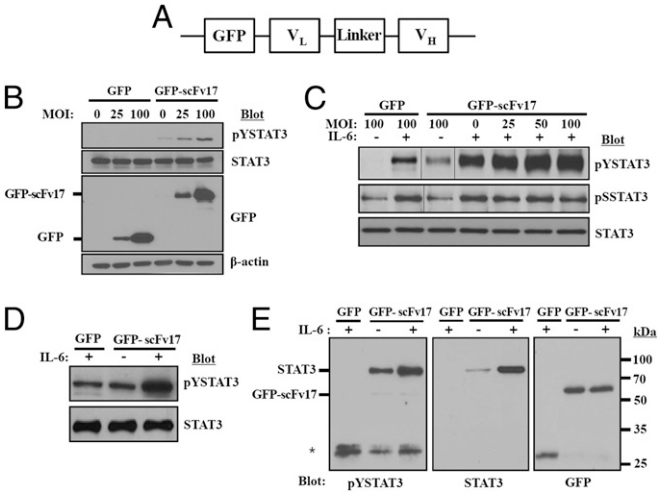

Fig. 1. Effect of GFP–scFv17 (intrabody 17) expression on the abundance of pYSTAT3 and pSSTAT3 and the selective binding of intrabody 17 to pYSTAT3 within HepG2 cells. (*A*) Domain organization of GFP–scFv17. (*B*) Effect of GFP–scFv17 expression on pYSTAT3 abundance in HepG2 cells. Cells infected with recombinant adenoviruses encoding GFP or GFP–scFv17 at various multiplicities of infection [MOI: 0, 25, or 100 plaque-forming units (pfu) per cell] were maintained in serum-free medium for 24 h, lysed, and subjected to immunoblot analysis with antibodies to pYSTAT3, STAT3, GFP, and β-actin (loading control). The positions of GFP–scFv17 and GFP are indicated. (*C*) Effect of GFP–scFv17 expression on the abundance of pYSTAT3 or pSSTAT3 in IL-6–stimulated HepG2 cells. Cells infected with adenoviruses for GFP (100 pfu per cell) or for GFP–scFv17 (0, 25, 50, or 100 pfu per cell) were incubated with or without IL-6 (50 ng/mL) for 30 min, after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins. Some lanes are spliced together from the same immunoblot; these alterations are indicated by a vertical line between the lanes. (*D* and *E*) Selective binding of GFP–scFv17 to pYSTAT3 inside of HepG2 cells. Cells infected with adenoviruses for GFP or GFP–scFv17 (100 pfu per cell) were incubated in the absence or presence of IL-6 (50 ng/mL) for 30 min, after which cell lysates were directly subjected to immunoblot analysis with antibodies to pYSTAT3 or to STAT3 (*D*), or they were first subjected to immunoprecipitation with antibodies to GFP and the resulting precipitates were subjected to immunoblot analysis with antibodies to pYSTAT3, STAT3, or GFP (*E*). The asterisk indicates nonspecific binding.

band containing STAT3 proteins phosphorylated only on Ser^{727} or phosphorylated on both Tyr^{705} and Ser^{727} (Fig. S3). The intensity of the upper bands was not significantly affected by GFP–scFv17 expression (Fig. S3).

To confirm the specific binding of GFP–scFv17 to pYSTAT3, we subjected lysates of IL-6–stimulated or -unstimulated HepG2 cells expressing GFP or GFP–scFv17 to immunoprecipitation with antibodies to GFP. The resulting immunoprecipitates were then subjected to immunoblot analysis with antibodies to pYSTAT3, STAT3, or GFP. Although immunoblot analysis of cell lysates indicated that the abundance of pYSTAT3 in IL-6–stimulated GFP-expressing cells was similar to that in unstimulated GFP–scFv17-expressing cells (Fig. 1*D*), bands immunoreactive with anti-pYSTAT3 and with anti-STAT3 were detected in the immunoprecipitates from the latter cells but not in those from the former (Fig. 1*E*). In addition, the intensity of these immunoreactive bands was greater for the immunoprecipitates from IL-6–stimulated GFP–scFv17-expressing cells than for those from unstimulated GFP–scFv17-expressing cells (Fig. 1*E*). These results suggested that GFP–scFv17 molecules are sufficiently expressed inside the cells to forms a stable complex with pYSTAT3 even after the stimulation with IL-6.

