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Txk, a member of the non-receptor tyrosine kinase of the Tec family, forms a complex with poly(ADP-ribose) polymerase 1 and elongation factor 1α and regulates interferon-γ gene transcription in Th1 cells

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Summary

We have found previously that Txk, a member of the Tec family tyrosine kinases, is involved importantly in T helper 1 (Th1) cytokine production. However, how Txk regulates interferon (IFN)-γ gene transcription in human T lymphocytes was not fully elucidated. In this study, we identified poly(ADP-ribose) polymerase 1 (PARP1) and elongation factor 1α (EF-1α) as Txk-associated molecules that bound to the Txk responsive element of the IFN-γ gene promoter. Txk phosphorylated EF-1α and PARP1 formed a complex with them, and bound to the IFN-γ gene promoter *in vitro*. In particular, the N terminal region containing the DNA binding domain of PARP1 was important for the trimolecular complex formation involving Txk, EF-1α and PARP1. Several mutant Txk which lacked kinase activity were unable to form the trimolecular complex. A PARP1 inhibitor, PJ34, suppressed IFN-γ but not interleukin (IL)-4 production by normal peripheral blood lymphocytes (PBL). Multi-colour confocal analysis revealed that Txk and EF-1α located in the cytoplasm in the resting condition. Upon activation, a complex involving Txk, EF-1α and PARP1 was formed and was located in the nucleus. Collectively, Txk in combination with EF-1α and PARP1 bound to the IFN-γ gene promoter, and exerted transcriptional activity on the IFN-γ gene.

Keywords: cytokines, T cells, Th1/Th2 cells, transcription factors

Introduction

Naive T cells differentiate into T helper 1 (Th1) or Th2 cells, in which both cytokine receptor-mediated signalling and T cell receptor (TCR) signalling play important roles. Interleukin (IL)-12 and interferon (IFN)-γ determines Th1 differentiation controlling the expression of transcription factor, T-bet [1,2], while GATA3 is involved importantly in the IL-4-mediated Th2 cell development [3,4]. The Runx1 transcription factor, which is expressed in naive T cells and repressed by T cell signalling, inhibits the differentiation of naive T cells into Th2 cells by suppressing GATA3 expression [5].

In addition to the Th1/Th2 differentiation, distinct signalling molecules and transcription factors contribute to the different cytokine expression in Th1 and Th2 cells. For instance, CARMA-1 (CARD11) and PDK1 have been reported as important molecules for activation of c-Jun N-terminal kinase (JNK) and nuclear factor (NF)-κB induced by TCR signalling [6–10]. Loss-of-function studies of CARMA-1 and downstream transcription factors, including JNK and NF-κB, demonstrate a Th2-dominant shift of immune response, suggesting an important role of CARMA-1 and these transcription factors for Th1 immune response [11,12]. In addition, calcipressin (Csp)-1 knockout mice impaired IFN-γ but not IL-4 production from T cells by down-regulating T-bet upon TCR stimulation [13]. However, to date, precise molecular mechanisms responsible for the differentiation and development of polarized Th1 responses are not clarified fully in humans.

The Tec family has emerged as a subfamily of non-receptor tyrosine kinases, consisting of Tec, Btk, Itk/Tsk/Emt, Bmx and Txk/Rlk. Generally, Tec family tyrosine kinases play an important role in actin cytoskeleton reorganization in response to TCR/CD28 signalling [14–16]. As well as cytoskeletal reorganization, they contribute to cell signalling for the development, activation and survival of lymphocytes [14–19]. Itk, the T cell-associated Tec family kinase, has been suggested for the involvement of Th2 cell development [18].

Txk/Rlk is involved in signalling pathways of lymphocyte activation, and presumed to function *in vivo* as an important signalling mediator [19–21]. Schneider *et al*. suggested that TCR can utilize mouse Rlk (as well as ZAP-70) in the phosphorylation of key sites in the adaptor protein, Src homology

2 (SH2) domain-containing leukocyte phosphoprotein (SLP-76), leading to up-regulation of the Th1-preferred cytokine IL-2 [22]. Similarly, Rajagopal *et al*. identified the T cell-specific adaptor protein, Rlk/Itk-binding protein (RIBP), which binds to mouse Rlk/Txk and modulates production of IL-2 and IFN-γ [23]. We have reported recently that Txk is expressed in Th1/Th0 cells and regulates specifically IFN-γ gene transcription, suggesting that Txk acts as a Th1 cell-specific transcription factor [24–26]. However, how Txk regulates IFN-γ gene transcription in human T lymphocytes is not elucidated fully. In this study, we identified the Txk and its associated molecules that bound to the Txk responsive element of the IFN-γ gene promoter, and demonstrate that Txk, PARP1 and EF-1α formed a complex to exert their transcriptional activity on the IFN-γ gene.

Materials and methods

Plasmid vectors

Human Txk cDNA was provided by Dr G. W. Litman, University of South Florida [24]. Txk cDNA was ligated into a mammalian expression vector, pME18S (provided by Dr K. Maruyama, Tokyo Medical and Dental University) and a His-tagged prokaryotic expression vector, pQE31 (pQE-txk-wt; Qiagen, Valencia, CA, USA) [26]. pQE-txk-kd (amino acids 1–387) was the kinase domain deleted Txk, and lacked the C-terminal region of Txk (amino acid 388–527) containing the majority of the kinase domain.

The full-length poly(ADP-ribose) polymerase 1 (PARP1) expression vector, pTP, was a gift from Dr Masutani (National Cancer Center Research Institute, Tokyo, Japan). The N-terminal and C-terminal regions of PARP1 cDNA were ligated into pGEX-5X-1 (Amersham Biosciences, Little Chalfont, UK) and the resulting plasmids were designated p glutathione-S-transferase (GST)–PARP1N (1–339) and pGST–PARP1C (338–1014), respectively.

Full-length human EF-1α cDNA [American Type Culture collection (ATCC) no. MGC16449] was ligated into pQE30 [pQE-EF-1α (1–462); Qiagen]. We developed the pGST EF-1α vector similarly.

The point mutant of Txk was created using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), forming pQE-txk K299E and pQE-txk Y91A. Their sequences have been verified by DNA sequencing.

