Identification of the kinase that activates a nonmetazoan STAT gives insights into the evolution of phosphotyrosine-SH2 domain signaling

Tsuyoshi Araki^a, Takefumi Kawata^b, and Jeffrey G. Williams^{a,1}

^aDivision of Cell and Developmental Biology, Wellcome Trust Biocentre, College of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom; and ^bDepartment of Biology, Faculty of Science, Toho University, Funabashi, Chiba 274-8510, Japan

Edited* by James E. Darnell, The Rockefeller University, New York, NY, and approved May 15, 2012 (received for review February 16, 2012)

SH2 domains are integral to many animal signaling pathways. By interacting with specific phosphotyrosine residues, they provide regulatable protein–protein interaction domains. *Dictyostelium* is the only nonmetazoan with functionally characterized SH2 domains, but the cognate tyrosine kinases are unknown. There are no orthologs of the animal tyrosine kinases, but there are very many tyrosine kinase-like kinases (TKLs), a group of kinases which, despite their family name, are classified mainly as serine-threonine kinases. STATs are transcription factors that dimerize via phosphotyrosine–SH2 domain interactions. STATc is activated by phosphorylation on Tyr922 when cells are exposed to the prestalk inducer differentiation inducing factor (DIF-1), a chlorinated hexaphenone. We show that in a null mutant for Pyk2, a tyrosine-specific TKL, exposure to DIF-1 does not activate STATc. Conversely, overexpression of Pyk2 causes constitutive STATc activation. Pyk2 phosphorylates STATc on Tyr922 in vitro and complexes with STATc both in vitro and in vivo. This demonstration that a TKL directly activates a STAT has significant implications for understanding the evolutionary origins of SH2 domain–phosphotyrosine signaling. It also has mechanistic implications. Our previous work suggested that a predicted constitutive STATc tyrosine kinase activity is counterbalanced in vivo by the DIF-1-regulated activity of PTP3, a Tyr922 phosphatase. Here we show that the STATc–Pyk2 complex is formed constitutively by an interaction between the STATc SH2 domain and phosphotyrosine residues on Pyk2 that are generated by autophosphorylation. Also, as predicted, Pyk2 is constitutively active as a STATc kinase. This observation provides further evidence for this highly atypical, possibly ancestral, STAT regulation mechanism.

SH2 domains form part of a paradigmatic “writer/reader/eraser” control module (1, 2) in which the writers are tyrosine kinases (TKs), the readers are SH2 domains, and the erasers are protein tyrosine phosphatases (PTPs). In principle no one element of such a three-component system can function usefully independently of the other two. So how could such a three-component system have evolved? Here the fungi and the amoebozoan *Dictyostelium* have been informative.

Metazoa possess large numbers of TKs, but the only unicellular organisms known to possess them are choanoflagellates, unicellular close relatives of the Metazoa (3, 4). Although neither *Dictyostelium* nor fungi possess TKs, they do have PTPs, thus suggesting that tyrosine phosphorylation arose in a common ancestor of fungi and Amoebozoa, mediated perhaps by dual-specificity kinases and regulated by dephosphorylation via PTPs (2). There are no phosphotyrosine-binding SH2 domains in fungi, but there is a sequence-related domain within the SPT6 protein that binds phospho-serine and that may have been ancestral to modern SH2 domains (5). In the fungi, therefore, phosphorylation of tyrosine appears to be used solely as a direct modulator of enzymatic function, but in *Dictyostelium* there are 13 SH2 domain proteins of widely varying function (6). Four are close orthologs of the metazoan STATs (7).

Metazoan STATs are phosphorylated most frequently by JAK family members (8–10). JAKs contain a TK domain, a

pseudokinase domain, an SH2 domain, and a FERM domain that mediates receptor binding (11). Activation is initiated when a cytokine binds to and induces the clustering of its cognate cell surface receptor. This clustering results in the activation of specific JAKs that are constitutively associated with the receptor chains. The JAKs then undergo autophosphorylation, and they also phosphorylate a specific tyrosine residue on the receptor. This phosphorylated residue acts as a docking site for the SH2 domain of the STAT. The JAK then tyrosine phosphorylates the STAT at a site near its C terminus, and this phosphorylation directs SH2 domain-mediated binding of a homodimerizing or heterodimerizing partner STAT.

Dictyostelium STATc, like metazoan STATs 1 and 3 (12), can be activated by either ligand stimulation or hyperosmotic stress (13, 14). The activating ligand for STATc is differentiation inducing factor (DIF-1), a chlorinated polyketide that is produced by the prespore cells and that induces prestalk-specific gene expression (15, 16). STATc tyrosine phosphorylation seems to be regulated, via DIF-1 and hyperosmotic stress, by the controlled dephosphorylation of transiently phosphorylated STATc molecules, i.e., in the manner suggested by Lim and Pawson's (2) hypothesis. The evidence for this regulation is as follows. The cognate tyrosine phosphatase, PTP3, was identified by substrate trapping using STATc, and the expression of a dominant negative form of PTP3 leads to constitutive phosphorylation of Tyr922 of STATc (17). Moreover, in response to DIF-1 or stress, PTP3 is phosphorylated on two serine residues, and this modification correlates with a reduction in PTP3 activity (17).

