RESEARCH ARTICLE **(Open Access)**

STAT1 plays an important role in the cell cycle

GLORIA DIMCO[1], RICHARD A. KNIGHT[2], DAVID S. LATCHMAN[2], ANASTASIS STEPHANOU[2]

[1]Medical Molecular Biology Unit, Institute of Child Health, University College London, London, UK

*Corresponding author; E-mail: gloriadimco@gmail.com

Abstract

The STAT protein family members are among the best studies of the latent cytoplasmic signal-dependent transcription factors that play important roles in development, cell growth and homeostasis. Recently, we have reported that STAT1 and STAT3 have opposing effects in apoptosis in cardiac myocytes exposed to ischaemia/reperfusion injury, which are dependent on the C-terminal phosphorylation domains of these factors. However, very little is known about the factors that interact with phosphorylated STAT1 or STAT3 in the heart, which may mediate their antagonistic effects. Thus, in the present proposal we will use mass spectrometry MALDI-TOF-base proteomic technology to identify novel binding partners for the activated forms of STAT1 or STAT3 in the ischaemic heart. The proposed studies will enable us to characterize novel factors involved in STAT1 and STAT3 protein interactions and may lead to better understanding of the STAT-dependent transcriptional pathways in the stress-induced myocardium and cell cycle.

Keywords: STAT1, STAT1-GST, Emi1, IFNγ, cancer.

1. Introduction

STATs are a family of latent cytoplasmic transcription factors that can be activated by a variety of tyrosine kinases in response to different cytokines, growth factors, and peptide ligands binding to their respective cell surface receptors (2, 11). These proteins have dual roles as cytoplasmic signalling proteins and nuclear transcription factors that activate many genes fundamental in development, cell growth, proliferation, apoptotic cell death, angiogenesis and immune responses (12, 13) by either binding directly to DNA or acting in conjuction with co-activator proteins. The signal transducer and activator of transcription 1 (STAT1) protein is essential in IFNγ signalling. Binding of IFNγ to its receptor results in Janus kinase-mediated phosphorylation of a specific tyrosine (Tyr-701) residue in the C-Terminal transcriptional domain of STAT1 (9). STAT1 is further phosphorylated, at least in part by p38 MAPK (3) and ERK pathway (8), on serine (Ser-727) residue and this process has been suggested to enhance its maximal transcriptional activity (14). The transcriptional activity of all STATs depends mainly on the carboxy-Terminal TAD that binds co-activators. It is well established that in addition to tyrosine, serine phosphorylation within the TAD promotes transcriptional activity and enhances the expression of selected genes (4, 8). STAT1 Ser-727 phosphorylation is mediated by several kinases including the ERK pathways (8) and p38 mitogen-activated protein kinase (p38 MAPK) (3) and phosphorylation of both tyrosine and serine residues appears to be required for maximal STAT1 transcriptional activity (14). Similarly, the MEK kinase-1-ERK activated pathway has also been shown to phosphorylate STAT3 on Ser-727 (1). The stress activated JNK kinase pathway also induces serine phosphorylation of STAT3 in Ser-727 although this seems to negatively regulate tyrosine phosphorylation and DNA binding and transcriptional activities of STAT3 in a JNK specific manner (10). In this study, in order to identify additional novel proteins that interact with activated STAT1, we performed GST pull-down assays in un-treated or IFNγ treated $STAT1^{-/-}$ MEF cells expressing STAT1-GST, followed by analysis of interacting proteins using MALDI Q-TOF Mass Spectrometry. A mass map was generated in order to identify the protein of interest by searching MS-Fit protein sequence database. Emi1 was identified as a potential STAT1 interacting partner. Therefore, it was hypothesized that the identification of other novel STAT1 binding partners may give further insight in STAT1 transcriptional and non-transcriptional roles in development, cell growth and homeostasis. Most of the results report on novel STAT1 interacting proteins.