Transfection into Jurkat cells

Purified plasmids were transfected into Jurkat cells by electroporation, as described previously [24]. In brief, 10 μg pME18S-Txk (Txk transfection) or pME18S (empty vector; mock transfection) were transfected; 48 h after transfection, Jurkat cells were stimulated with 1 μg/ml phytohaemagglutinin (PHA) and cultured for various periods.

Antibodies

Anti-PARP1 antibodies were purchased from Sigma Chemicals (St Louis, MO, USA) and from MP Biomedicals (Aurora, OH, USA). Anti-EF-1α monoclonal antibody was from Upstate (Lake Placid, NY, USA); anti-phosphotyrosine antibody, PY20, was from Santa Cruz Biotechnology. We developed anti-human Txk antibody by immunizing rabbits with a peptide (CSVQKRQMRTQISLSTDEEL) [26]. Anti-GST polyclonal antibody was from Amersham Biosciences and anti-poly(ADP-ribose) monoclonal antibody was from Trevigen (Gaithersburg, MD, USA).

Immunoblotting analysis

The cells and recombinant proteins were lysed with buffer containing 50 mM Tris, pH 8·0, 1% Nonidet P-40, 150 mM NaCl, and the protease inhibitors as described previously [26]. Equivalent amounts of proteins were resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and blocked with 3·5% bovine serum albumin (BSA). Detection was conducted as described previously [26].

Expression and purification of recombinant proteins

Expression of Txk proteins were induced by stimulation with isopropyl β-D-thiogalactopyranoside (IPTG; Takara Bio, Shiga, Japan) of pQE-txk-wt for 4 h at 37°C. The recombinant proteins were purified by using Ni-NTA beads. Expression of PARP1 protein was induced by stimulation with 3β-indoleacrylic acid (IAA; Sigma Chemicals) of pTP for 4 h at 30°C. pGST-PARP1N and pGST–PARP1C were stimulated with IPTG, and the recombinant mutant proteins were purified by using glutathione beads.

In vitro kinase assay

Recombinant Txk was incubated with adenosine triphosphate (ATP) (0·25 mM) in kinase buffer [40 mM HEPES, pH 7·4, 100 mM NaCl, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM dithiothreitol (DTT)] for the indicated time. The proteins were analysed by immunoblotting with antibodies against Txk, EF-1α, PARP1 and phosphotyrosine.

Protein–protein binding assay

In a His pull-down assay, His-tagged Txk-coupled beads were incubated with soluble full-length PARP1 in assay buffer. In a GST pull-down assay, GST-tagged mutant PARP1 protein (derived from pGST–PARP1N and pGST–PARP1C)-coupled beads were incubated with soluble Txk and/or EF-1α. Proteins co-precipitated with relevant tagged protein were subjected to immunoblotting analysis.

Poly(ADP-ribosyl)ation assay

In vitro poly(ADP-ribosyl)ation of Txk was performed using Txk-wt-coupled beads and 5 μg of recombinant PARP1 (R&D Systems, Minneapolis, MN, USA), which were incubated for 30 min in reaction buffer containing 25 mM Tris-HCl, 10 mM $MgCl_2$, 50 μM $ZnCl_2$, 250 μM β -NAD (Sigma Chemicals) and 20 ng of activated DNA (R&D systems), pH 8·0.

DNA–protein binding assay

A gel shift assay was performed using the digoxigenin (DIG) gel shift kit (Boehringer Mannheim Biochemica, Mannheim, Germany). In brief, DIG-labelled DNA fragments were incubated for 15 min with recombinant proteins. Protein–DNA complexes were separated from free probe on a polyacrylamide gel. Thereafter, the gels were transferred electrically to nylon membrane and detected by chemiluminescence. We verified that a 10-fold excess of specific cold oligonucleotide competed with the binding of the protein to the DIG-labelled probe, whereas similar excess from another site would not compete (Fig. 5).

DNA probes

The probes were derived from sequences present in the IFN-γ promoter region [24,25] and irrelevant promoter regions. Actual DNA sequences synthesized were as follows: IFN-γ gene (designated as IFN-γ −53/−39), −56 to −36 region, ACGTAATCCTCAGGAGACTTC. As a control, OCT-2 A, GGAGTATCCAGCTCCGTAGCATGCAAATCCTCTGG was used.

Cytokine production and enzyme-linked immunosobent assay (ELISA)

Normal PBL were pretreated with PJ34 (Merck, Tokyo, Japan) for 1 h and were stimulated with PHA for 24 h. Cytokines of the culture supernatants were assessed using commercial ELISA kits (human IFN-γ and human IL-4 ultrasensitive; Biosource International, Camarillo, CA, USA).

Multi-colour confocal analysis of molecular interactions among, Txk, PARP1 and EF-α

DsRed monomer, cyan fluorescent protein (CFP) and GFP-labelled protein were generated from pDsRedmonomer, pECFP and pEGFP vectors, respectively (Clontech, Palo Alto, CA, USA). The plasmid was transfected into Cos7 cells by electroporation. After 24 h incubation, the cells were assayed for fluorescence. To activate Txk, active Fyn (FynY531F, a constitutive active form of Fyn) and as a negative control, inactive Fyn (FynK299M, a kinase negative mutant) (gifts from Dr Toyoshima, University of Tokyo) were transfected simultaneously. Spectral imaging was performed with LSM510META (Carl Zeiss, Jena, Germany).

Results

The IFN-γ promoter −53/−39 region binding protein complex includes Txk, PARP1 and EF-1α

We found that Txk expression is restricted to Th1/Th0 cells with IFN-γ production and that Txk protein binds directly to the IFN-γ promoter/enhancer region (−53/−39) to exert its positive effect on IFN-γ gene transcription. The IFN-γ promoter (−53/−39) oligoDNA was labelled with DIG and was reacted with nuclear proteins of Txk-transfected Jurkat cells stimulated with PHA for 1–4·5 h. Thereafter, DNA binding proteins were recovered by biotinylated anti-DIG antibody and streptavidin Dynabeads, followed by magnet separation. The DNA–protein complexes were washed extensively and loaded onto SDS-PAGE, and the IFN-γ promoter (−53/−39) region-binding proteins were detected by silver staining. A 50-kDa (two arrows) and 110-kDa protein (one arrow) bound to the IFN-γ promoter (−53/−39) region were detected reproducibly together with the Txk protein (Fig. 1a). As control DNA, calf thymus DNA was sonicated; approximately 50 base pairs (bp) DNA fragment were recovered and treated similarly. No specific proteins bound to the control DNA. The110 kDa and 50 kDa proteins were electrotransferred and their sequences determined by proteinase digestion and subsequent high performance liquid chromatography (HPLC) analysis. The amino acid sequences of peptides derived from the 110 kDa protein were NREELGFRPEYS and IFPPETSASVAA. Those from the 50 kDa protein were YYVTIIDAPGHR and HINIVVIGHVD. The homology analysis of the peptide sequences revealed that the 110 kDa protein and 50 kDa protein were human PARP1 [27–42] and EF-1α [43–51], respectively.