An essential key to understanding STATc activation is to identify the kinase that tyrosine phosphorylates it. *Dictyostelium* has no recognizable orthodox TKs, but it possesses a large contingent of TK-like kinases (TKLs) (18). Although they are intermediate in sequence between TKs and serine/threonine kinases, the metazoan TKL proteins generally are considered to be serine/threonine kinases (18, 19). However, six of the 66 *Dictyostelium* TKLs have been shown to phosphorylate tyrosine residues selectively (Fig. S14). VSK3 was identified bioinformatically and is a transmembrane kinase with a role in phagocytosis (20). The other kinases were identified by expressing cDNA libraries in *Escherichia coli* and monitoring phosphotyrosine production in individual bacterial colonies using an antibody (21–23). Zak1 and Zak2 (originally termed “Pyk4”) phosphorylate GSK3 on tyrosine residues, increasing its activity (23–25). The Pyk3-null mutant displays

Author contributions: T.A., T.K., and J.G.W. designed research; T.A. and T.K. performed research; and T.A., T.K., and J.G.W. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

Freely available online through the PNAS open access option.

¹To whom correspondence should be addressed. E-mail: j.g.williams@dundee.ac.uk.

See Author Summary on page 11072 (volume 109, number 28).

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1202715109/-DCSupplemental.

prolonged activation of STATc after exposure to DIF-1, but the mechanism is unknown (26). The Pyk1 disruptant has reduced spore viability (27); hence the protein sometimes is termed “SplA” (for “Spore lysis A”). Pyk2 was isolated in the same cDNA screen as Pyk1 but was not analyzed further (21). Here we characterize Pyk2, genetically and biochemically, and show that it is a direct, constitutive activator of STATc.

Results

Pyk2-Null Mutant Is Defective in DIF-1-Induced Activation of STATc.

As part of a functional screen of the TKL family, designed to identify potential STAT TKs, we analyzed Pyk2 (21). Pyk2 is a predicted 127-kDa protein with a single TKL domain located close to the C terminus (Fig. S1B). Most of the protein outside the TKL domain is composed of simple repeat sequences, a feature typical of many *Dictyostelium* proteins. It does not have a predicted signal peptide or transmembrane domains. As measured by RNA-Seq and displayed on dictyExpress (<http://dictybase.org/>), expression of Pyk2 increases from a low level during growth and rises throughout development before decreasing late during culmination. It is not cell-type specific in its expression. To generate a null mutant, the kinase domain was replaced with a hygromycin-resistant cassette (Fig. S1B). The resultant mutant, the *pyk2*[−] strain, develops with correct kinetics to produce fruiting bodies that are morphologically normal.

Activation of STATc by DIF-1 was analyzed in the *pyk2*[−] mutant by monitoring phosphorylation on Tyr922 using a STATc phosphorylation-specific monoclonal antibody. In the assay cells are starved in shaken suspension for 4 h and then are induced with DIF-1 at 100 nM. In parental Ax2 cells the activation of STATc by DIF-1 is rapid and transient, with tyrosine phosphorylation and nuclear accumulation peaking after about 3 min of induction (Fig. 1A and ref. 28). In the *pyk2*[−] mutant there is no detectable activation by DIF-1. Nuclear accumulation of STATc in the *pyk2*[−] strain is, as would be expected from its profound tyrosine phosphorylation defect, not detectable in DIF-1-treated cells (Fig. 1B). We also analyzed the tyrosine phosphorylation and nuclear accu-

mulation of STATc in migrating slugs; as expected, the mutant strain is defective in both processes (Fig. S2).

Although Pyk2 is absolutely required for DIF-1 activation of STATc, Pyk2 is not essential for stress activation; inactivating *pyk2* retards stress-induced activation only slightly (Fig. S3A). Thus, the *pyk2*[−] mutant genetically uncouples the DIF-1 and stress STATc activation pathways. We assume that another, semiredundant kinase is primarily responsible for stress activation of STATc.

DIF-1 also acts later in development to induce expression of the *ecmA* gene. The *ecmA* gene is inducible in *pyk2*[−] cells suggesting that a different signaling pathway is involved (Fig. S3B). [Note: The absolute extent of expression actually is somewhat higher in untreated and DIF-induced *pyk2*[−] cells than in control parental cells. These results are in accordance with previous evidence (28), confirmed in Fig. S3B, showing that STATc is a repressor of *ecmA* gene expression. In conjunction they suggest that the elevated *ecmA* expression in the *pyk2*[−] strain is the result of the requirement for Pyk2 for STATc activation.] Another of the four *Dictyostelium* STATs, STATa, is activated when cells are induced with cAMP (7). This activation occurs normally in the *pyk2*[−] strain, again implying separate signaling pathways (Fig. S3C).

Overexpression of Pyk2 Causes Constitutive STATc Activation. If Pyk2 forms part of the DIF-1–STATc signaling pathway, its overexpression might be expected to cause constitutive STATc activation. This prediction was tested using a strain expressing Myc-Pyk2, an N-terminally Myc-tagged form of Pyk2 under the transcriptional control of a semiconstitutive actin promoter. Parental Ax2 and Myc-Pyk2-transformed cells, termed “Myc-Pyk2 OE” (for “overexpressor”) cells, were starved and shaken in suspension for 4 h and then were left untreated or were exposed to DIF-1. There is constitutive activation of STATc, such that STATc is tyrosine phosphorylated to a higher level in the uninduced Myc-Pyk2 OE cells than in parental cells exposed to DIF-1 (Fig. 2A). As would be expected from its high level of tyrosine phosphorylation, STATc is enriched in the nuclei of Myc-Pyk2 OE cells, even in the absence of inducer (Fig. 2B).

