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Immunochemical Identification of a Stat3 Analogue in Streptomycetes

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Dedicated to the memory of Professor Vera Johanides

Summary

The STAT (**s**ignal **t**ransducers and **a**ctivators of **t**ranscription) family of transcription factors are present in species as diverse as mammals, insects, plants and slime moulds. They play key roles in growth factor-regulated signaling pathways. Here we report the identification of a Stat-like protein in the prokaryote *Streptomyces*. Antibodies specific for mouse Stat3 cross-react with proteins in extracts of various *Streptomyces* sp. Furthermore, we demonstrate that, like eukaryotic Stat proteins, the Stat-like protein found in *Streptomyces* is phosphorylated on tyrosine. These results show that the Stats may play roles in cellular regulation in streptomycetes and suggest a bacterial evolution of these proteins.

Key words: *Streptomyces*, Jak-STAT, Stat3, tyrosine phosphorylation, prokaryotic signaling, antibody

Introduction

In all organisms protein phosphorylation plays a cardinal role in the regulation of cellular processes. Many signaling pathways are controlled by alteration in the phosphorylation state of tyrosine, serine, or threonine. Until recently, it was believed that protein phosphorylation on these amino acids was a characteristic of eukaryotic organisms. However, there have been numerous reports of tyrosine phosphorylation in the *Bacteria* and *Archaea*. Such modifications have been demonstrated in association with a variety of developmental changes in different bacterial species *(1)*.

In particular, *Streptomyces* sp. seem to possess many of the signaling molecules that were once thought to exist only in eukaryotes. Numerous serine-threonine protein kinases (STPK) have been cloned from a variety of streptomycetes *(1)*, or uncovered by the genomic sequencing of *Streptomyces coelicolor* A3 *(2)* (D. Hopwood, personal communication). In addition, genes encoding eukaryotic-like phosphatases of various types have been discovered in *Streptomyces (2)*. The eukaryotic-like kinases and phosphatases have *in vitro* kinase or phosphatase activity, and may play roles in bacterial developmental and metabolic processes. Other domains

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involved in eukaryotic signal transduction, such as the SH3, and FHA protein-protein interaction domains have also been found in actinomycetes *(3,4)*. The FHA domains of prokaryotes bind to phosphoserine and phosphothreonine residues, as is the case in eukaryotes *(5)*. Tyrosine phosphorylation on numerous proteins has also been shown in *Streptomyces*, a process which is regulated by the growth phase, changes in culture medium (*6*) and is affected by cAMP levels *(7)*.

The Jak-STAT signaling cascade is a key pathway in eukaryotes in which tyrosine phosphorylation plays a central role. Stats (**s**ignal **t**ransducers and **a**ctivators of **t**ranscription) are a family of latent cytoplasmic transcription factors that are activated by phosphorylation on a single tyrosine residue (near residue 700) in response to extracellular ligands. At least seven mammalian Stats have been identified. Stats 1, 2, 4, and 6 have relatively restricted functions, whereas Stat3 and Stat5 have broader functions. STAT signaling is involved in the regulation of numerous cellular functions such as embryonic development, cell death, and cell migration (reviewed in *8,9)*. An active Stat dimer is formed *via* the reciprocal interactions between the SH2 domain of one monomer and the phosphorylated tyrosine of the other *(10)*. Stat homo- or heterodimers accumulate in the nucleus and activate transcription. The discovery of Stats in *Drosophila (11), Caenorhabditis elegans (12), Anopheles (13), Danio (14), Xiphophorus (15), Dictyostelium discoideum (16)*, and in higher plants *(17)* implies an ancient evolutionary origin for these functions.

We report the presence of a Stat-like protein in *Streptomyces* sp. detected using antibodies specific for the critical tyrosine residues and the surrounding motif of mouse Stat3. This protein was determined to be phosphorylated on tyrosine. These results suggest that a Stat, or »Stat-like« proteins may exist in *Streptomyces*, and raises fundamental questions as to the origin and evolution of the Stat family of proteins.