Phosphorylation of PARP1 and EF-1α by Txk

To examine whether Txk phosphorylates tyrosine residues in PARP1 and EF-1α, we made wild-type Txk (Txk-wt; amino acids, 1–527), kinase domain-deleted Txk (Txk-kd; amino acids, 1–387), and catalytic-inactivated Txk (Txk-K299E and Txk-Y91A) using the pQE vector and His-tag system (Fig. 1b).

Kinase activity of Txk-wt and Txk-kd was assessed by detecting autophosphorylation of tyrosine residue. Txk-wt was incubated with or without ATP for 30 min. Anti-His immunoblotting confirmed the amounts of each Txk protein in the reaction (Fig. 2a, lower panel). In the presence of exogenous ATP (0·25 mM), Txk-wt phosphorylated itself by its own kinase activity, confirming that Txk-wt has kinase

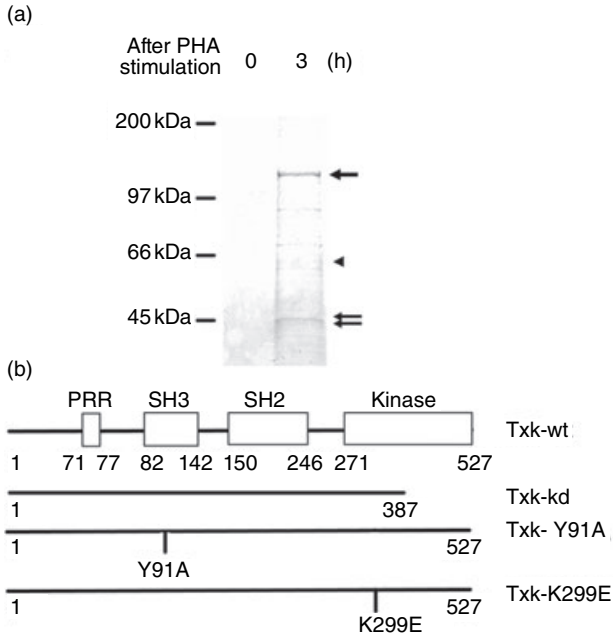

Fig. 1. Identification of T cell nuclear proteins that bind to interferon (IFN)-γ promoter (−53/−39) region together with Txk. Jurkat cells were transfected with pME18S-Txk-wt (wild-type Txk) expression vector, and were cultured for 48 h. Thereafter, the cells were activated with phytohaemagglutinin (PHA) or kept unstimulated. IFN-γ promoter region (core region, −53/−39; actual synthetic oligo DNA, −56/−36) to which Txk bound was digoxigenin (DIG)-labelled, and was incubated with nuclear proteins of the Txk transfected Jurkat cells in the presence of 5 μg/reaction poly(dI-dC)(dI-dC). The DNA-binding nuclear proteins were recovered by using anti-DIG antibody and appropriate-Dynabeads, followed by magnetic separation. As control DNA, calf thymus DNA was sonicated, approximately 50 base pairs (bp) DNA fragment were recovered by glass beads and treated similarly. The DNA–protein complexes were washed extensively and were loaded onto sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The results shown are representative of three independent experiments with essentially the same result. (a) IFN-γ promoter (−53/−39) region oligoDNA binding proteins were detected by silver staining. A 50-kDa (two arrows) and an 110-kDa protein (one arrow) bound to the IFN-γ promoter region oligoDNA were reproducibly detected. An arrowhead indicates Txk protein. The 110 kDa and 50 kDa proteins were electrotransferred and their sequences determined by proteinase digestion and subsequent high performance liquid chromatography (HPLC) analysis. The peptide sequences of the 110 kDa protein were NREELGFRPEYS and IFPPETSASVAA, indicating human PARP1. Those of the 50 kDa protein were YYVTIIDAPGHR and HINIVVIGHVD, indicating EF-1α. (b) Schematic representation of the domain structure of Txk and its mutants. Wild-type Txk (Txk-wt; amino acids, 1–527; His-Txk-wt ~63 kDa) contains proline-rich region (PRR), Src homology region 3 (SH3), SH2 and kinase domain. Kinase domain-deleted mutant Txk (Txk-kd; amino acids, 1–387; His-Txk-kDa ~46 kDa) lacks a vast majority of kinase domain. Txk-Y91A was a point mutant whose autophosphorylation site Y residue was substituted with A. Txk-K299E was a point mutant Txk whose ATP binding K residue was substituted with E.

activity (arrow, upper panel of Fig. 2a). In contrast, Txk-kd incubated with ATP did not phosphorylate itself, indicating that Txk-kd lacked kinase activity (arrowhead, upper panel, Fig. 2a).

PARP1 includes the DNA binding domain (amino acids, 1–339) and the automodification and catalytic domains (amino acids, 338–1014). We next evaluated whether the phosphorylation of PARP1 was induced by Txk. Wild-type PARP1, the GST tagged N terminal region of PARP1 (GST–PARP1N; amino acids, 1–339) and the GST-tagged C terminal region of PARP1 (GST–PARP1C; amino acids 338–1014) were produced by *Escherichia coli* (Fig. 2b). Each of the recombinant PARP1 protein and Txk-wt were incubated with ATP for 1–2 h. In the presence of ATP, Txk-wt phosphorylated GST–PARP1N (arrowhead, upper panel, Fig. 2c). Phosphorylation of GST–PARP1C was evident by the incubation with Txk-wt (arrowhead, lower panel of Fig. 2c). Similarly, Txk-wt tyrosine phosphorylated wild-type PARP1 (data not shown). Thus Txk has the ability to phosphorylate PARP1.