Fig. 1. Characterization of the *pyk2*[−] strain. (A) Activation of STATc in parental Ax2 and *pyk2*[−] cells. Parental Ax2 cells and *pyk2*[−] cells were developed in shaken suspension for 4 h and then were treated with DIF-1 at 100 nM for the indicated times. Tyrosine phosphorylation of STATc was assayed by Western blot (IB) using phospho-STATc antibody CP22 (pSTATc, Top), and the same blot was reprobed with total-STATc antibody 7H3 (STATc, Middle). (Bottom) The combined data from several such experiments are quantified. (B) Nuclear translocation of STATc in parental and *pyk2*[−] cells. Cells developed as in A were treated with DIF-1 at 100 nM for 3 min. The nuclear accumulation of STATc was assayed immunohistochemically using total STATc antibody. Ethanol-treated cells were used as controls, because ethanol is the vehicle for DIF-1. There are many fewer labeled nuclei in the null strain. (Scale bar: 10 μm.)

Fig. 2. Characterization of a Pyk2 overexpressor strain. (A) STATc activation in parental and Pyk2-overexpressing cells. STATc activation was assayed in Ax2 cells and in Ax2 cells transformed with Myc-Pyk2 (Myc-Pyk2 OE cells) using DIF-1 at 100 nM, after 3 min of induction and as in Fig. 1A. When it is overexpressed in this way, Pyk2 interacts detectably with the monoclonal antibody used to monitor tyrosine phosphorylation of STATc. Hence we also confirmed the identity of STATc by analyzing the Myc-Pyk2 construct in a STATc-null background. (B) Nuclear localization of STATc in parental and Pyk2 OE cells. STATc nuclear translocation was assayed in cells developed in suspension induced with DIF-1, as in Fig. 1B, using Ax2 cells or Myc-Pyk2 OE cells. Note that the total STATc antibody, which is N-terminal epitope directed, was used to detect STATc; it does not cross-react with Pyk2. (Scale bar: 10 μm.)

Unexpectedly CP22, the monoclonal antibody used to detect the Tyr922-phosphorylated form of STATc, also detects a band migrating at lower relative mobility (upper arrowhead in Fig. 2A, *Top*). This protein does not derive from the gene encoding STATc, because it is present in a STATc-null background (Fig. 2A). Parental Ax2 samples do not display the band; it is observed only when the antibody is used to analyze samples from Myc-Pyk2 OE cells. Also, it migrates at the position of Myc-Pyk2, detected on the same Western blot by reprobing with the 9E10 Myc antibody. These data can be explained most easily by the CP22 antibody cross-reacting with one or more phosphotyrosine residues in the Myc-Pyk2 protein at a level that is not detectable in the much less highly expressed endogenous Pyk2 protein. Further evidence supporting this notion is presented below.

Pyk2 Is Associated with STATc Constitutively in Vivo and Binds to It in Vitro. Pyk2 could form part of a signal transduction cascade that activates a downstream TK to phosphorylate STATc or could itself be a direct modifier of STATc. In the latter case, Pyk2 might be predicted to form a stable complex with STATc. This possibility was investigated by performing immunoprecipitation (IP) with extracts from Myc-Pyk2 OE cells, using the 9E10 Myc antibody as the immunoprecipitating antibody and analyzing the precipitate for STATc using the 7H3 total STATc antibody (Fig. 3A). STATc is not detectable in parental Ax2 cell extracts incubated with the Myc antibody or in mock precipitates of cell extracts omitting the Myc antibody. In Myc-Pyk2 OE cells STATc is constitutively coprecipitated; i.e., STATc is present at equal levels in immunoprecipitates derived from cells in the presence or absence of DIF-1 (Fig. 3A). These results were confirmed when

the protocol was reversed, i.e., when IP was performed using the 7H3 STATc antibody, and Myc-Pyk2 was detected using 9E10 (Fig. 3B). Thus, Pyk2 and STATc interact constitutively in *Dictyostelium* lysates. Also, in support of the suggestion concerning the binding of the CP22 phospho-STATc antibody to Myc-Pyk2 (Fig. 2A), the immunoprecipitated Myc-Pyk2 is bound by the 4G10 general phosphotyrosine antibody (Fig. 3A).

We further investigated the interaction between Pyk2 and STATc by performing GST pull-down experiments. GST-Pyk2 is a fusion between GST and Pyk2 cloned in a bacterial expression vector. When used in affinity chromatography with *Dictyostelium* extracts, GST-Pyk2 pulls down STATc (Fig. 3C). The binding again is constitutive; i.e., the amount of STATc bound is similar in control and DIF-treated cells. The converse experiment, using GST-STATc to pull down Myc-Pyk2, gives analogous results (Fig. 3D).

Pyk2 Tyrosine Phosphorylates STATc on Tyr922. If Pyk2 is the enzyme that phosphorylates STATc, then Pyk2 produced in *E. coli* would be expected to phosphorylate STATc on Tyr922. This notion was tested using GST-Pyk2 expressed in *E. coli* and purified by glutathione affinity chromatography. The substrate was His-STATc, and the reaction was assayed using the CP22 phospho-STATc antibody. The bacterially expressed kinase tyrosine phosphorylates His-STATc in an ATP-dependent manner, but control GST protein does not (Fig. 4A). To confirm this result and to study the regulation of Pyk2 activity, we established an immunoprecipitation kinase assay using Myc-Pyk2 produced in *Dictyostelium*.