Materials and Methods

Preparation of cell-free extract

Cultures of *Streptomyces* were grown in S-medium, a rich liquid medium (2 % glucose, 0.4 % yeast extract, 1 % malt extract, 0.3 % glycine, pH=7) inoculated with *Streptomyces* and vigorously shaken at 30 °C until the cells were grown to late-exponential phase. The biomass was then collected by centrifugation at 7 000 x g in a Sorvall GSA rotor (DuPont) at 4 °C, and washed twice with wash buffer (5 mM Tris, pH=7.5, 0.5 mM EDTA, 5 % glycerol, 0.5 mM DTT), resuspended in lysis buffer (50 mM Tris pH=7.5, 150 mM NaCl, 1 % TritonX-100, 1 mM PMSF, 0.1 mM sodium vanadate) and disrupted by sonication. Cell debris was removed by centrifugation at 12 000 x g and the supernatant was used as cell-free extract. Protein concentration was determined by absorbance measurement at 280 nm.

Western blotting

Western blots were carried out by previously described methods (*6*). Anti-phosphotyrosine antibody 4G10 (Upstate biotechnology) used in this study, is highly specific for phospho-tyrosine present in peptides or proteins and it does not react with phosphoserine or phosphothreonine. For Western blotting the antibody was diluted at 1:10 000. Anti-Stat3 (New England Biolabs) recognizes Stat3, and was diluted at 1:1 000. Anti-phosphotyrosine Stat3 (Tyr-705) (New England Biolabs) detects Stat3 only when modified by phosphorylation at tyrosine and was used at a 1:5 000 dilution. Cell lysates of human neurogenic cells (SK-N-MC) treated with or without Ciliary Neurotrophic Factor (CNTF), were used as control cell extracts for the primary antibody. As further controls, identical experiments were performed where 0.1 M O-phospho-L-serine (Sigma) or 0.1 M O-phospho--L-threonine were included in the solution containing anti--pStat3. Similarly 5 mg/mL of the peptide (SAAPYLKTK) was included in the incubation mixture containing anti--Stat3. Immunoblots were also performed where the primary antibody was omitted.

Immunoprecipitation

Cell lysates (1 mg total protein) were incubated with anti-pStat3, anti-Stat3, or 4G10 monoclonal antibodies (1:100 dilution), and immune complexes were collected on protein A-Sepharose beads. The immunoprecipitate beads were then boiled in SDS sample buffer and subjected to SDS-PAGE and Western blotting.

Results

Identification of anti-Stat3, and anti-phosphoStat3 reactive proteins in Streptomyces sp.

Anti-phosphoStat3 antibody (anti-pStat3) is directed against a peptide sequence corresponding to residues 701-709 (SAAPYLKTK) of mouse Stat3 when the Tyr705 is phosphorylated. Using cell-free extracts of *S. coelicolor*, *S. griseus*, and *S. ambofaciens* that were harvested at late--log phase, two proteins migrating at approximately 83 and 75 kDa reacted strongly with the anti-pStat3 antibody. In cell-free extracts obtained from *S. lavendulae* and *S. rimosus* anti-pStat3 detected only one protein of about 75 kD (Fig. 1A). Only one protein also reacted with anti-pStat3 in extracts of *S. capreolus*, migrating at 67 kD which is smaller than that of other strains. No cross-reactive proteins were observed on incubation with secondary antibody alone (data not shown).

To confirm that the anti-pStat3 antibody was reacting with a sequence containing phosphotyrosine, and not phosphoserine or phosphothreonine, an anti-pStat3 immunoblot was performed in the presence of 0.1 M O-phospho-L-serine or 0.1 M O-phospho-L-threonine. No difference in signal pattern or intensity was observed in reactions containing SK-N-MC cell extract or *Streptomyces* cell extracts versus immunoblots with anti-pStat3 antibody alone (data not shown).

Using anti-Stat3 antibody directed against an extended sequence of mouse Stat3 (residues 686-709), that detects this sequence regardless of its phosphorylation state, reactive proteins were identified in all *Streptomyces* tested except *S. capreolus* (Fig. 1B). As in experiments with the anti-phosphoStat3 antibody, Western blotting using anti-Stat3 antibody was performed on extracts of *Streptomyces* grown to late log phase. In *S. coelicolor* and