2. Material and Methods

Cell culture and reagents. MEFs wild-type (+/+) and STAT1 knockout (-/-) were kindly provided by David E. Levy and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Human IFNγ was purchased from Sigma (UK). Rabbit anti-STAT1 antibody was purchased from Santa Cruz Biotechnology. STAT1-pGEX-5X-2 was a gift from Dr. Behrmann (Insitut für Biochemie, Germany).
Detection of proteins following SDS-PAGE. Coomassie staining. The SDS-PAGE mini gel was transferred from the electrophoresis apparatus and incubated with 0.2% Coomassie gel stain (0.2% (w/v) Coomassie, 40% (v/v) methanol, 10% (v/v) acetic acid) for 30 minutes at room temperature with continuous shaking.
The proteins were visualized by de-staining with Coomassie destain (50% (v/v) methanol, 10% (v/v) acetic acid) overnight with continuous shaking until the protein bands appeared blue and the background staining had gone. The de-staining procedure involved several changes of the Coomassie destain solution. However, the number of changes was reduced by placing a piece of absorbent paper in the corner of the tray to absorb the Coomassie blue stain left in the matrix. The gel was then dried on a gel dryer at 60-80ºC for 30 minutes and kept as a permanent record. ***Silver staining.*** Following electrophoresis, analytical gels were incubated in a solution containing methanol: acetic acid: ddH_2O (50: 10: 40 v/v) for at least 1 hour. The gel was then sensitised in a solution containing 6.8% (w/v) sodium acetate, 5% (w/v) sodium thiosulfate and 30% (v/v) ethanol for 30 minutes followed by 3 x 5 minutes washes in ddH_2O.
The gel was then incubated in 0.25% (w/v) silver nitrate containing 0.04% (v/v) formaldehyde for 30 minutes. Gels were then washed 3 x 3 minutes in ddH_2O and the solution was replaced with 2.5% (w/v) sodium carbonate containing 0.02% (v/v) formaldehyde and incubated with gentle agitation until protein spots were visible. The process was terminated by incubating the gel in 5% (v/v) acetic acid for 10 minutes. Gels were scanned immediately using the Bio-Rad GS-800 Densitometer (Bio-Rad, UK).
Matrix-Assisted Laser Desorption Ionisation Waters 'Premier' quadrupole time of flight mass spectrometer (MALDI Q-TOF MS). Peptide analysis. Mass spectrometry was carried out on a MALDI Q-TOF MS instrument, fitted with a reflectron and a 337 nm UV laser (TOF Spec E. MicroMass, Manchester, UK). Peptide analysis were performed in positive ion mode with the following voltages, source 20 Kilo Volts (kV), extraction 19.95kV, focus 16.5 kV, reflectron 25kV and a pulse voltage of 2900V.
Spectra were acquired by averaging over a period of 5 scans of highest signal. Data were acquired in reflectron mode, operating over a mass range of 6000 m/z with matrix suppression set at 650 Daltons (Da) using Mass Lynx software (MicroMass, UK). **MS data analyses.** Data analysis was carried out using MassLynx and BioLynx data analysis software. Database searching and mass mapping studies were performed using Protein Prospector database software ('MS-FIT') at University of San Francisko (http://falcon.ludwig.ucl.ac.uk/mshome3.2.htm). Peptide mass calculations were performed using PAWS proteomic analysis software (http://prowl.rockfeller.edu/).
GST Pull-down assays. Glutathione-S-transferase (GST)-fusion proteins were purified from bacteria by using glutathione-Sepharose beads as instructed by the manufacturer (Amersham Pharmacia). *In vitro* translation reactions were carried out using the TNT T7 system (Promega). Briefly, STAT1-GST fusion proteins or GST alone (control) bound to gluatathione sepharose beads were washed in NENT buffer (100mM NaCl, 1mM EDTA, 20mM Tris pH 8.0, 1% igepal and 0.5% milk powder) after incubation (with rotation) at room temperature for 30 min. Non-specific binding was blocked by incubation in 20% milk powder in NENT buffer for 15 min at room temperature. Following washes, (1x in NENT buffer (without added milk powder) and 1x in Transcription wash buffer (20mM HEPES, 60mM NaCl, 1mM DDT, 6mM $MgCl_2$, 8.2% glycerol, 0.1mM EDTA), the beads were incubated with equal amounts of each of the *in vitro* translated proteins in 100ul of Transcription buffer for 1 h at room temperature with rotation.
Following 5x washes in NENT buffer (without added milk powder) to remove unbound proteins, the beads with interacting proteins were solubilised in SDS loading buffer, heated to 95^0C for 5 min and resolved on a 12% SDS polyacrylamide gel, which was then dried and exposed to radiographic film. The resolved bands were quantified by densitometry and IVT proteins retained by the fusion protein expressed as a percentage of the equivalent amounts of *in vitro* translated proteins (input), which were also run on a similar gel.