Again, the substrate was produced in *E. coli*, and phosphorylation on Tyr922 was the readout. However, we found in pre-

Fig. 3. Evidence that Pyk2 and STATc interact physically. (A and B) Co-IP of STATc and Pyk2. Ax2 cells or Myc-Pyk2 OE cells were developed in suspension as in Fig. 2A and were induced for the indicated times with DIF-1. (A) Lysates were immunoprecipitated using the 9E10 Myc antibody (+Ab) or were mock precipitated (–Ab). The precipitates were analyzed by Western blot using the total STATc antibody 7H3 and also with 9E10. The Myc antibody analysis confirms that the IP enriches for Myc-Pyk2. The 7H3 antibody analysis shows that STATc is coprecipitated only when Myc-Pyk2 is present and Myc antibody is added. DIF-1 treatment did not significantly affect the amount of STATc recovered. The same samples were reanalyzed with the 4G10 general phosphotyrosine antibody, and this analysis shows that Myc-Pyk2 is constitutively phosphorylated on tyrosine. (B) The reverse protocol to that in A was applied to Ax2 and Myc-Pyk2 OE lysates; i.e., the samples were precipitated with 7H3, and the Western blot was analyzed using 9E10 and, as a precipitation control, with 7H3. (C and D) GST pull-down assays of STATc and Pyk2. Ax2 cells and Myc-Pyk2 OE cells at 4 h of development in suspension were left untreated or were exposed to DIF-1 (100 nM) for 3 min. Cells then were lysed, and the extracts were subjected to pull-down assay. (C) For the Ax2 samples, GST-Pyk2 was used in the pull-down assay, and STATc was detected using the 7H3 total STATc antibody. (D) For the Myc-Pyk2 OE cells, GST-STATc was used in the pull-down assay, and the Myc-Pyk2 protein was detected using the 9E10 Myc antibody.

Fig. 4. In vitro tyrosine phosphorylation of STATc by Pyk2. (A) Tyrosine phosphorylation of STATc using recombinant GST-Pyk2. GST-Pyk2 fusion proteins, produced in *E. coli*, were purified by glutathione affinity chromatography and were used in a kinase reaction with His-STATc or His-STATcR831A, a mutant form with an inactivating substitution in the SH2 domain, as substrate. The reaction products were assayed for STATc tyrosine phosphorylation by Western blotting using phospho-STATc antibody. (B) Tyrosine phosphorylation of STATc was assayed immunologically using immunopurified Myc-Pyk2. Ax2 cells transformed with Myc-Pyk2 (Myc-Pyk2 OE cells) or with its kinase-dead form, Myc-Pyk2K880A (Myc-Pyk2K880A OE cells) were lysed at 4 h of suspension development, and enzyme immunopurified from them was assayed for STATc TK activity. In addition to the substrate, GST-STATcΔ, a major low-mobility species, Myc-Pyk2, is autophosphorylated. Neither protein species is observed when the kinase-dead K880A form of Myc-Pyk2 is used as enzyme. (C) Time course of the activation of STATc by DIF-1. A time course of the DIF-1 induction of STATc tyrosine phosphorylation activity by Myc-Pyk2 was generated as in B.

liminary experiments that the optimal assay substrate is GST-STATcΔ; which contains the approximate C-terminal half (amino acids 463–932) of STATc, encompassing the SH2 domain and the site of tyrosine phosphorylation (13). Uninduced cell lysates were immunoprecipitated with the 9E10 Myc antibody, and a kinase reaction was performed using GST-STATcΔ as the substrate. The reaction products were analyzed by Western blot using the CP22 antibody. The immunoprecipitated Myc-Pyk2 tyrosine phosphorylates STATc, and again the reaction is totally ATP dependent (Fig. 4B). As a further negative control, a “kinase-dead” construct, Myc-Pyk2K880A, was analyzed in parallel. This construct bears an alanine substitution of the lysine residue equivalent to K72 of PKA, an essential residue in the ATP binding site (29). This protein, Myc-Pyk2K880A, is inactive in the in vitro assay (Fig. 4B).

As a further check of the specificity of the reaction, we used a modified detection protocol (Fig. S4). Here the analysis involved parallel isotopic kinase labeling of two substrates: GST-STATc (full-length STATc) and its mutant form, GST-STATcYF. The latter construct bears a substitution of Tyr922 by phenylalanine. If the only position of labeling is Tyr922, there should be no radioactive signal on the gel at the migration position of GST-STATcYF. As expected, no signal was observed, and again, in this assay, the kinase-dead form of Pyk2 is totally inactive.

We determined the effect of inducing cells with DIF-1 before lysis and assay. The reactions were analyzed either by isotopic labeling (Fig. S4) or, over a time course of DIF-1 induction, immunologically (Fig. 4C). Neither assay method detected any significant increase in Pyk2 kinase activity in cells exposed to DIF-1. Thus, as assayed in vitro, the kinase activity of cells treated with DIF-1 is not increased over the basal level observed in uninduced cells.

Tyrosine Phosphorylation Activity of Pyk2 Is Essential for Its Interaction with STATc. To characterize the reaction mechanism further, we repeated the STATc co-IP analysis using Myc-Pyk2K880A, the kinase-dead form of Myc-Pyk2. There is no apparent interaction with STATc (Fig. 5A). Thus, formation of an in vivo complex with STATc requires that Myc-Pyk2 act as a kinase. A pull-down assay strengthened this conclusion, be-

cause it failed to detect an interaction between Myc-Pyk2K880A with GST-STATc (Fig. 5B).

Pyk2 Autophosphorylates on Tyrosine, and This Autophosphorylation Is Essential for Interaction with STATc. In the immunoprecipitated kinase assay the CP22 phospho-STATc antibody did not bind to Myc-Pyk2K880A, the kinase-dead form of Myc-Pyk2 (Fig. 4B). Also, in the co-IP experiments, analysis using the 4G10 general phosphotyrosine antibody showed no tyrosine phosphorylation of Myc-Pyk2K880A (Fig. 5A). The simplest explanation of these two observations is that the tyrosine phosphorylation of Myc-Pyk2 is caused by autophosphorylation. In confirmation of this notion, GST-Pyk2 purified from *E. coli* binds to 4G10 antibody, but the kinase-dead form does not (Fig. 5B).