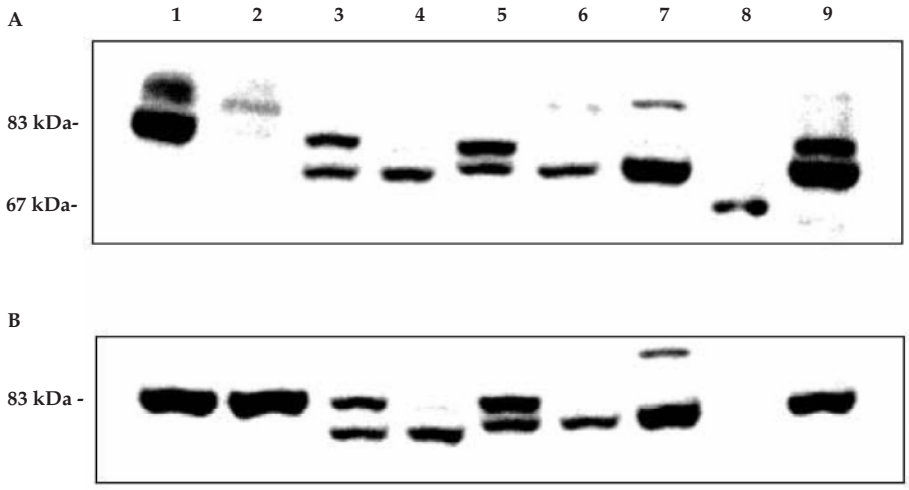

Fig. 1. Western blot analysis of the cell extracts from *Streptomyces* species. The blot was probed with anti-phosphoStat3 antibody (A), or anti-Stat3 antibody (B). The lanes contain the following cell lysates: lane 1, SK-N-MC + CNTF; lane 2, SK-N-MC; lane 3, *S. coelicolor*; lane 4, *S. lavendulae*; lane 5, *S. ambofaciens*; lane 6, *S. rimosus*; lane 7, *S. lividans*; lane 8, *S. capreolus*; lane 9, *S. griseus*.

S. ambofaciens two proteins reacted, one migrating at 83 kDa, and another at about 75 kDa. In *S. lavendulae*, *S. rimosus*, *S. griseus* and *S. lividans* only one strongly cross-reacting protein was observed migrating at about 75 kDa.

All of the cross-reactive protein bands observed in the anti-Stat3 Western blot corresponded with those observed in the anti-pStat3 Western blots. No cross-reactive proteins were observed in anti-pStat3 or anti-Stat3 Western blots of cell extracts of *Escherichia coli* or *Bacillus subtilis* (data not shown).

Anti-pStat3 and anti-Stat3 antibodies precipitate a 75 kDa reactive protein

To establish the specificity of the anti-pStat3 and anti-Stat3 antibodies, immunoprecipitations were performed on late-log extracts of *S. coelicolor* and *S. griseus* using these antibodies and then subjecting these precipitation reactions to Western blotting using the same respective antibodies. Anti-pStat3 antibody precipitated a protein of 75 kDa from both extracts of *S. coelicolor* and *S. griseus* (Fig. 2A). Anti-Stat3 antibody precipitated a protein of the same molecular mass, but to a lesser extent (Fig. 2B).

Anti-pStat3 immunoprecipitates a tyrosine-phosphorylated protein

To confirm that the anti-pStat3 antibody precipitated a protein that was phosphorylated on tyrosine, immunoprecipitations were performed using anti-pStat3 and 4G10 antibodies and subjected to Western blotting with 4G10 or anti-pStat3 antibody, respectively. A protein migrating at the same molecular weight, 75 kDa, was precipitated from *S. griseus* and *S. coelicolor* late-log extracts by anti-pStat3 antibody that cross-reacted with 4G10 antibody (Fig. 3). Furthermore, a protein of the same molecular mass was observed to be precipitated by 4G10 antibody that cross-reacted with anti-pStat3 antibody (Fig. 3).