3. Results and Discussion

Optimization of conditions for STAT1-GST protein expression. In order to optimize the inducible levels of STAT1-GST in DH5α and BL21 bacterial strains, three different temperatures ranges following induction of STAT1-GST with IPTG at different times were tested prior to analysis of protein-protein interactions. 1.0 OD_{600nm} unit of bacterial cells were resuspended in Laemmli buffer and subjected to Western blotting with anti-STAT1 antibody. The Western blot results revealed that STAT1-GST protein is best expressed in DH5α bacterial strain at 37^0C after 3 hours of IPTG induction.

Assessing minimum protein concentration for the identification of interacting proteins. To obtain information on the minimum concentration of STAT1-GST protein required for successful identification of an interacting protein from an in-gel protease digestion, a concentration gradient of 0.1, 0.2, 0.4, 0.5, 1, 3 and 5µg of BSA was subjected to 12% SDS-PAGE and the protein concentration was approximated by comparative staining against BSA. The results showed that STAT1-GST and GST proteins have an intensity of staining similar to 5µg of BSA. This was confirmed by quantifying the scanned image using ImageQuant software, which gave almost identical pixel intensity for STAT1-GST and GST and 5µg of BSA. From this result, it was therefore concluded that 5µg of protein was sufficient to generate positive protein identification using GST pull-down assay.

Assessment of staining methods for protein visualization. Staining techniques were assessed to identify which staining method gave reproducibility with high sensitivity. 1-D SDS-PAGE was used to assess the sensitivity of two types of staining method. The non-colloidal Coomassie blue (R250) (Sigma, Poole, UK) was compared to silver staining (Sigma, Poole, UK). Coomassie blue staining demonstrated a lower detection limit, while silver staining demonstrated an extremely efficient sensitivity. Thus, silver staining method showed much better sensitivity and would therefore enable the visualization of proteins of considerably lower abundance. Proteins with low abundance are often tightly regulated and so any effects on these proteins could be potentially very important. It was decided that Coomassie blue would not be used for analytical gel staining, but could be used for staining of preparative SDS-PAGE to aid in the generation of peptide mass maps.

Pull-down assay to test for interaction of STAT1-GST with proteins from MEF whole cell lysates. STAT1-GST was used to identify novel interacting proteins with whole cell lysates isolated from MEF cells treated with IFNγ (50ng/ml) at different time points. STAT1-GST fusion protein was prepared by cloning STAT1 cDNA encoding the protein into the pGEX-5X2-2 vector up-stream of a GST sequence (provided by Behrman, JBC, 280:21700, 2005). GST alone was also prepared (negative control) to detect GST specific binding proteins in MEF cells treated with IFNγ by pre-clearing the whole cell lysate. The fusion proteins were expressed as described in materials and methods and immobilized on Glutathionine Sepharose 4B beads. Following 6 washes to remove unbound proteins, the bound complexes were then resolved on a 12% SDS-PAGE. As shown in Figure 1, several non-specific proteins interacted with GST protein alone (lanes 8-12), while many proteins interacted with STAT1-GST fusion protein (lanes 2-6), ranging in size from 16-175kDa (predominant bands are indicated by boxes with their estimated apparent molecular mass). In this way, bands present only in the STAT1-GST pull-downs, but not in the GST pull-downs suggest a number of potential STAT1 interacting proteins present.