We also investigated the role of Pyk2 autophosphorylation biochemically by performing enzymatic dephosphorylation (Fig. 5C). The GST-Pyk2 fusion protein was bound to glutathione beads, and the coupled beads either were left untreated or were digested with T-cell PTP (TC PTP), a tyrosine-specific protein phosphatase. As a specificity control, a parallel reaction was performed in the presence of sodium orthovanadate, an inhibitor of tyrosine phosphatases. The bead complexes were used in pull-down reactions using *Dictyostelium* extracts, and the resultant Western blot was analyzed using the 7H3 total STATc antibody. Treatment of beads with the tyrosine phosphatase greatly reduces the amount of STATc pulled down, and sodium orthovanadate restores binding. Thus, autophosphorylation of Pyk2 is essential for efficient binding to STATc.

Interaction of STATc with Tyrosine-Phosphorylated Pyk2 Is Mediated by the STATc SH2 Domain. To investigate the possibility of a direct interaction between the SH2 domain of STATc and phosphotyrosine on Pyk2, we created mutant forms of STATc. In STATcR831A an invariant and essential SH2 domain arginine residue (R831, equivalent to R175 of v-src), is mutated to alanine. We also analyzed a mutant of STATc in which Tyr922 is replaced with phenylalanine. When the SH2 domain-mutant protein GST-STATcR831A is used in pull-down assays with control extracts or extracts from DIF-1-treated cells, there is no detectable Myc-Pyk2 binding (Fig. 6A). However, GST-STATcY922F, in

Fig. 5. Determination of the role of Pyk2 autophosphorylation. (A) Analysis of the interaction of kinase-dead Myc-Pyk2 with STATc by co-IP assay. Myc-Pyk2 OE or Myc-Pyk2K880A OE cells were induced with DIF-1, and then Pyk2 was immunoprecipitated with 9E10 Myc antibody and analyzed for STATc binding using the 7H3 total STATc antibody as in Fig. 3A. As a loading control, the blot was reprobed for Myc-Pyk2 using 9E10 Myc antibody. The same samples were reanalyzed with the 4G10 general phosphotyrosine antibody; this analysis shows that Myc-Pyk2 is constitutively phosphorylated on tyrosine. Presumably because of their difference in phosphorylation status, the parental and kinase-dead forms of Myc-Pyk2 display a small difference in mobility indicated by a double arrowhead. (B) Analysis of the interaction of wild-type and kinase-dead GST-Pyk2 with STATc. Ax2 cells were developed for 4 h and then were left untreated or were exposed to DIF-1 as in Fig. 4B. Cells were lysed, and the extracts were subjected to pull-down assay using GST-Pyk2 or GST-Pyk2K880A (its kinase-dead form) and assaying STATc by Western blot as in Fig. 3C. The blot was reprobed with a GST antibody as a loading control. (C) Determination of the phosphatase sensitivity of GST-Pyk2 binding to STATc. GST-Pyk2 was bound to glutathione beads, and the beads either were left untreated or were digested with the TC PTP tyrosine phosphatase. A parallel reaction was performed on GST-Pyk2 beads in the presence of sodium orthovanadate, an inhibitor of the enzyme. The various beads were used in pull down of STATc in extracts from cells left untreated or induced with DIF-1 and were assayed as in Fig. 3B. The blot was reprobed with a GST antibody as a loading control, and the same samples were reanalyzed with the 4G10 general phosphotyrosine antibody.

which the mutation is in the site of tyrosine phosphorylation, binds to Myc-Pyk2 normally. Thus, STATc tyrosine phosphorylation is not essential for the interaction, but the STATc SH2 domain is required.

Although the above result shows that the STATc SH2 domain is required for binding to Pyk2, the assays use *Dictyostelium* lysates; hence, in principle, another tyrosine-phosphorylated protein could be acting as an adaptor to mediate the interaction. Therefore we analyzed the interaction of recombinant His-STATc and recombinant autophosphorylated GST-Pyk2. The two bacterially produced proteins interact in a pull-down assay, with GST-Pyk2 immobilized on beads and His-STATc in solution (Fig. 6B). This binding is reduced strongly when GST-Pyk2 is used to pull down the SH2 domain-mutant form of STATc, His-STATcR831A. Again, prior dephosphorylation of GST-Pyk2 by TC PTP reduces binding, and this effect is reversed by sodium orthovanadate. These results indicate that there is a direct SH2 domain-phosphotyrosine interaction between Pyk2 and STATc. We also used His-STATcR831A as a substrate in enzymatic assays with recombinant GST-Pyk2; in the experiment in which His-STATc was tyrosine phosphorylated by GST-Pyk2, His-STATcR831A was not phosphorylated (Fig. 4A). Thus, an intact STATc SH2 domain is required for STATc to act as a substrate for Pyk2.

Discussion

STAT Activation by a TKL. One principal finding is that, in *Dictyostelium*, a TKL is the direct, upstream activator of a STAT, a function that is performed by TKs in the Metazoa. Several lines of evidence combine to show that (i) Pyk2 is essential for DIF-1 activation of STATc; (ii) overexpression of Pyk2 causes constitutive activation of STATc; (iii) Pyk2 coimmunoprecipitates with STATc in *Dictyostelium* lysates, and the two proteins interact in both configurations of a pull-down assay; and (iv) both recombinant and immunopurified Pyk2 correctly phosphorylate STATc on Tyr922.