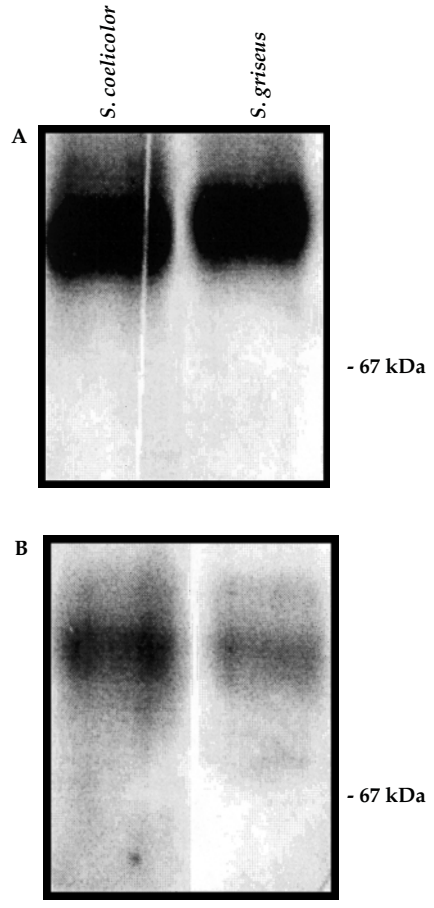

Fig. 2. Western blot analysis of *S. coelicolor* and *S. griseus* cell free extracts immunoprecipitated and subsequently blotted with anti-phosphoStat3 antibody (A) or anti-Stat3 antibody (B)

Anti-pStat3 is specific for the sequence SAAPYLKTK

To establish the specificity of the anti-Stat3 antibody for the sequence SAAPYLKTK, and investigate the possibility that the observed signals could be due to a

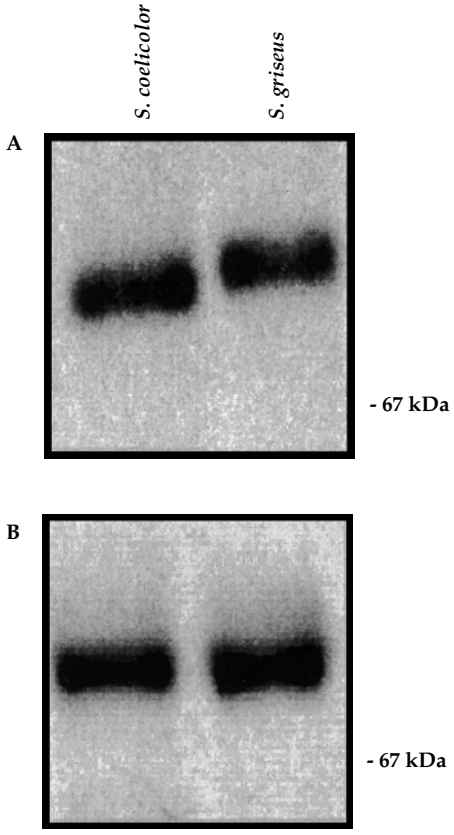

Fig. 3. Western blot analysis of *S. coelicolor* and *S. griseus* cell free extracts immunoprecipitated with anti-phosphoStat3 antibody and blotted with anti-phosphotyrosine antibody (4G10) (A), or immunoprecipitated with anti-phosphotyrosine antibody (4G10) and blotted with anti-phosphoStat3 antibody (B)

non-specific reaction, an immunoblot of *Streptomyces* cell extracts was performed using anti-Stat3 antibody in the presence of the peptide SAAPYLKTK (5 mg/mL). This competition resulted in a total absence of bands in late-log cell extracts of *S. coelicolor*, *S. lividans* and *S. lavendulae* (data not shown).

Discussion

Our studies provide evidence that *Streptomyces* sp. possesses proteins related to eukaryotic Stat3 proteins by immunological cross-reaction with both anti-Stat3 and anti-pStat3 antibody (specific for Stat3 only when the critical tyrosine residue, Tyr 705 in human Stat3, is phosphorylated). Furthermore, we present evidence that this protein is indeed phosphorylated on tyrosine as it was detected by Western blotting with 4G10 antibody following immunoprecipitation from *S. griseus* and *S. coelicolor* by anti-pStat3 antibody, or conversely by experiment of blotting with anti-pStat3 antibody following immunoprecipitation by 4G10 antibody.

Phosphorylation on a single tyrosine residue found near amino acid 700 is obligatory for Stat activation. This phosphorylation results in dimerization of two Stat proteins via reciprocal SH2 mediated interactions. Only following this dimerization can the Stat proteins activate transcription *(8).* We expect that the »Stat3-like« protein from *Streptomyces* sp. possesses a single tyrosine residue, analogous to eukaryotic Stats3, which is critical for its activation.