We then studied tyrosine phosphorylation of EF-1α by Txk (Fig. 2e). EF-1α (Fig. 2d; schematic representation of the domain structure of EF-1α) was incubated with Txk in the presence of ATP. Txk-wt phosphorylated tyrosine residue of EF-1α. These results suggested that Txk phosphorylated tyrosine both of PARP1 and EF-1α.

Phosphorylation-dependent complex formation of full-length PARP1 with Txk

Because Txk phosphorylates tyrosine residues of PARP1 and EF-1α, it is of interest to test whether the proteins make a complex upon phosphorylation. We first tested whether full-length PARP1-wt bound to Txk. Unlabelled full-length PARP1 was incubated with Txk-wt and Txk-kd in the presence of ATP (Fig. 3a). Txk-wt and Txk-kd, both of which were tagged with polyhistidine, were precipitated by the addition of Ni-NTA resin. The co-precipitated proteins were analysed by immunoblotting with anti-PARP1 antibody. The lower panel indicates amounts of Txk-wt and Txk-kd proteins detected by anti-Txk antibody (Fig. 3a, lower panel). Txk-wt co-precipitated PARP1 in the presence of ATP (upper panel, Fig. 3a). Txk-kd did not precipitate PARP1. In the absence of ATP, no binding protein was detected (data not shown). Taken together, these findings suggest that phosphorylation of PARP1 was important for the binding of PARP1 with Txk.

Trimolecular complex formation including Txk, EF-1α and PARP1 in the presence of ATP

Txk-wt and EF-1α were admixed with GST–PARP1N (Fig. 3b,i), GST–PARP1C (Fig. 3b,ii) and GST (Fig. 3b,iii). The mixtures were incubated for 60 min in the presence of