In HepG2 cells, IFN-γ induces tyrosine phosphorylation of STAT1 but not that of STAT3, whereas IL-6 induces tyrosine phosphorylation of STAT3 and to a much lesser extent that of STAT1 (12, 13). Immunoblot analysis of HepG2 cells stimulated with IFN-γ or IL-6 revealed that the commercial antibodies to pYSTAT3 recognized pYSTAT3 but not pYSTAT1 (Fig. S4*A*). Whereas the abundance of pYSTAT3 was increased by GFP–scFv17 expression in cells incubated with or without IL-6, GFP–scFv17 expression had no effect on the abundance of pYSTAT1 (Fig. S4*B*). Furthermore, expression of GFP–scFv17 had no effect on expression of the gene for IFN regulatory factor 1 (IRF1), a downstream target of STAT1, in HepG2 cells incubated with or without IL-6 (Fig. S4*C*). These results suggested that intrabody 17 binds pYSTAY3 but not pYSTAT1.

Furthermore, the ability of GFP–scFv17 to recognize pYSTAT3 selectively over pYSTAT2 was demonstrated in HeLa cells stimulated with IFN-α (Fig. S5*A*), over pYSTAT4 in NK92 cells stimulated with IL-12 (Fig. S5*B*), over STAT5 in A431 cells stimulated with EGF (Fig. S5*C*), and over pYSTAT6 in HeLa cells stimulated with IL-4 (Fig. S5*D*). In all of these experiments, pYSTAT3 abundance was increased following GFP–scFv17 expression with or without stimulant, whereas GFP–scFv17 expression had no effect on the abundance of the other pYSTATs.

Intrabody 17 Blocks the Nuclear Translocation of pYSTAT3 and Expression of APR Proteins in HepG2 Cells Stimulated with IL-6 as Well as in Mouse Liver Stimulated with IL-6 or LPS. The effect of intrabody expression on the IL-6–induced nuclear translocation of STAT3 in HepG2 cells was examined by immunofluorescence analysis with antibodies to STAT3 or pYSTAT3. For unstimulated cells expressing either GFP or GFP–scFv17, most immune reactivity for anti-STAT3 was detected in the cytosol, with only a small amount apparent in the nucleus (Fig. 2*A*). Stimulation with IL-6 resulted in a marked increase in anti-STAT3 immunoreactivity in the nucleus in GFP-expressing cells but not in GFP–scFv17-expressing cells (Fig. 2*A*). Immunoreactivity for anti-pYSTAT3 was detected predominantly in the nucleus of IL-6–stimulated GFP-expressing cells but was apparent in the cytosol of unstimulated or IL-6–stimulated GFP–scFv17-expressing cells (Fig. 2*B*). We also examined the intracellular distribution of STAT3 and pYSTAT3 by immunoblot analysis of whole cell lysates as well as of separated nuclear and cytosolic fractions (Fig. 2*C*). In agreement with the immunofluorescence results, pYSTAT3 generated in GFP-expressing cells in response to IL-6 stimulation was found exclusively