Western blotting with anti-pStat3 and antiStat3 antibody of cell extracts from *S. coelicolor*, *S. lavandulae*, *S. ambofaciens*, *S. rimosus*, *S. lividans* and *S. griseus* revealed two cross-reactive proteins of approximately 75 kDa and 83 kDa. In higher eukaryotes there are at least seven different Stat family members and multiple splice variants of each protein which share a common carboxy-terminal activating tyrosine residue *(8).* Our results show that *Streptomyces* have Stat3-like proteins similar in the amino acid sequence surrounding a phosphorylated tyrosine. As immunoprecipitations from *S. coelicolor* and *S. griseus* with anti-pStat3 and anti-Stat3 antibody reveal only one reactive protein (75 kDa), the 83 kDa reactive form seen in Streptomycete cell free extracts may be more divergent from mouse Stat3 than the 75 kDa form. Alternatively, since eukaryotic Stat3 is known to undergo additional phosphorylation on serine 727 which causes the protein to be retarded in an SDS-PAGE resulting in the appearance of a Stat3 doublet *(18)*, it is possible the streptomycete STAT3-like protein undergoes two types of post-translational modification similar to its mammalian counterpart; serine phosphorylation is a common theme in prokaryotic signaling.

The differences in size of the various anti-Stat3 reactive proteins among the *Streptomyces* sp. tested is not unexpected given the genomic and proteomic diversity known to exist between different strains of streptomycetes. Signaling proteins are especially dissimilar between even closely related *Streptomyces* sp. Members of the Bld protein family involved in developmental regulation are as little as 20 % identical between different *Streptomyces* species. Other signaling proteins such as AfsK, an STPK from *S. coelicolor* and *S. griseus*, although structurally similar, have different functions *(19).*

Tyrosine kinases and tyrosine phosphorylation of cell signaling proteins are believed to have evolved at the same time as eukaryotic multicellularity. The results from studies of *Myxococcus xanthus*, *Pseudomonas* sp., *Nostoc commune*, *Anabaena* sp., *Mycobacterium tuberculosis*, *Streptomyces* sp., and representatives of *Archaea*, where all three components of a protein tyrosine phosphorylation/dephosphorylation network have been identified, strongly suggest that tyrosine phosphorylation evolved much earlier *(2).* Our results suggest that one process for phosphotyrosine-mediated signaling in *Streptomyces* involves a protein that is related to the STAT family in eukaryotes.

The possible existence of a Stat3 homologue in *Streptomyces* reveals this gene to be of more ancient origin than is currently believed. The discovery of Stat in *Dictyostelium (16),* an organism that lacks the full complement of metazoan signaling pathways, such as src-like or receptor like tyrosine kinases, confirms that the Stat family of proteins have ancient origins. Furthermore, there is evidence of proteins with a Stat-like structure in higher plants *(17).* The *Dictyostelium* Stat and the plant Stat-like proteins both posses SH2 phosphotyrosine binding domains, and are the most primitive such domains known so far. No prokaryotic SH2 domain has so far been identified. Streptomycetes are bacteria which

undergo complex physiological and morphological differentiation cycles, resembling that of multi-cellular organisms such as fungi, or even *Dictyostelium* itself, thus it is not surprising that it possesses a Stat-like protein. The streptomycete Stat-like protein may represent the most ancient setting for an SH2 domain. Furthermore, we suggest that the study of signal transduction processes in the streptomycetes and their relationship to differentiation are likely to be valuable biochemical and evolutionary models for eukaryotic processes.

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Imunokemijska identifikacija Stat3-analoga u streptomiceta

Sažetak

Porodica transkripcijskih faktora STAT (signalni transduktori i aktivatori transkripcije) prisutna je u raznim vrstama kao što su sisavci, insekti, biljke i sluzave plijesni. STAT faktori su bitni za signalne putove regulirane faktorom rasta. U radu je prikazana identifikacija proteina sličnog Statu u prokariotu vrste *Streptomyces*. Antitijela specifična na Stat3 miša unakrsno su reagirala s proteinima u ekstraktima raznih vrsta *Streptomyces*. Nadalje je pronađeno da je protein sličan Statu nađen u streptomicetama fosforiliran na tirozinu, kao i eukariotski Stat protein. Dobiveni rezultati pokazuju da Stat proteini vjerojatno sudjeluju u staničnoj regulaciji streptomiceta i upućuju na bakterijsko podrijetlo tih proteina.