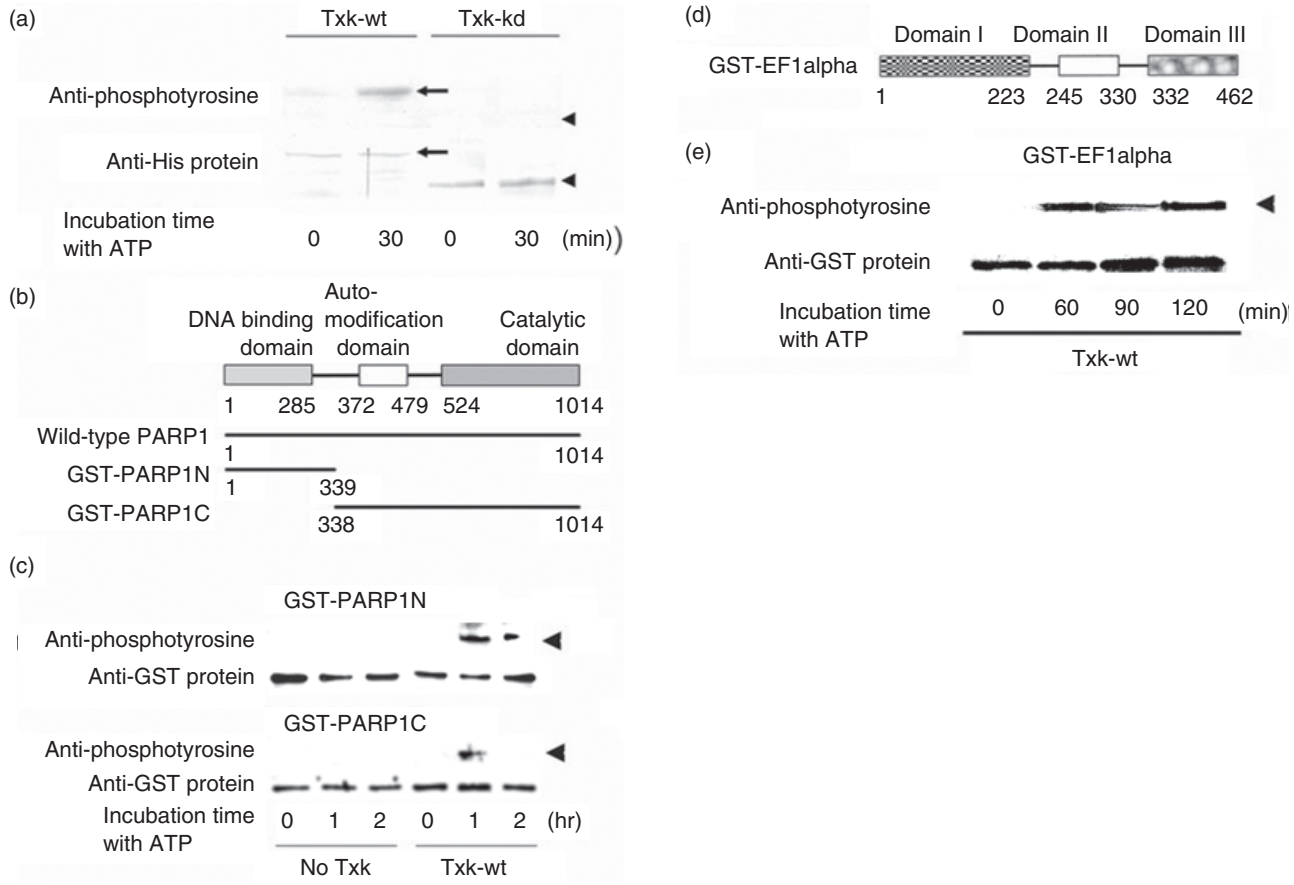

Fig. 2. Phosphorylation of poly(ADP-ribose) polymerase 1 (PARP1) and elongation factor 1α (EF-1α) by wild-type Txk. (a) Kinase activity of Txk-wt and kinase domain deleted mutant Txk (Txk-kd) detected by autophosphorylation. Txk-wt and Txk-kd were incubated with adenosine triphosphate (ATP) for 30 min, and were then analysed with immunoblotting. The upper panel was anti-phosphotyrosine (PY20) immunoblotting. The membrane was re-probed with anti-His protein antibody (the lower panel), indicating amounts of Txk in the reactions. In the presence of exogenous ATP Txk-wt phosphorylated itself (~63 kDa) by its own kinase activity, confirming that Txk-wt has kinase activity (arrow). Txk-kd (~43 kDa) incubated with ATP for 30 min did not phosphorylate itself, indicating that Txk-kd lacked kinase activity (arrowhead). (b) Schematic representation of the domain structure of PARP1 and its deletion mutants. PARP1 includes the DNA binding domain (amino acids, 1–339) and the automodification domain and catalytic domain (amino acids, 338–1014). Wild-type PARP1 (PARP1-wt; ~113 kDa), glutathione-S-transferase (GST)-tagged N terminus region of PARP1 (GST–PARP1N; amino acids, 1–339; ~64 kDa) and GST-tagged C terminus region of GST (GST–PARP1C; amino acids, 338–1014; ~101 kDa) were produced by *Escherichia coli*. (c) Phosphorylation of PARP1 by wild-type Txk. GST–PARP1N and Txk-wt were coincubated with ATP for 0–1–2 h. GST-tagged protein was recovered by the addition of glutathione beads, and was analysed with anti-phosphotyrosine immunoblotting, followed by anti-GST protein immunoblotting. In the presence of ATP, Txk-wt phosphorylated GST–PARP1N (arrowhead). Similarly, phosphorylation of GST–PARP1C was evident by co-incubation with Txk for 1 h (arrowhead). (d) Schematic representation of the domain structure of EF-1α. EF-1α includes domains 1, 2 and 3. We prepared both His-tagged wild-type EF-1α (~51 kDa) and GST-tagged wild-type EF-1α (~76 kDa) by culturing *E. coli*. (e) Phosphorylation of EF-1α by Txk-wt. GST-EF-1α was incubated with Txk-wt in the presence of ATP for 0–120 min, and glutathione-binding proteins were recovered. Arrowhead indicates phosphorylated GST-EF-1α.

ATP. The binding proteins were recovered by the addition of glutathione-sepharose beads. Anti-GST protein immunoblotting indicated amounts of GST-tagged protein in the reaction. EF-1α and Txk-wt that had co-precipitated with GST tagged protein were detected by anti-EF-1α antibody (upper panel) and anti-Txk antibody (middle panel), respectively. GST–PARP1N co-precipitated Txk-wt when the two proteins were co-incubated (middle panel of Fig. 3b,i). Furthermore, GST–PARP1N co-precipitated Txk-wt and EF-1α when the three molecules were co-incubated (upper and middle panels of Fig. 3b,i). GST–PARP1C bound to Txk-wt when the two proteins were co-incubated (middle panel of Fig. 3b,ii). In contrast, GST protein failed to bind neither protein (Fig. 3b,iii). Thus, Txk-wt formed the trimolecular complex involving GST–PARP1N and EF-1α. Collectively, it was suggested that Txk bind to PARP1 and EF-1α to form a trimolecular complex (Fig. 3b,i). The trimolecular complex formation required