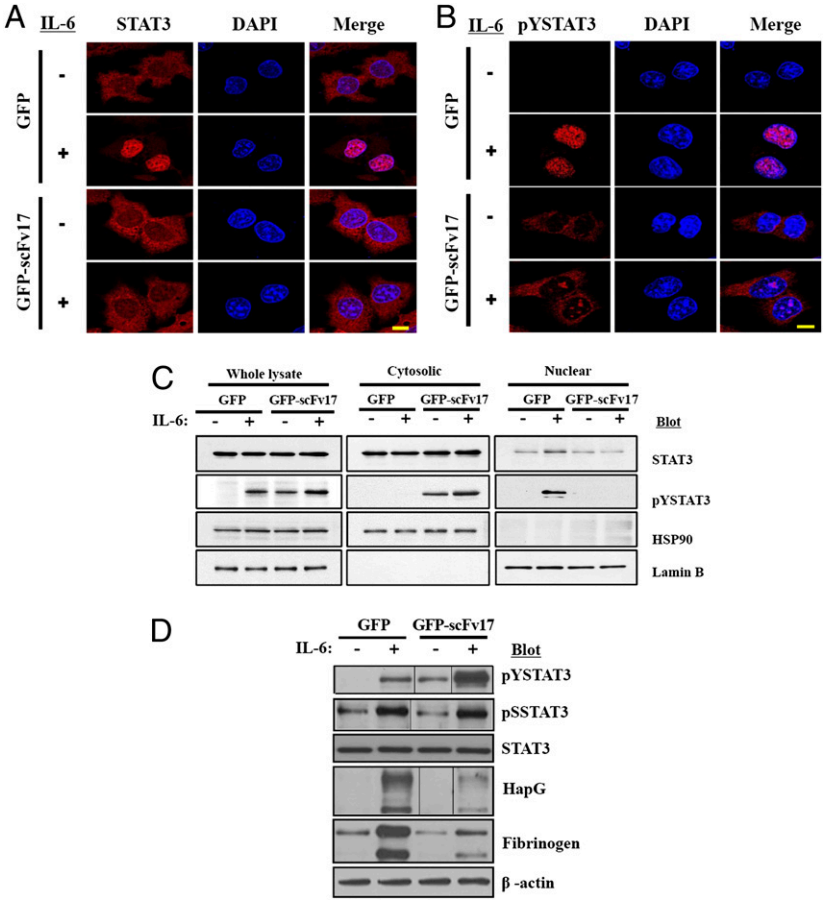

Fig. 2. GFP–scFv17 blocks the nuclear translocation of pYSTAT3 and expression of APR proteins in HepG2 cells stimulated with IL-6. (*A* and *B*) HepG2 cells infected for 24 h with adenoviruses encoding GFP or GFP–scFv17 (50 pfu per cell) were incubated in the absence or presence of IL-6 (100 ng/mL) for 30 min and then subjected to immunofluorescence staining with antibodies to STAT3 (*A*) or with those to pYSTAT3 (*B*). Nuclei were also stained with 4′,6-diamidino-2-phenylindole (DAPI). (Scale bars, 10 μm.) (*C*) Whole cell lysates, cytosolic, and nuclear fractions prepared from HepG2 cells infected and stimulated as in *A* and *B* were subjected toimmunoblot analysis with antibodies to the indicated proteins. HSP90 and lamin B were examined as cytosolic and nuclear markers, respectively. (*D*) HepG2 cells infected as in *A* and *B* were incubated in the absence or presence of IL-6 (50 ng/mL) for 30 min, after which cell lysates were subjected to immunoblot analysis with antibodies to pYSTAT3, pSSTAT3, or STAT3 or with those to haptoglobin (HapG), fibrinogen, or β-actin, respectively. The two bands recognized by anti-HapG are the glycosylated and nonglycosylated forms of the β-chain, and the two bands recognized by antifibrinogen are the α- and β-chains. Some lanes are spliced together from the same immunoblot; these alterations are indicated by a vertical line between the lanes.

in the nucleus, whereas that generated in IL-6–stimulated GFP–scFv17-expressing cells remained in the cytosol. Immunoblot analysis with antibodies to STAT3 revealed the IL-6–induced translocation of STAT3 to the nucleus in GFP-expressing cells, whereas such translocation was not evident in GFP–scFv17-expressing cells. These results thus suggested that intrabody 17 is an effective inhibitor of the nuclear translocation of pYSTAT3.

IL-6 induces the production and secretion of APR proteins such as haptoglobin, fibrinogen (α-, β-, and γ-chains), and serum amyloid A proteins (SAA1–SAA3) in hepatocytes (14). The binding of dimeric pYSTAT3 to GAS elements in the promoters of such APR genes plays a key role in their transcriptional induction by IL-6 (1). We next examined the effect of GFP–scFv17 expression on the IL-6–induced production of APR proteins in HepG2 cells. Immunoblot analysis showed that IL-6 markedly increased the production of haptoglobin (glycosylated and unglycosylated β-chains) and fibrinogen (α- and β-chains) in GFP-expressing cells (Fig. 2*D*). Expression of GFP–scFv17 almost completely blocked the induction of these proteins by IL-6, whereas it increased the abundance of pYSTAT3 (Fig. 2*D*). The induction of APR proteins by IL-6 was also blocked by RNAi-mediated knockdown of STAT3 and by the chemical inhibitor stattic (Fig. S6), the latter of which selectively inhibits the function of the SH2 domain of STAT3 (15). Expression of GFP–scFv17 did not affect the serine phosphorylation of STAT3 induced by IL-6 (Fig. 2*D*), whereas stattic did markedly inhibit this effect of IL-6 (Fig. S6; see also Fig. 4*C*).

To test in vivo function of intrabody 17, adenoviruses encoding GFP or GFP–scFv17 were injected through tail vein into mice to achieve overexpression of intrabody 17 in the liver, and then mice were further injected with IL-6 (Fig. 3). As observed in HepG2 cells, the abundance of pYSTAT3 was increased by GFP–scFv17 expression in the livers of mice injected with or without IL-6, and the IL-6–induced nuclear translocation of pYSTAT3 was prevented by GFP–scFv17 expression (Fig. 3*A*).