There are TKLs in amoebozoans, plants, and animals but not in yeast, suggesting that the TKLs were lost selectively during

fungal evolution (19). Given that the TKLs are intermediate in their signature motifs between TKs and serine/threonine kinases, it appears probable that metazoan TKs evolved from an ancestral TKL protein (18). The SH2 domains of the STATs are significantly divergent from most other SH2 domains, and this divergence has led to the suggestion that STATs arose early in the evolution of phosphotyrosine signaling (30). This notion is concordant with their status as “fast track” plasma membrane-to-nucleus signal transducers; presumably, the generation of such a simple pathway required minimal evolutionary innovation. Therefore it is tempting to speculate that the TK–SH2 domain “writer–reader” combination may have coevolved from an ancestral TKL–STAT partnership.

Is There a Conserved Motif for Tyrosine-Selective TKLs? Interestingly, five of the six biochemically proven tyrosine-selective kinases, including Pyk2, share a di-peptide sequence, (T/S)S, within kinase subdomain VIb (Fig. S1A), the motif that is most discriminatory between animal TKs and serine/threonine kinases (31). A different criterion, the absence of a serine or a threonine at a position equivalent to Thr201 in PKA, has been applied to the same protein set, but when this criterion is used as a discriminator three of the six register as serine/threonine kinase (18). Thus, the sequence HRDL (T/S)S may act as a signature motif for identifying tyrosine-selective kinases in other organisms. Two additional *Dictyostelium* TKLs, DDB_G0283397 and DDB_G0278521, share this consensus sequence, but as yet neither is characterized.

Mechanism of STATc Activation. We suggest that, as the mechanism of STATc activation by Pyk2, the STATc SH2 domain binds to a site of tyrosine autophosphorylation in Pyk2. We assume that the STATc bound to Pyk2 and the free STATc are in equilibrium and that the relative levels of kinase and phosphatase activity determine the proportion in each pool. Pyk2 activity is held to be constitutive. Activation is initiated when PTP3 is down-regulated by DIF-1–induced serine phosphorylation (32). Once enough free phosphorylated STATc has accumulated, STATc dimerization occurs via the STATc SH2 domain, and this dimerization precludes

Fig. 6. Determination of the role of the STATc SH2 domain. (A) Pull-down analysis of *Dictyostelium*-expressed Myc-Pyk2 by mutant forms of STATc. Myc-Pyk2 OE cell lysates from control cells or cells induced with DIF-1 were subjected to pull-down assay using GST-STATc or its R831A- and Y922F-mutant forms as in Fig. 3B. The blot was reprobed with a GST antibody as a loading control. (B) Pull-down analysis of recombinant Pyk2, STATc, and its R831A-mutant form. GST-Pyk2, His-STATc, and His-STATcR831A, all expressed in *E. coli*, were subjected to affinity chromatography using their respective tags. The GST-Pyk2 fusion protein was attached to the glutathione beads, and aliquots were treated with TC PTP with or without sodium orthovanadate, as in Fig. 5C. The His-tagged STATc proteins were used in binding to the GST-Pyk2/glutathione beads as indicated. The recovered STATc protein was assayed by Western blot using a His antibody, or a GST antibody as indicated.

any further interaction with Pyk2. The evidence for this assumption is that (i) Pyk2 is tyrosine phosphorylated constitutively in vivo, and this phosphorylation is the result of autophosphorylation; (ii) because Pyk2 is autophosphorylated, we were able to demonstrate a direct interaction between STATc and GST-Pyk2 using bacterially expressed fusion proteins; (iii) the interaction of STATc with Pyk2, in co-IP and pull-down analyses is dependent on the kinase activity of Pyk2; (iv) enzymatic tyrosine dephosphorylation of GST-Pyk2 reduces its binding to STATc; and (v) the SH2 domain of STATc is essential for interaction with Pyk2.

Such a mechanism is significantly different from the canonical metazoan JAK-STAT pathways in which STATs bind to docking sites on the cytokine receptor rather than on the JAK receptor and bind only after stimulation with ligand. Instead, the mechanism is more similar to that used in growth factor-stimulated STAT activation. After binding to its ligand, the EGF receptor autophosphorylates on two tyrosine residues that function as docking sites for STAT3 (33). Similarly, the PDGF- β receptor binds to and directly activates STAT5 (34). Thus, in the proposed STATc mechanism, as in the two growth factor receptor pathways, the STAT interacts directly with the kinase rather than with a separate receptor. This similarity between the Metazoa and a nonmetazoan is consistent with an ancient evolutionary origin for a direct interaction. However, our further observation that the *Dictyostelium* kinase is constitutively active highlights the key difference with the metazoan systems: The regulation of STATc activation by DIF-1 is mediated by controlled dephosphorylation by PTP3 rather than by the activation of a TK (17). Therefore, as suggested by Lim and

Pawson (2), such a mechanism may constitute an evolutionary precursor to the metazoan paradigm.

Materials and Methods

Cell Culture, Development, and Induction. *Dictyostelium* strain Ax2 (Gerisch isolate) cells were grown axenically at 21 °C in HL-5 medium (35). For induction, cells were developed in KK2 buffer (16.5 mM KH_2PO_4 , 3.8 mM K_2HPO_4 , pH 6.2) at a concentration of 1×10^7 cells/mL by shaking for 4 h at 200 rpm when DIF-1 (Enzo Life Sciences) was added to 100 nM.

