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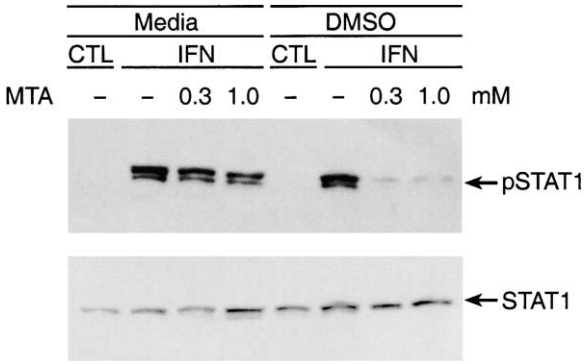

Figure 1. Effect of MTA on STAT1 Tyrosine Phosphorylation

MTA was dissolved either in DMSO or directly in tissue culture medium. MTA was added to the cells 1 hr prior to stimulation with IFNβ (1,000 u/ml) for 30 min. Cell lysates were probed with p(Y701)STAT1 or STAT1 antisera.

Response to Matters Arising

In their Matters Arising article, Meissner et al. present several experiments that call into question our conclusions from the results reported in our original manuscript (Mowen et al., 2001). We clearly acknowledge the importance of these new findings, but at the same time have reservations about the extent to which these results invalidate our conclusions of STAT1 arginine methylation.

We had employed monoclonal antibodies against dimethylarginine (DMA) to demonstrate methylation of STAT1 in vivo. Since the antibody was ineffective in Western blots, we performed immunoprecipiations, followed by anti-STAT1 immunoblotting. Specificity of the antibodies was demonstrated by the fact that only a methylated STAT1 N-terminal domain could successfully compete for binding to the DMA antibody. Other labs have in the meantime reproduced these findings for STAT1, STAT3, and STAT6 (Chen et al., 2004; Duong et al., 2004; Rho et al., 2001). However, despite the use of high-stringent immunoprecipitation condition, coimmunoprecipitation of STAT proteins with associated, arginine-methylated proteins can naturally not be definitively excluded in these experiments. However, Rho et al. reported the arginine methylation of STAT3 (Rho et al., 2001). This study not only used the DMA antibody for immunoprecipitation followed by STAT3 Western blotting but also successfully employed the DMA antibody for Western blotting of STAT3 immunoprecipitates to demonstrate STAT3 arginine methylation (Rho et al., 2001, Figure 2).

In the metabolic labeling experiments using L-[*methyl*-^{3}H]methionine, the incorporation of radioisotope is not restricted to arginine residues, as lysines as well as COOH termini can also be methylated. It would therefore be helpful to know whether the authors were able to detect radioisotope incorporation into other proteins known to be arginine methylated on a single residue (e.g., Sam68, CBP, EWS).

Dr. Vinkemeier's group further reports that in their hands the methylation inhibitor MTA blocks tyrosine phosphorylation of STAT1, which is in striking contrast to our findings. The authors further state that "… the same observations were also made for STAT6 (Chen et al., 2004)." Meissner et al. incubated the cells with MTA in DMSO, whereas we had solubilized MTA in tissue culture media. While somewhat unexpected, this difference in solvents used seems to make a significant difference (see Figure 1): when dissolved in DMSO, MTA does indeed block STAT1 tyrosine phosphorylation. However, when MTA is dissolved in media (as was done in our original studies to completely block IFNα/β-mediated ISG induction), no inhibition of STAT1 tyrosine phosphorylation is observed. While we cannot offer an explanation for these different, solvent-dependent effects of MTA, the finding nevertheless invalidates the notion that mere inhibition of STAT1 tyrosine phosphorylation accounts for the inhibitory effects of MTA on IFNα/β-induced gene transcription. In addition, the paper reporting STAT6 arginine methylation quoted in this context by the authors clearly shows that STAT6, *but not STAT1* tyrosine phosphorylation is inhibited by the methyltransferase inhibitors (Chen et al., 2004: Figure 6B). Lastly, MTA-mediated inhibition of STAT1 tyrosine phosphorylation cannot explain the increased PIAS association with hypomethylated STAT1 we and others have observed (Duong et al., 2004). The fact that MTA also blocks an NF-κB luciferase does not justify the conclusion that it is a nonspecific transcriptional inhibitor. The inhibitory effect of MTA on LPS-mediated NF-κB activation has been previously reported; however, the same paper provides clear evidence for significantly increased IL-10 production under these conditions (Hevia et al., 2004). We have also shown in our manuscript that c-*fos* induction by serum is not affected by MTA. Lastly, in a follow-up paper published in 2002 we had used different methylation inhibitors, which like MTA caused abrogation of IFNα/β-induced transcription without blocking STAT1 tyrosine phosphorylation (Zhu et al., 2002).

Our own mass spectrometry data, which had identified peptides of the appropriate molecular mass (Supplemental Figure S1 at http://www.cell.com/cgi/content/full/119/5/589/DC1/), and the concurring results we had obtained from the mutational analysis of STAT1 R31 may have mislead us into the conclusion that R31

is the site of methylation. The alternative interpretation of the results we had obtained with the STAT1 R31 mutants as offered by Dr. Vinekmeier's group is certainly compelling (although it does not disprove STAT1 methylation). It is nevertheless surprising that STAT1 R31 mutants or NH2-terminal deletions act as gain-of-function mutants in the presence of endogenous wild-type STAT1 (Mowen et al., 2001; Shuai et al., 1996).

In summary, we concur that the new evidence presented by Meissner et al. clearly merits further investigations and a re-evaluation of our initial conclusions from our results. The alternative interpretations of the data obtained from our original studies (e.g., R31 mutants, MS) offered here do call into question whether STAT1 is methylated on R31. However, it is important to note that arginine methylation of STAT1 at a different residue cannot be excluded on the basis of the presented experiments.

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