The expression of APR genes in the liver was measured in mice injected with IL-6 for 0, 2, and 6 h by RT-PCR analyses of the mRNAs for five APR genes: haptoglobin, fibrinogen, and SAA1–SAA3. IL-6 induced increases in the expression of these five genes in GFP-expressing mice (Fig. 3*B*). Expression of GFP–scFv17 almost completely blocked the induction of these five mRNAs (Fig. 3*B*).

In the liver, lipopolysacchride (LPS) activates the resident macrophage Kupffer cells. Activated Kupffer cells produce proinflammatory cytokines including IL-1β, IL-6, and tumor necrosis factor α (TNFα) (16), which results in increased expression of APR genes in liver. The effect of intrabody 17 on the expression of APR genes in the liver was measured in GFP- or GFP–scFv17-expressing mice after injection of LPS for 0, 8, and 16 h. As in the case of IL-6 injection, LPS injection induced the nuclear translocation of pYSTA3 and the expression of the five APR genes in GFP-expressing mice (Fig. 3 *C* and *D*), and the expression of intrabody 17 blocked the LPS-induced nuclear translocation of pYSTAT3 as well as the LPS-induced expression of hepatic APR genes. We noted, however, that the pattern of APR down-regulation resulting from the expression of the intrabody differs significantly between IL-6– and LPS-injected mice. Whereas expression of all five hepatic APR genes was nearly completely suppressed in IL-6–injected mice, their expression was inhibited to widely varying extents in LPS-injected mice. Haptoglobin, fibrinogen, and SAA1–SAA3 expression levels were inhibited by ∼80%, ∼95%, ∼50%, ∼70%, and ∼15%, respectively, when measured 8 h after LPS injection (Fig. 3*D*). These results provide a striking illustration of the utility of the intrabody in evaluating the differential impact of pYSTAT3 on the expression of distinct genes in response to different stimuli.

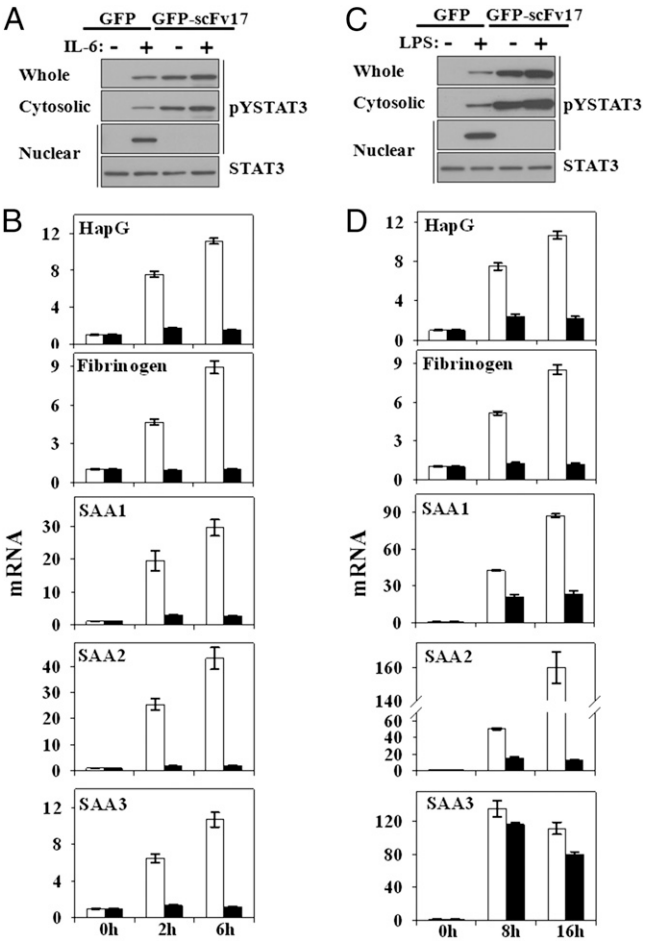

Fig. 3. GFP–scFv17 blocks the IL-6– or LPS-induced expression of APR genes in mice. (*A* and *C*) Adenoviruses encoding GFP or GFP–scFv17 were injected into mice, and then mice were further injected with either IL-6 (*A*) or LPS (*C*), and their livers were collected at indicated times. Whole liver lysates, cytosolic, and nuclear fractions prepared from the liver were subjected to immunoblot analysis with antibodies to pYSTAT3 or STAT3. (*B* and *D*) Total RNA prepared from the liver treated as in *A* (*B*) or in *C* (*D*) was subjected to quantitative RT-PCR analysis for determination of the relative amounts of haptoglobin (HapG), fibrinogen, and SAA1–SAA3 mRNAs using specific primers listed in Table S1 in GFP- (open bars) or GFP–scFv17 (solid bars)-expressing mice. Data are means ± SD (n = 4).