Immunological Analyses. The CP22 phospho-STATc antibody was used to detect STATc Tyr922 phosphorylation by Western blot analysis. STATc immunostaining and non-phospho-specific Western blot analyses were performed using 7H3 total STATc antibody (32). Anti-phosphotyrosine antibody 4G10 (Millipore) was used to monitor general phosphotyrosine modification, anti-Myc antibody 9E10 was used for Myc-tagged proteins, and anti-polyhistidine peroxidase-conjugated antibody (Sigma-Aldrich) was used for His-tagged proteins. IP of Myc-tagged proteins was performed essentially as described in ref. 17; 1–4 mL of cell suspension at 10^7 cells/mL was harvested and lysed in 1 mL mNP40 lysis buffer, [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% (vol/vol) Nonidet P-40, 50 mM NaF, 2 mM EDTA (pH 8.0), 2 mM Na-pyrophosphate, 2 mM benzamide, 1 $\mu\text{g}/\text{mL}$ pepstatin, 1 mM PMSF, and Complete EDTA-free protease inhibitor mixture (Roche Diagnostics)] for 10 min on ice. After pre-clearing by centrifugation, the supernatant was incubated with anti-Myc antibody or anti-total STATc antibody for 30 min at 4 °C with gentle rocking, followed by another hour of incubation with Dynabeads Protein-G (Life Technologies) for the 9E10 Myc antibody or with Protein-G agarose (Roche Diagnostics) for total STATc antibody. Beads were washed four times in mNP40 buffer, and proteins were eluted by boiling in SDS gel sample buffer.

Construction of the Pyk2 Disruption Vector and Tagged Proteins. In the disruption construct, Pyk2 sequences encompassing the TKL domain were replaced with a hygromycin-resistance cassette (Fig. S1B). For the Myc-Pyk2 construct, Pyk2 was tagged at its N terminus by addition of the 11-aa Myc epitope, EQKLISEEDLN. The Myc-Pyk2 fusion was cloned under the transcriptional control of the semiconstitutive actin 15 promoter and was inserted into a G418 resistance vector. For GST-STATc constructs, STATc (full length: amino acids 1–932 or Δ : amino acids 463–932) was tagged at its N terminus by insertion into the *E. coli* expression vector pGEX-5x-1 (GE Healthcare). For His-STATc, STATc was tagged at its N terminus in the *E. coli* expression vector pET-28a (Merck). Point-mutant forms of Pyk2 and STATc were generated using a PCR-based method. Bacterially expressed tagged proteins were purified with glutathione-Sepharose 4B (GE Healthcare) or TALON metal affinity resin (Clontech Laboratories).

GST Pull-Down Assays. GST-Pyk2 or GST-STATc proteins (typically 10 μg) were mixed with glutathione beads (200- μL packed volume) in GST-buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1.0 mM EGTA, 1.0% (vol/vol) Triton X-100, 5 mM DTT, and Complete Protease inhibitor mixture] for 1 h at 4 °C. After two washings in GST-buffer and twice in mNP40 buffer, the GST-protein/glutathione-Sepharose complex (50- μL packed volume per sample) was mixed with cell lysate ($1\text{--}4 \times 10^7$ cells in 1 mL) in mNP40 buffer (17). After incubation at 4 °C for 1 h with gentle rocking, the beads were washed four times in mNP40 buffer. Samples were prepared by boiling in SDS gel sample buffer. For an in vitro binding assay between GST-Pyk2 and His-STATc, the GST-Pyk2/glutathione complex was mixed and incubated with purified His-STATc in mNP40 buffer at 4 °C for 2 h. After four washings in mNP40 buffer, proteins were eluted in SDS gel sample buffer.

In Vitro Kinase Assay with Bacterially Expressed Proteins. Bacterially expressed GST-Pyk2 and His-STATc proteins were used for the in vitro kinase assay. GST-Pyk2 protein (0.1 μg per reaction) was incubated with His-STATc (0.2 μg per reaction) as the substrate and 0.2 mM ATP in Hepes kinase buffer [20 mM Hepes-NaOH (pH 7.5), 30 mM NaCl, 10 mM MgCl_2 , 0.02% Nonidet P-40, 1 mM Glycylglycyl-L-proline, and 1 mM DTT] at 21 °C for 30 min. The kinase reaction was terminated by boiling in SDS gel sample buffer.

IP Kinase Assay Using Myc-Pyk2. Myc-Pyk2 was immunopurified as described above, but, instead of being washed four times in mNP40 buffer, the Myc-Pyk2/Dynabead complex was washed twice in mNP40 high-salt washing buffer containing 1 M NaCl instead of 150 mM NaCl and was washed two more times in mNP40 buffer without EDTA. For immunological detection of the kinase activity, the complex was incubated in Hepes kinase buffer with GST-STATc, which contains the approximate C-terminal half (amino acids 463–932) of

STATc as the substrate (typically 1.0 μ g per reaction, purified from bacteria) and 0.2 mM ATP at 21 °C for 30 min. The reaction was terminated by boiling in SDS gel sample buffer. GST-STATc Δ protein was subjected to SDS/PAGE and detected with the CP22 phospho-STATc antibody after Western blotting. For isotopic labeling, the kinase reaction was performed as above but using 32 P- γ -ATP (0.185 MBq per reaction) (PerkinElmer) mixed with unlabeled ATP (final concentration: 0.2 mM) and bacterially expressed GST-STATc (full length) or Y922F proteins as substrate. After separation by SDS gel electrophoresis, kinase activity was detected by autoradiography.

TC PTP Digestion. The GST-Pyk2/glutathione-Sepharose complex was washed twice in GST buffer and twice in TC PTP buffer (New England Biolabs). The GST-Pyk2 beads (packed volume, 100 μ L) were incubated in 400 μ L of TC PTP buffer containing 100 U (2 μ L) of TC PTP (New England Biolabs) for 30 min at 30 °C. Sodium orthovanadate at a final concentration of 1 mM was used as the TC PTP inhibitor.

ACKNOWLEDGMENTS. The work was funded by Wellcome Trust Program Grant 082579 (to J.G.W.) and Grant-in-Aid 24510307 from the Japan Society for the Promotion of Science (to T.K.).