Identification of novel STAT1 interacting proteins by MALDI-TOF MS. MS was employed to determine the identity of STAT1 interacting proteins obtained from 1-D SDS-PAGE silver staining. After gel electrophoresis separation of the bound proteins, 6 bands were subjected to in-gel protease digestion with trypsin. The molecular masses of the tryptic peptides of each protein band were measured by MALDI Q-TOF MS and a mass map was generated by Dr. Kevin Mills (Biochemistry Research Group, Clinical and Molecular Genetics Unit, Institute of Child Health). The resulting peptide mass map was used to identify the protein of interest by searching MS-Fit protein sequence database. This program determines the best match between a theoretically constructed map for each protein in the database and the experimentally determined peptide mass map. Typically, a minimum of four peptides are required for positive protein identification. The search resulted in the identification of Emi1 protein (band 2) and five candidate proteins (band 3) as potential STAT1 interacting partners. In addition, STAT1-GST (band 1, positive control), Actin (band 4), Glutathione S-transferase (band 5) and Keratin (band 6) proteins were identified (Figure 2). It should be noted that only Ubiquinone biosynthesis methyltransferase COQ5 and Emi1 proteins sequences present in the MS-Fit database were from mouse, while the other proteins sequences identified

were from rat. It is possible that a higher probability hit might have been achieved if a mouse sequence had been present in the database. However, although peptides were detected by MALDI Q-TOF MS for these novel proteins, it will be necessary to perform GST pull-down assays of these IVT ^{35}S methionine-labelled proteins to confirm that these proteins interact with STAT1.

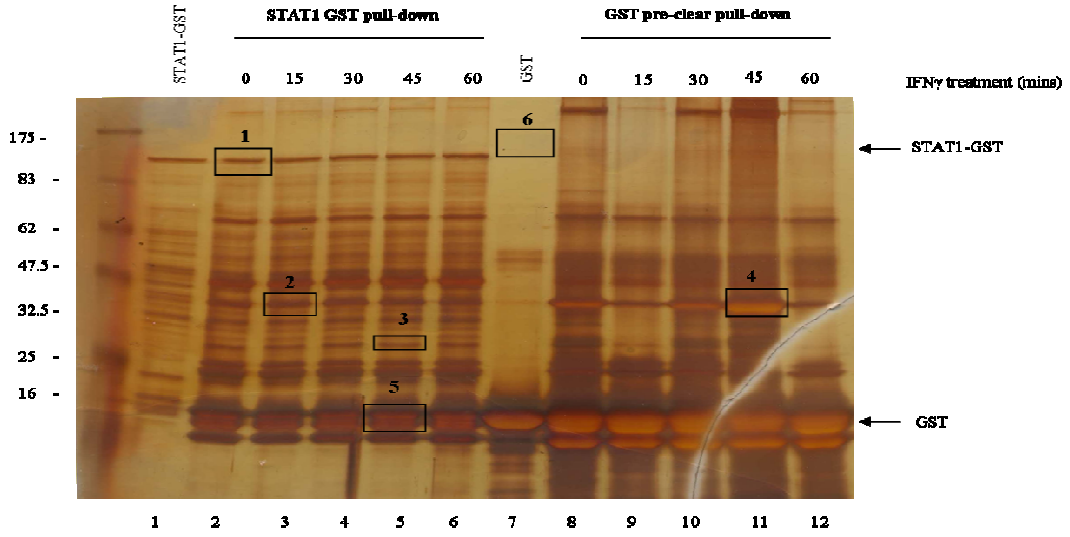

Figure 1. Identification of STAT1-GST and GST binding proteins from MEF whole cell lysates.