Intrabody 17 Does Not Affect Induction of c-Fos Gene Expression by PMA in HepG2 Cells. Transcription of the c-Fos gene is markedly increased by phorbol 12-myristate 13-acetate (PMA) in HepG2 cells (17). Treatment of HepG2 cells with PMA was also previously shown to result in the accumulation of pSSTAT3 but not in that of pYSTAT3 (17). The upstream regulatory region of the c-Fos gene contains several regulatory sequences including the sis-inducible element (SIE), which is thought to bind pSSTAT3 (17). The SIE does not resemble a canonical STAT binding site, however, and binding of pSSTAT3 to this element might require its interaction with other nuclear proteins that are bound at the gene promoter. Nevertheless, pSSTAT3 alone was found to be sufficient to induce the accumulation of c-Fos mRNA (18). PMA activates mitogen-activated protein kinsae (MAPK) signaling, and PMA-induced pSSTAT3 accumulation is sensitive to the inhibition of MAPK kinase (MEK) by PD98059, suggesting that extracellular signal-related kinase (ERK), a member of the MAPK family, is responsible for STAT3 phosphorylation on serine (17–19).

We investigated the effect of GFP–scFv17 on the PMA-induced expression of the c-Fos gene in HepG2 cells. Consistent with previous observations (17), PMA induced the accumulation of pSSTAT3, but not that of pYSTAT3, and this pSSTAT3 accumulation was inhibited by the MEK inhibitor PD98059 (Fig. 4*A*). The increase in the abundance of pYSTAT3 induced by GFP–scFv17 expression was not affected by PMA alone or together with

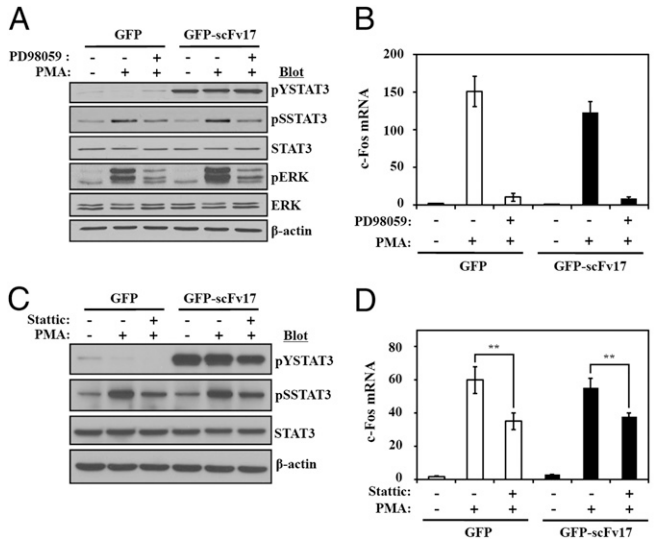

Fig. 4. Effect of GFP–scFv17 expression on PMA-induced pSSTAT3 accumulation and c-Fos gene expression in HepG2 cells. (*A* and *B*) HepG2 cells infected with adenoviruses encoding GFP or GFP–scFv17 (50 pfu per cell) were incubated with or without PD98059 (50 μM) or PMA (100 ng/mL) for 30 min, after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins (*A*) or the relative amount of c-Fos mRNA in the cells was measured by reverse transcription (RT) and real-time PCR analysis (*B*). The mRNA data are means ± SD from three independent experiments. (*C* and *D*) HepG2 cells infected as in *A* and *B* were incubated with or without stattic (100 μM) or PMA (100 ng/mL) for 30 min, after which cell lysates were subjected to immunoblot analysis (*C*) or the relative amount of c-Fos mRNA in the cells was determined (*D*). The mRNA data are means ± SD from three independent experiments. $^{**}P < 0.01$.

PD98059 (Fig. 4*A*). PMA induced a pronounced increase in the amount of c-Fos mRNA in GFP-expressing HepG2 cells, and this effect was largely abolished by PD98059 (Fig. 4*B*). Expression of GFP–scFv17 inhibited the PMA-induced accumulation of c-Fos mRNA by only ∼20% (Fig. 4*B*), with this effect likely being attributable to the retention of some pSSTAT3 molecules in the cytosol by GFP–scFv17 as a result of their also being phosphorylated on tyrosine. Stattic markedly inhibited PMA-induced pSSTAT3 accumulation in both GFP- and GFP–scFv17-expressing HepG2 cells, whereas GFP–scFv17 expression did not affect serine phosphorylation of STAT3 (Fig. 4 *B* and *C*). In accordance with these results, stattic inhibited the PMA-induced accumulation of c-Fos mRNA by a greater extent, ∼40% in GFP-expressing cells and ∼30% in GFP–scFv17-expressing cells, than did GFP–scFv17 expression (∼10% in these experiments) (Fig. 4*D*).