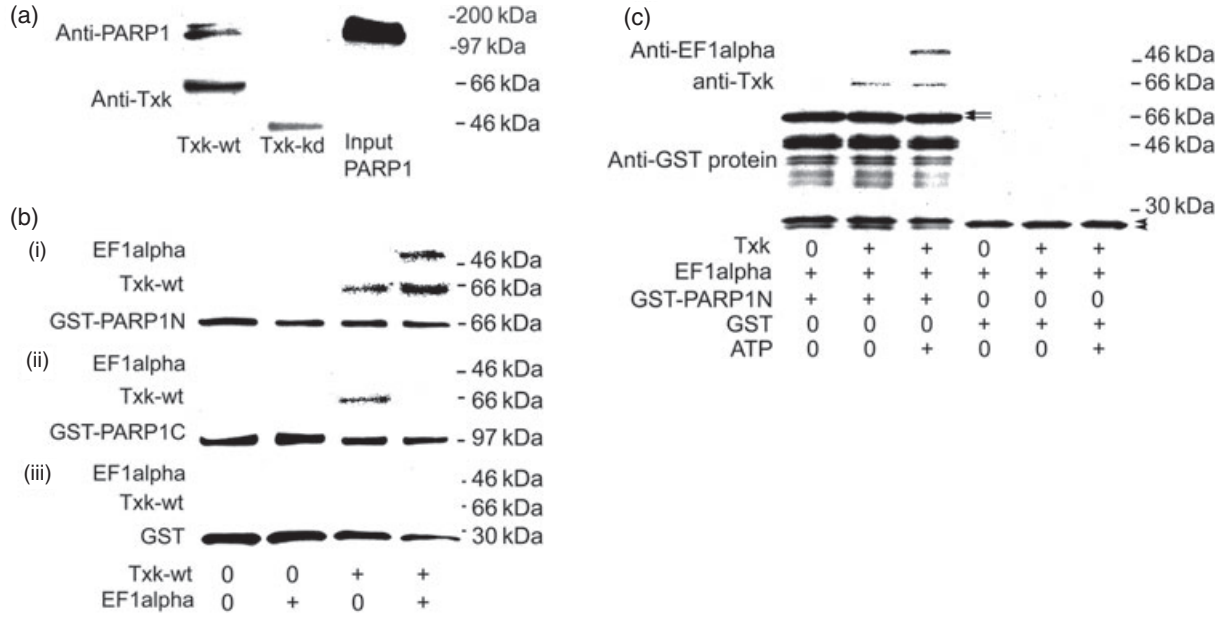

Fig. 3. Phosphorylation dependent trimolecular complex formation of poly(ADP-ribose) polymerase 1 (PARP1), elongation factor 1α (EF-1α) and Txk *in vitro*. (a) Phosphorylation-dependent interaction of unlabelled full-length PARP1 with Txk. Full-length wild-type PARP1 was incubated with Txk-wt and Txk-kd in the presence of adenosine triphosphate (ATP). Txk-wt and Txk-kd, both of which were tagged with poly Histidine, were precipitated by the addition of Ni-NTA resin. After extensive washing, the binding proteins to the beads were analysed by immunoblotting with anti-Txk antibody. The membrane was next analysed with anti-PARP1 antibody. Lower panel indicates amounts of Txk-wt and Txk-kd proteins detected by anti-Txk antibody. Txk-wt co-precipitated PARP1 in the presence of adenosine triphosphate (ATP). Txk-kd did not precipitate PARP1. In the absence of ATP, no binding protein was detected, thus, the results were omitted. (b) Trimolecular complex formation including Txk-wt, EF-1α and PARP1 in the presence of ATP. Txk-wt and EF-1α were incubated with glutathione-S-transferase (GST)–PARP1N (a), GST–PARP1C (b) and GST (c) for 60 min in the presence of ATP. The binding proteins were recovered by the addition of glutathione-sepharose beads. 'Anti-GST protein' indicates amounts of GST-tagged protein. EF-1α and Txk-wt that had co-precipitated with GST-tagged protein were detected by anti-EF-1α antibody (upper panel) and anti-Txk (middle panel) antibody, respectively. (i) GST–PARP1N co-precipitated Txk-wt when the two proteins were co-incubated. Furthermore, GST–PARP1N co-precipitated Txk-wt and EF-1α when the three proteins were co-incubated. (ii) GST–PARP1C bound to Txk-wt when the two proteins were co-incubated. (iii) GST protein (~26 kDa) did not bind either protein. Txk-wt formed the trimolecular complex involving GST–PARP1N and EF-1α, suggesting that Txk bound to EF-1α together with PARP1N (which includes DNA binding domain). (c) To further confirm phosphorylation dependency of the trimolecular complex formation, a mixture of Txk-wt, GST–PARP1N (two arrows) or GST (two arrowheads) and EF-1α was incubated with or without ATP for 60 min, and glutathione-sepharose beads was applied to the mixture. The bead-binding proteins were analysed similarly. EF-1α and Txk-wt bound to PARP1N only in the presence of ATP, suggesting that trimolecular complex formation was phosphorylation-dependent.

exogenous ATP, because the trimolecular complex was not formed without ATP (Fig. 3c).

To confirm phosphorylation dependency of the trimolecular complex formation, a mixture of GST–PARP1N and EF-1α was incubated with Txk-wt and its mutants for 60 min, and glutathione sepharose beads were applied to each mixture. The bead-binding proteins were analysed similarly. Again, Txk-wt bound to EF-1α and GST–PARP1N in the presence of ATP (Fig. 4a). Txk-kd, which lacked kinase activity, did not form a trimolecular complex (Fig. 4a). Txk-K299E and Txk-Y91A, both of which lacked autophosphorylation activity [26], did not form the trimolecular complex. As a control protein GST was treated similarly, and neither protein bound to GST (Fig. 4b). These results suggest that EF-1α and Txk-wt bound to PARP1N only when Txk has kinase activity and that (auto)phosphorylation of Txk may be important for trimolecular complex formation.

IFN-γ promoter binding activity of the trimolecular complex involving Txk, PARP1 and EF-1α

Firstly, we confirmed that protein complex formed in PHA stimulated Jurkat cells bound to the IFNγ promoter (−53/−39) region. We have demonstrated that this complex involved Txk, PARP1 and EF-1α (Fig. 1a). Nuclear proteins of the PHA stimulated Jurkat cells were collected at the indicated time and incubated with DIG-labeled oligoDNA in the presence of poly(dI-dC)(dI-dC). Binding of the protein complex involving Txk, PARP1 and EF-1α to the IFNγ promoter region was detected after PHA stimulation (Fig. 5a). We next performed a gel shift assay to determine whether the trimolecular complex involving Txk bound to the IFN-γ promoter (−53/−39) region. A mixture of the recombinant proteins was tyrosine phosphorylated in the presence of ATP for 60 min. Thereafter, the mixture was subjected to a gel

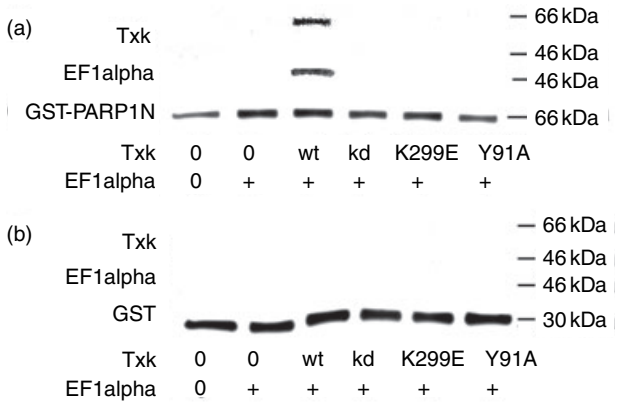

Fig. 4. Phosphorylation dependency of trimolecular complex formation of Txk with poly(ADP-ribose) polymerase 1 (PARP1) and elongation factor 1α (EF-1α). (a) To further confirm phosphorylation dependency of the trimolecular complex formation, a mixture of glutathione-S-transferase (GST)–PARP1N and EF-1α was incubated with Txk-wt or its mutants for 60 min, and glutathione-sepharose beads were applied to the mixture. The binding proteins were analysed similarly. Anti-GST immunoblotting indicated the amounts of GST–PARP1N in the reactions. Anti-Txk and anti-EF-1α indicated the co-precipitated Txk and EF-1α, respectively. EF-1α and Txk-wt bound to PARP1N only when Txk had kinase activity. Mutant Txk which lacked kinase activity did not form a trimolecular complex. (b) As a control protein, GST was analysed similarly.

shift assay using a double-stranded oligoDNA that corresponded to −53 to −39 of the human IFN-γ gene promoter. A combination of GST–PARP1N, Txk-wt and/or EF-1α was incubated with the DIG-labelled oligoDNA in the presence of poly(dI-dC)(dI-dC). The DNA–protein complex appeared in the reaction consisting of three recombinant proteins (arrow, lane 4, Fig. 5b).

Next, the protein mixture was incubated with the DIG-labelled probe in the presence of a 10-fold molar excess of the unlabelled relevant oligoDNA and the unlabelled irrelevant Oct-2 competitor oligoDNA [25] (lanes 5 and 6, Fig. 5b). The DNA–protein complex disappeared specifically by introduction of the relevant oligoDNA (lane 5), but not by the introduction of irrelevant Oct-2 oligoDNA (lane 6), indicating the specificity of the binding (arrow, specific binding of trimolecular complex to the promoter region). It has been reported that PARP1 itself has non-specific DNA binding capability. Two arrows (below the specific bands) indicate non-specific binding of GST–PARP1N to the probe (lanes 2, 3, 4, 5 and 6). Thus, the trimolecular complex bound specifically to the IFN-γ promoter region.