GST fusion proteins containing STAT1 and GST alone were purified from *E. coli* using Glutathione Sepharose 4B beads. Cells were treated with IFNγ (50ng/ml) for 0, 15, 30, 45 and 60 minutes. WCL proteins interacting with 5µg of purified STAT1-GST fusion protein and GST protein alone were analyzed by 12% SDS-PAGE and silver staining. Lane 1 and 7 show STAT1-GST and GST proteins alone. Lanes 2-6 show proteins retained following incubation with STAT1-GST fusion protein. Lanes 8-12 show proteins retained following incubation with GST alone. The presence of possible STAT1 interacting proteins are annotated by boxes around specific bands.

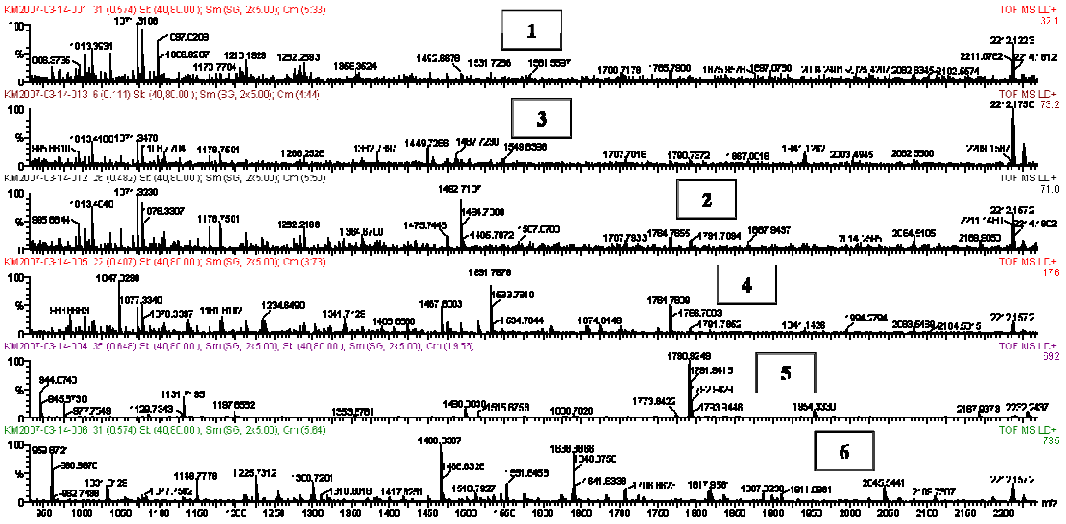

Figure 2. MALDI Q-TOF mass spectrum of the tryptic in-gel digestion of STAT1-GST, PRP18, Emi1, Actin, Glutathione S-transferase and Keratin proteins.

Bands identified to interact with STAT1-GST protein were subjected to 1-D SDS-PAGE followed by in-gel protease digestion with trypsin. A peptide mass map was generated and all proteins were successfully identified using MALDI Q-TOF MS indicating that this method was optimal for the detection of peptide identification from in-gel digestions.

4. Conclusions

The work presented in this study details a second approach to identify additional interacting partners of activated STAT1, using *in vitro* GST pull-down assays from whole cell lysates followed by MALDI Q-TOF Mass Spectrometry. The data generated suggested that 1-D PAGE and silver staining was a preferred technique for the identification of potential STAT1 binding partners. Our data demonstrated that full length STAT1 can interact with Emi1 protein. Since Emi1 is a key regulator of both G1/S transition and mitotic entry by stimulating the accumulation of cyclin A and cyclin B through inhibition of APC/C, these results demonstrate that a functional interaction between STAT1 and Emi1 may have consequences for the prevention of Emi1 activity on APC/C, leading to G1/S cell cycle arrest. Meanwhile, evidence has emerged that significant overexpression of Emi1 is present in different tumors (5, 6). Examination of hEmi1 mRNA levels in a panel of 250 tumors revealed that 30-40% of tumors show a substantial increase in expression compared to matched normal tissue (7). Indeed, hEmi1 overexpression can result in aberrant chromosome segregation, suggesting that hEmi1 overexpression in tumors might contribute to genomic instability by subverting early mitotic events, as well as the balance of the S-phase-promoting transcriptional program (7). Studies have also suggested a requirement for Cdh1 in G1 cell cycle arrest and the DNA damage-induced G2 checkpoint (7). Thus, alterations in hEmi1 expression might be expected to affect the level or timing of APC/C activity, resulting in genomic instability through several mechanisms. Therefore, our finding that STAT1 interacts with Emi1 represents a potential important insight into the molecular pathway underlying tumorigenesis and provide a possible cell cycle model for the development and progression of cancer. Further studies will be necessary to determine whether these correlations indicate a critical function for STAT1 and Emi in tumor progression.