Intrabody 17 Does Not Affect the Abundance of Mitochondrial pSSTAT3 in PMA-Stimulated HepG2 Cells. In addition to its presence in the cytosol and nucleus, STAT3 is also found in mitochondria of primary tissues and cultured cells (6, 7). In mouse liver and heart, for example, ∼10% of STAT3 is localized to mitochondria (6). Mitochondrial STAT3 is required for optimal electron transport function of complexes I and II, and it supports Ras-dependent malignant transformation, with these mitochondrion-specific functions being dependent on pSSTAT3 (6–8). We therefore examined the effect of GFP–scFv17 expression on mitochondrial STAT3 by isolating mitochondrial and cytosolic fractions from PMA-stimulated HepG2 cells. The abundance of STAT3 in the mitochondrial fraction was affected by neither PMA treatment nor GFP–scFv17 expression (Fig. 5). The abundance of pSSTAT3 in mitochondria, although relatively low, was increased by PMA treatment but was not affected by GFP–scFv17 expression (Fig. 5), suggesting that the mitochondrial localization and serine phosphorylation of STAT3 are independent of its tyrosine-phosphorylation–related functions.

Effect of Intrabody 17 Expression on Cell Viability and Gene Expression. Earlier studies with tissue-specific STAT3-deficient mice indicated that in most cell types STAT3 activation leads to suppression of apoptosis, and STAT3 inhibition by stattic or RNAi-mediated knockdown resulted in decreased cell viability. We evaluated the effect of GFP–scFv17 expression, stattic, and RNAi knockdown on the viability of HepG2 cells maintained in DMEM media supplemented with 10% FBS (Fig. S7). Cell viability was markedly reduced by stattic (∼35% and ∼90% at 2 μM and 20 μM, respectively) and STAT3 knockdown (∼60%) but reduced by less than 10% by GFP–scFv17 expression, demonstrating a differential role of pYSTAT3 in cell survival.

Finally, we identified genes whose expression levels were significantly changed by GFP–scFv17 expression or RNAi knockdown of STAT3, compared with expression in their respective controls in IL-6–stimulated HepG2 cells (Fig. S8 and Dataset S1). In IL-6–stimulated HepG2 cells, intrabody expression and STAT3 knockdown affected the mRNA levels of 35 genes and 146 genes, respectively, with 23 genes common to the two groups.

Discussion

STAT3 is a cellular regulator that exhibits functional versatility. Transcriptional activity of STAT3 independent of pYSTAT3 was demonstrated by mutation of Tyr^{705} to Phe (Y705F) and expression of the mutant protein in STAT3-null cells, which resulted in a more than twofold change in the abundance of >1,000 mRNAs (20). In addition to growth factors and cytokines, STAT3 is activated by oncoproteins and carcinogens. It is capable of inducing cell transformation and tumorigenesis, with STAT3 target genes being implicated in many processes associated with tumorigenesis, including cell proliferation, migration, and invasion as well as apoptosis, inflammation, and angiogenesis (2). These effects of STAT3 are mediated in part through its interaction with many other proteins, with ∼40 such proteins including other transcriptional factors, cytosolic proteins, and mitochondrial proteins having been identified (3). The functions of pYSTAT3, pSSTAT3, and USTAT3 thus depend on the extracellular stimulus, cell type, and activation status of the cell, with the role of each STAT3 species in a given biological process having been difficult to evaluate.

Approaches based on gene targeting or RNAi, which entirely remove the protein target from a cell, are not informative for evaluation of the function of multiple modified forms of a protein like STAT3. Expression of mutant forms of STAT3 such as Y705F or S727A can provide insight but requires elimination of endogenous STAT3 in the cells under study. Intrabodies have been developed, especially as therapeutic agents, for neutralization of the function of target proteins (21). The utility of this approach was recently highlighted by the preparation

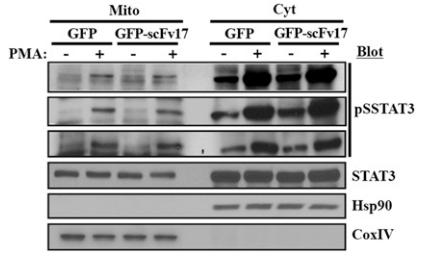

Fig. 5. Effect of GFP–scFv17 expression on PMA-induced accumulation of pSSTAT3 in mitochondria. HepG2 cells infected with adenoviruses encoding GFP or GFP–scFv17 (50 pfu per cell) were incubated in the absence or presence of PMA (100 ng/mL) for 30 min, after which mitochondrial (Mito) and cytosolic (Cyt) fractions were prepared from the cells and subjected to immunoblot analysis with antibodies to the indicated proteins. Blots of pSSTAT3 from three independent experiments are shown. HSP90 and cytochrome oxidase IV (CoxIV) were probed as cytosolic and mitochondrial markers, respectively.