Involvement of poly(ADP-ribosyl)ation of trimolecular complex including Txk by PARP1 in the Th1 cell function

Because Txk formed a trimolecular complex with PARP1 and EF-1α, it was interesting to test whether PARP1

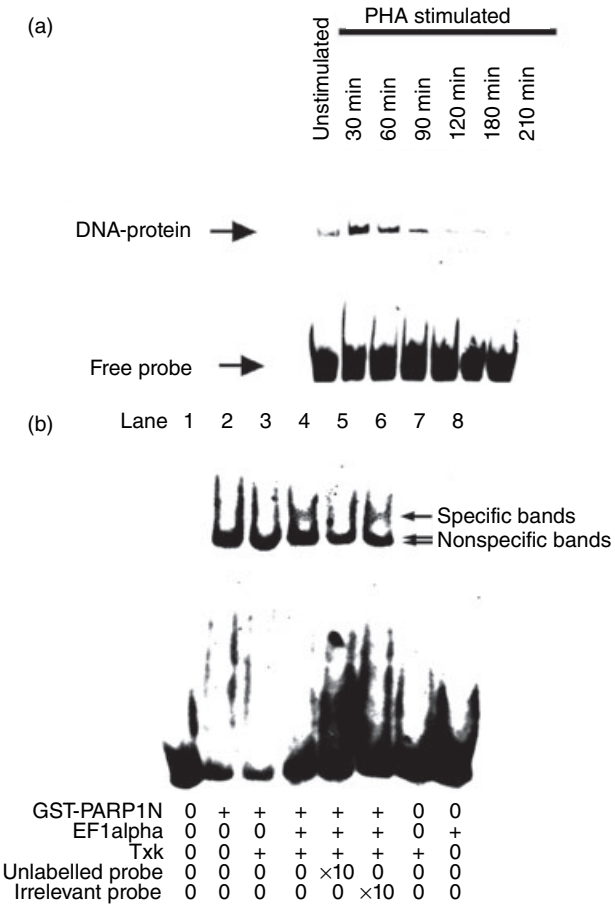

Fig. 5. Binding activity of the trimolecular complex consisting of Txk, poly(ADP-ribose) polymerase 1 (PARP1) and elongation factor 1α (EF-1α) to interferon (IFN)-γ promoter −53/−39 region. IFN-γ promoter region (core region; −53 to −39) was labelled with digoxigenin (DIG). (a) Nuclear proteins of the PHA stimulated Jurkat cells were collected at the indicated time and were incubated with DIG-labelled oligoDNA in the presence of poly(dI-dC)(dI-dC). Binding of protein complex to IFNγ promoter region was detected after PHA stimulation transiently. (b) We next performed a gel shift assay to determine whether the trimolecular complex involving Txk bound to the IFN-γ promoter region. A mixture of 50 ng GST-PARP1N, 25 ng Txk-wt and 25 ng EF-1α was incubated with the DIG-labelled oligoDNA in the presence of 1 μg poly(dI-dC)(dI-dC). To confirm binding specificity, the protein mixture was incubated with the DIG-labelled probe in the presence of a 10-fold molar excess of unlabelled relevant oligoDNA (−53/−39 probe) (lane 5) and unlabelled Oct-2 competitor oligoDNA (lane 6). The DNA–protein complex was disappeared specifically by introduction of the relevant oligoDNA indicating the specificity of the binding (arrow, specific binding of trimolecular complex to the promoter DNA). Two arrows (below the specific bands) indicate non-specific binding of glutathione-S-transferase (GST)–PARP1N to the probe, which appeared in the presence of GST–PARP1N.

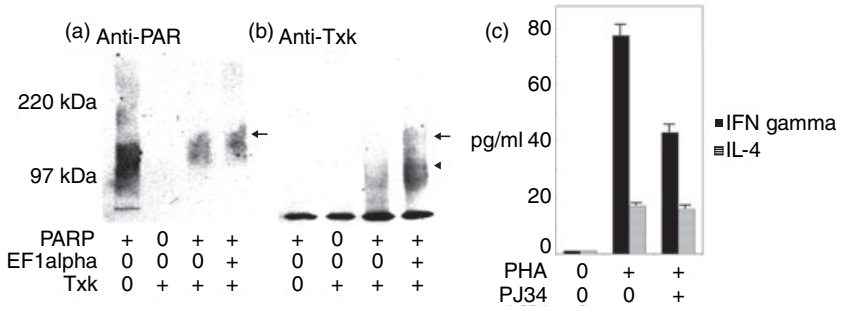

Fig. 6. Poly(ADP-ribosyl)ation of Txk by poly(ADP-ribose) polymerase 1 (PARP1). (a,b) *In vitro* poly(ADP-ribosyl)ation assay was carried out. His-tagged Txk was incubated with or without elongation factor 1α (EF-1α) in the presence of catalytically active full-length PARP1. Thereafter, Txk was recovered as Ni-NTA resin-binding proteins. The precipitated proteins were analysed with immunoblotting. (a) Immunoblotting with anti-poly(ADP-ribose) antibody (anti-PAR) to detect poly(ADP-ribosyl)ated proteins. Arrow indicates the heavily poly(ADP-ribosyl)ated protein, appearing in the presence of Txk, EF-1α and PARP1. (b) The same membrane was re-probed with anti-Txk antibody. Arrow indicates that the precipitated Txk was poly(ADP-ribosyl)ated by PARP1. Arrowhead indicates Txk with less and/or without poly(ADP-ribosyl)ation. (c) Effects of PARP1 inhibitor on the cytokine production of human peripheral blood lymphocytes (PBL). Culture supernatants of phytohaemagglutinin (PHA)-stimulated PBL in the presence/absence of 4000 nM PJ34 were assessed for cytokine production. PJ34 treatment of normal PBL reduced interferon (IFN)-γ production of PBL specifically. Interleukin (IL)-4 production was affected marginally by the PARP1 inhibitor.

poly(ADP-ribosyl)ated Txk in this condition. Thus, an *in vitro* poly(ADP-ribosyl)ation assay of Txk was carried out. Txk was incubated with or without EF-1α in the presence of catalytically active full-length PARP1 (PARP1-wt). His-tagged Txk was precipitated by using Ni-NTA-resin. The poly(ADP-ribosyl)ation of resin binding Txk was tested by immunoblotting with anti-poly(ADP-ribose) antibody (anti-PAR) (Fig. 6). Figure 6a shows the poly(ADP-ribosyl)ated proteins; Fig. 6b indicates immunoblotting with anti-Txk antibody of the same membrane. The size of the Txk (arrow) was nearly 200 kDa, suggesting that the complex may be heavily poly(ADP-ribosyl)ated (arrow). A part of Txk was not or was less poly(ADP-ribosyl)ated (arrowhead, Fig. 6b). These results suggest that Txk was poly(ADP-ribosyl)ated by PARP1.