- Pawson T, Gish GD, Nash P (2001) SH2 domains, interaction modules and cellular wiring. *Trends Cell Biol* 11:504–511.
- Lim WA, Pawson T (2010) Phosphotyrosine signaling: Evolving a new cellular communication system. *Cell* 142:661–667.
- King N, Hittinger CT, Carroll SB (2003) Evolution of key cell signaling and adhesion protein families predates animal origins. *Science* 301:361–363.
- King N, et al. (2008) The genome of the choanoflagellate *Monosiga brevicollis* and the origin of metazoans. *Nature* 451:783–788.
- Dengl S, Mayer A, Sun M, Cramer P (2009) Structure and in vivo requirement of the yeast Spt6 SH2 domain. *J Mol Biol* 389:211–225.
- Eichinger L, et al. (2005) The genome of the social amoeba *Dictyostelium discoideum*. *Nature* 435:43–57.
- Kawata T (2011) STAT signaling in *Dictyostelium* development. *Dev Growth Differ* 53:548–557.
- Bromberg J, Darnell JE, Jr. (2000) The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19:2468–2473.
- Chatterjee-Kishore M, van den Akker F, Stark GR (2000) Association of STATs with relatives and friends. *Trends Cell Biol* 10:106–111.
- Schindler C, Levy DE, Decker T (2007) JAK-STAT signaling: From interferons to cytokines. *J Biol Chem* 282:20059–20063.
- Ghoreschi K, Laurence A, O'Shea JJ (2009) Janus kinases in immune cell signaling. *Immunol Rev* 228:273–287.
- Gatsios P, et al. (1998) Activation of the Janus kinase/signal transducer and activator of transcription pathway by osmotic shock. *J Biol Chem* 273:22962–22968.
- Fukuzawa M, Abe T, Williams JG (2003) The *Dictyostelium* prestalk cell inducer DIF regulates nuclear accumulation of a STAT protein by controlling its rate of export from the nucleus. *Development* 130:797–804.
- Araki T, et al. (2003) A STAT-regulated, stress-induced signalling pathway in *Dictyostelium*. *J Cell Sci* 116:2907–2915.
- Kay RR, Thompson CRL (2001) Cross-induction of cell types in *Dictyostelium*: Evidence that DIF-1 is made by prespore cells. *Development* 128:4959–4966.
- Williams JG, et al. (1987) Direct induction of *Dictyostelium* prestalk gene expression by DIF provides evidence that DIF is a morphogen. *Cell* 49:185–192.
- Araki T, Langenick J, Gamper M, Firtel RA, Williams JG (2008) Evidence that DIF-1 and hyper-osmotic stress activate a *Dictyostelium* STAT by inhibiting a specific protein tyrosine phosphatase. *Development* 135:1347–1353.
- Goldberg JM, et al. (2006) The *dictyostelium* kinome—analysis of the protein kinases from a simple model organism. *PLoS Genet* 2:e38.
- Miranda-Saavedra D, Barton GJ (2007) Classification and functional annotation of eukaryotic protein kinases. *Proteins* 68:893–914.
- Fang J, et al. (2007) A vesicle surface tyrosine kinase regulates phagosome maturation. *J Cell Biol* 178:411–423.
- Tan JL, Spudich JA (1990) Developmentally regulated protein-tyrosine kinase genes in *Dictyostelium discoideum*. *Mol Cell Biol* 10:3578–3583.
- Adler K, Gerisch G, von Hugo U, Lupas A, Schweiger A (1996) Classification of tyrosine kinases from *Dictyostelium discoideum* with two distinct, complete or incomplete catalytic domains. *FEBS Lett* 395:286–292.
- Kim L, Liu J, Kimmel AR (1999) The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification. *Cell* 99:399–408.
- Kim L, Harwood AJ, Kimmel AR (2002) Receptor-dependent and tyrosine phosphatase-mediated inhibition of GSK3 regulates cell fate choice. *Dev Cell* 3:523–532.
- Kim L, et al. (2011) Combinatorial cell-specific regulation of GSK3 directs cell differentiation and polarity. *Dictyostelium Development* 138(3):421–430.
- Lee NS, Rodriguez M, Kim B, Kim L (2008) *Dictyostelium* kinase DPYK3 negatively regulates STATc signaling in cell fate decision. *Dev Growth Differ* 50:607–613.
- Nuckolls GH, Osherov N, Loomis WF, Spudich JA (1996) The *Dictyostelium* dual-specificity kinase splA is essential for spore differentiation. *Development* 122:3295–3305.
- Fukuzawa M, Araki T, Adrian I, Williams JG (2001) Tyrosine phosphorylation-independent nuclear translocation of a *Dictyostelium* STAT in response to DIF signaling. *Mol Cell* 7:779–788.
- Kamps MP, Taylor SS, Sefton BM (1984) Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. *Nature* 310:589–592.
- Chen X, et al. (1998) Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA. *Cell* 93:827–839.
- Hanks SK, Hunter T (1995) Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J* 9:576–596.
- Araki T, Williams JG (2012) Perturbations of the actin cytoskeleton activate a *Dictyostelium* STAT signalling pathway. *Eur J Cell Biol* 91:420–425.
- Shao H, Cheng HY, Cook RG, Tweardy DJ (2003) Identification and characterization of signal transducer and activator of transcription 3 recruitment sites within the epidermal growth factor receptor. *Cancer Res* 63:3923–3930.
- Valgeirsdóttir S, Pauku K, Silvennoinen O, Heldin CH, Claesson-Welsh L (1998) Activation of Stat5 by platelet-derived growth factor (PDGF) is dependent on phosphorylation sites in PDGF beta-receptor juxtamembrane and kinase insert domains. *Oncogene* 16:505–515.
- Watts DJ, Ashworth JM (1970) Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *Biochem J* 119:171–174.