of an intrabody that recognizes and stabilizes the conformation of the inactive form, but not the active form, of protein tyrosine phosphatase 1B, in an attempt to enhance insulin signaling (22). Intrabodies are also an attractive option for neutralizing the function of a particular modified form of a protein that is covalently modified at multiple sites, given that it is theoretically possible to develop an intrabody that binds to an individual modified site with high specificity, but which spares the function of others. This possibility has not previously been tested, however.

In the present study, we isolated a phage clone that produces a Fab (Fab17) that binds specifically to the pYSTAT3 peptide. The scFv–Fc17 fusion protein derived from the Fab17 clone was found to bind to the pYSTAT3 peptide with high affinity (K_d = 76 pM) and to react with pYSTAT3 selectively over USTAT3, pYSTAT1, and other tyrosine-phosphorylated proteins in lysates of IL-6–stimulated HepG2 cells. The effect of shielding the region of STAT3 containing phosphorylated Tyr^{705} was examined by expressing the intrabody derived from scFv–Fc17 fusion protein as a GFP fusion protein (GFP–scFv17) in HepG2 cells with the use of an adenoviral vector. Expression of intrabody 17 blocked the IL-6–induced translocation of pYSTAT3 to the nucleus as well as the IL-6–induced production of hepatic APR proteins (haptoglobin and fibrinogen) that are encoded by genes with promoters containing the canonical pYSTAT3 binding element (GAS). In contrast, GFP–scFv17 expression affected neither the accumulation of pSSTAT3 in response to IL-6 or PMA nor PMA-induced expression of the c-Fos gene. These observations are consistent with the previous suggestion that pSSTAT3 modulates c-Fos gene transcription by binding to the SIE located in the promoter region (17). Given that intrabody expression did not affect the abundance of pSSTAT3 in mitochondria of PMA-stimulated HepG2 cells, it also likely does not influence the mitochondrial function of STAT3, which has been shown to depend on pSSTAT3. Expression of the intrabody thus selectively blocks signaling downstream of pYSTAT3 without affecting pSSTAT3-dependent pathways. This pattern of inhibition differs from that of stattic, which has been thought to inhibit the function of pYSTAT3 but not that of pSSTAT3, given that it binds to the SH2 domain of STAT3, but which in the present study was found to inhibit pSSTAT3 accumulation in cells stimulated with IL-6 or PMA.

The utility of intrabody 17 in vivo was tested by expressing it in the livers of mice through tail vein injection of GFP–scFv17 adenoviruses followed by further injection of IL-6 or LPS. Intrabody expression down-regulated LPS- or IL-6–induced induction of APR products in the livers. However, the pattern of APR down-regulation differed significantly between IL-6– and LPS-injected mice. Whereas the expression of haptoglobin, fibrinogen, and SAA1–SAA3 was nearly completely suppressed in IL-6–injected mice, their expression was inhibited to widely varying extents (15–95%) in LPS-injected mice. These results provide an example of how the intrabody can be used in evaluating the differential impact of pYSTAT3 on the expression of distinct genes in response to various cytokines and growth factors. Indeed, an array-based analysis revealed that IL-6–induced gene expression pattern in GFP–scFv17-expressing HepG2 cells is quite different from that in STAT3-knockdown HepG2 cells, with the number of changes being much less in the intrabody-expressing cells. In another example for the utility of the intrabody, we showed that the viability of HepG2 cells is not significantly affectd by GFP–scFv17 expression, whereas the viability is drastically decreased when STAT3 function was inhibited by stattic or RNAi-mediated knockdown.

An undesirable effect of the tight binding of GFP–scFv17 to the tyrosine-phosphorylated site of pYSTAT3 was the accumulation of pYSTAT3 in the absence of external stimulation. The accumulation of pYSTAT3 was proportional to the level of intrabody expression. When the level of intrabody expression is high, this signal-independent accumulation of pYSTAT3 can thus result in the sequestration of a substantial proportion of STAT3 molecules in the cytosol and in their uncoupling from the pool sensitive to signal inputs. Despite this shortcoming, intrabodies are an attractive option for selective inhibition of the function of one specific covalently modified form of a protein among multiple such forms, because of the possibility of developing high-affinity binders and their intrinsic specificity. For a protein like STAT3, which functions in several different compartments of the cell, intrabodies can also be targeted to the compartment of interest. Intrabodies thus have great potential to increase our understanding of the cellular functions of distinct modified forms of a given protein in living cells.