5. Acknowledgements

We greatly appreciate Dr. Behrmann (Institut für Biochemie, Germany) for the STAT1-pGEX-5X-2 construct. We would also like the British Heart Foundation for funding the study (G.G., BHF Ph.D. studentship FS/04/021).

6. References

1. Chung J, Uchida E, Grammer T. C, and Blenis J: **STAT3 serine phosphorylation by ERK-dependent and independent pathways negatively modulates its tyrosine phosphorylation.** Molecular and Cellular Biology 1997, **17**(11):6508-6516.
2. Darnell JE, Kerr IM, Stark GR: **Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins.** Scienc*e* 1994, **264**(5164):1415-1421.
3. David M, Petricoin E. Benjamin C, Pine R, Weber MJ, Larner AC: **Requirement for MAP kinase (ERK2) activity in interferon alpha- and interferon beta-stimulated gene expression through STAT proteins.** Science 1995, **269**(5231):1721-1723.
4. Decker T, Kovarik P: **Serine phosphorylation of STATs.** Oncogene 2000, **19**(21):2628-2637.
5. Guardavaccaro D, Kudo Y, Boulaire J, Barchi M, Busino L, Donzelli M, Margottin-Goguet F, Jackson PK, Yamasaki L, Pagano M: **Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo**. Developmental Cell 2003, **4**(6):799-812.
6. Gutgemann I, Lehman, NL, Jackson PK, Longacre TA: **Emi1 protein accumulation implicates misregulation of the anaphase promoting complex/cyclosome pathway in ovarian clear cell carcinoma.** Modern Pathology 2008, **21**(4):445-454.
7. Hsu JY, Reimann JD, Sorensen CS, Lukas J, Jackson PK: **E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1).** Nature Cell Biology 2002, **4**(5):358-366.
8. Kovarik P, Mangold M, Ramsauer K, Heidari H, Steinborn R, Zotter A, et al: **Specificity of signaling by STAT1 depends on SH2 and C-terminal domains that regulate Ser727 phosphorylation, differentially affecting specific target gene expression.** EMBO Journal 2001, **20**:91-100.

9. Levy DE, Darnell JE Jr: **Stats: transcriptional control and biological impact.** Nature Reviews Molecular Cell Biology 2002, **3**:651-62.
10. Lim CP, Cao X: **Serine phosphorylation and negative regulation of Stat3 by JNK.** Journal of Biological Chemistry 1999, **274**(43):31055-31061.
11. Schindler C, Darnell JE: **Transcriptional responses to polypeptide ligands: the JAK-STAT pathway**. Annual Review of Biochemistry 1995, **64**:621-651.
12. Shuai K, Schindler C, Prezioso VR, Darnell JE, Jr: **Activation of transcription by IFN-gamma: tyrosine phosphorylation of a 91-kD DNA binding protein.** Scienc*e* 1992, **258**(5089): 1808-1812.
13. Yu H, Jove R: **The STATs of cancer--new molecular targets come of age.** Nature Reviews Cancer 2004, **4**(2):97-105.
14. Wen Z, Zhong Z, Darnell JE Jr: **Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation.** Cell 1995, **82**:241-50.