Materials and Methods

Antibodies to pYSTAT3, pSSTAT3, STAT3, pYSTAT1, STAT1, and to phosphorylated or total forms of ERK were obtained from Cell Signaling Technology; those to haptoglobin, fibrinogen, and β-actin were from Sigma-Aldrich; and those to phosphotyrosine (4G10) were from Upstate Biotechnology. Recombinant human IL-6 and antibodies to GFP were obtained from Abfrontier, and DAPI was from Roche Applied Science.

Details of cell culture, immunoprecipitation, statistical analysis, animal treatments, and antibody (Fab, scFv–Fc fusion protein, and intrabody) generation are given in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This study was supported by grants from the Korean Science and Engineering Foundation (National Honor Scientist program Grant 2006-05106 and Bio R&D Program Grant M10642040001-07N4204-00110 to S.G.R.).

1. Darnell JE, Jr. (1997) STATs and gene regulation. *Science* 277(5332):1630–1635.
2. Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: A leading role for STAT3. *Nat Rev Cancer* 9(11):798–809.
3. Aggarwal BB, et al. (2009) Signal transducer and activator of transcription-3, inflammation, and cancer: How intimate is the relationship? *Ann N Y Acad Sci* 1171:59–76.
4. He G, Karin M (2011) NF-κB and STAT3: Key players in liver inflammation and cancer. *Cell Res* 21(1):159–168.
5. Decker T, Kovarik P (2000) Serine phosphorylation of STATs. *Oncogene* 19(21): 2628–2637.
6. Wegrzyn J, et al. (2009) Function of mitochondrial Stat3 in cellular respiration. *Science* 323(5915):793–797.
7. Gough DJ, et al. (2009) Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science* 324(5935):1713–1716.
8. Reich NC (2009) STAT3 revs up the powerhouse. *Sci Signal* 2(90):pe61.
9. Liu L, McBride KM, Reich NC (2005) STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-alpha3. *Proc Natl Acad Sci USA* 102(23): 8150–8155.
10. Timofeeva OA, et al. (2012) Mechanisms of unphosphorylated STAT3 transcription factor binding to DNA. *J Biol Chem* 287(17):14192–14200.
11. Ogata A, et al. (1997) IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. *J Immunol* 159(5):2212–2221.
12. Stahl N, et al. (1995) Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science* 267(5202):1349–1353.
13. Haan S, Keller JF, Behrmann I, Heinrich PC, Haan C (2005) Multiple reasons for an inefficient STAT1 response upon IL-6-type cytokine stimulation. *Cell Signal* 17(12): 1542–1550.
14. Baumann H, Gauldie J (1994) The acute phase response. *Immunol Today* 15(2):74–80.
15. Schust J, Sperl B, Hollis A, Mayer TU, Berg T (2006) Stattic: A small-molecule inhibitor of STAT3 activation and dimerization. *Chem Biol* 13(11):1235–1242.
16. Morrison DC, Ryan JL (1987) Endotoxins and disease mechanisms. *Annu Rev Med* 38: 417–432.
17. Yang E, Lerner L, Besser D, Darnell JE, Jr. (2003) Independent and cooperative activation of chromosomal c-fos promoter by STAT3. *J Biol Chem* 278(18):15794–15799.
18. Sengupta TK, Talbot ES, Scherle PA, Ivashkiv LB (1998) Rapid inhibition of interleukin-6 signaling and Stat3 activation mediated by mitogen-activated protein kinases. *Proc Natl Acad Sci USA* 95(19):11107–11112.
19. Hodge C, et al. (1998) Growth hormone stimulates phosphorylation and activation of elk-1 and expression of c-fos, egr-1, and junB through activation of extracellular signal-regulated kinases 1 and 2. *J Biol Chem* 273(47):31327–31336.
20. Yang J, Stark GR (2008) Roles of unphosphorylated STATs in signaling. *Cell Res* 18(4): 443–451.
21. Lobato MN, Rabbitts TH (2003) Intracellular antibodies and challenges facing their use as therapeutic agents. *Trends Mol Med* 9(9):390–396.
22. Haque A, Andersen JN, Salmeen A, Barford D, Tonks NK (2011) Conformation-sensing antibodies stabilize the oxidized form of PTP1B and inhibit its phosphatase activity. *Cell* 147(1):185–198.