We next studied whether the PARP1 inhibitor affects cytokine production of human PBL. PBL was preincubated with PJ34, an inhibitor of PARP1, for 1 h. The PBL was then stimulated with PHA for 24 h. Cytokine production was assessed using commercial ELISA kits. PJ34 at a concentration of 4000 nM inhibited IFN-γ protein production potently, whereas IL-4 production was marginally affected (Fig. 6c). These results suggest that PARP1 was involved in Th1 cytokine production, but not Th2 cytokine production.

Confocal analysis of molecular complex formation upon activation of Txk, PARP1 and EF-1α

To evaluate molecular interactions among Txk, PARP1 and EF-1α, we constructed a panel of expression vectors labelled with GFP, DsRed monomer and CFP. Cos7 cells were electrotransfected with the combinations of plasmids. To achieve activation and phosphorylation of Txk, activated Fyn (FynY531F, a constitutive active form of Fyn) and as a negative control, inactivated Fyn (FynK299M, a kinase negative mutant), were transfected simultaneously. Ds-Red-labelled Txk was present in cytoplasm in the inactive condition, and the Txk accumulated in the nucleus when active Fyn was co-transfected (Fig. 7a). GFP-labelled PARP1 was located constitutively in the nucleus (Fig. 7b). When activated, PARP1 and Txk co-localized in the nucleus. Similarly, a vast majority of CFP-labelled EF-1α was located in the cytoplasm in the inactive condition, whereas EF-1α translocated into the nucleus upon activation. EF-1α and Txk co-localized in the nucleus when activated (Fig. 7c). When CFP-labelled EF-1α, GFP-labelled PARP1 and Ds-Red-labelled Txk were co-transfected with active Fyn, co-localization of the three molecules was detected in the nucleus (Fig. 7d).

It is important to confirm that Txk forms a complex with PARP1 and EF-1α in human T cells. Thus, Jurkat cells were transfected with Txk expression plasmid, and after stimulation with PHA the nuclear protein was collected. Proteins that bind to the IFN-γ promoter (−53/−39) region were purified and analysed using the immunoblotting method. The specific antibodies revealed that the DNA binding proteins were detected as PARP1 of 110 kDa (Fig. 7e) and EF-1α of 50 kDa (Fig. 7f). These results confirm that Txk, PARP1 and EF-1α form a complex and bind to the IFN-γ promoter (−53/−39) region in human T cells.

Discussion

Txk is expressed specifically in Th0 and Th1 cells and up-regulates IFN-γ gene transcription, but not IL-2 and IL-4 [24–26]. However, the Txk-mediated IFN-γ specific signal transduction pathway was not understood clearly. Here, we have demonstrated that Txk formed a protein complex on the IFN-γ promoter region which was identified as a Txk-responsive element [25], and that PARP1 and EF-1α were identified as components of the Txk binding protein

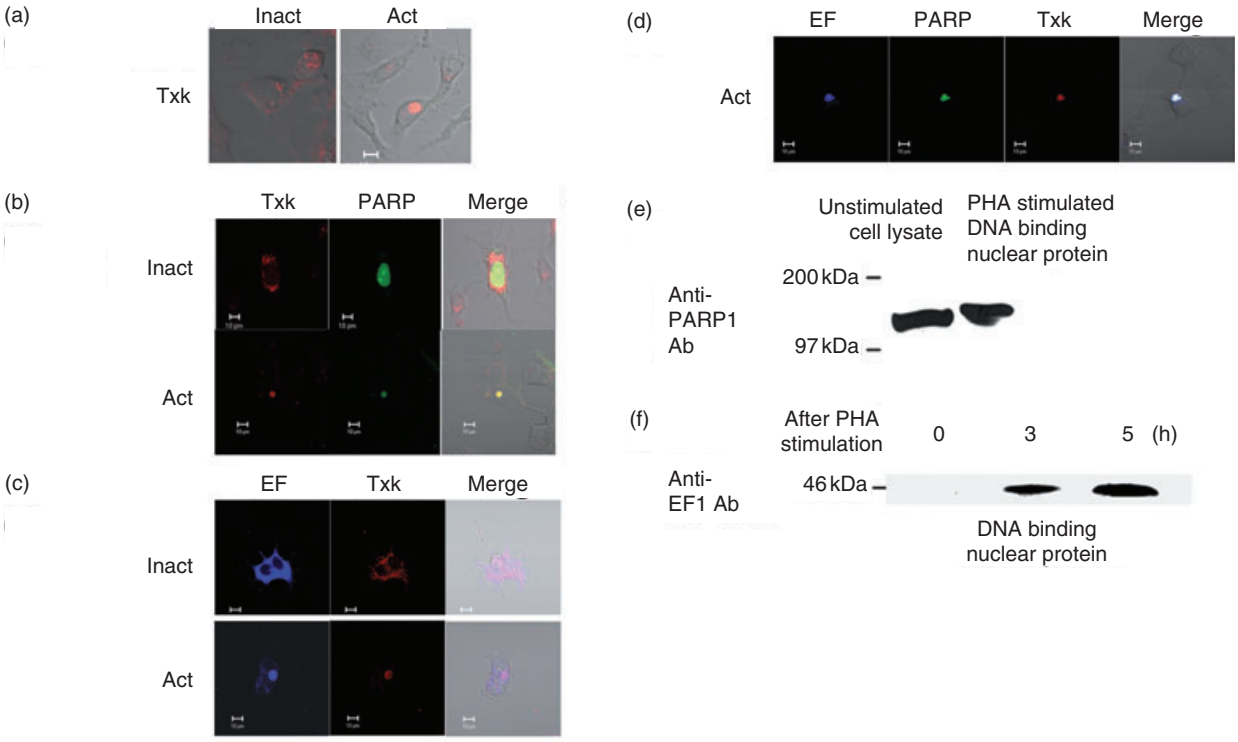

Fig. 7. Multi-colour confocal analysis of molecular complex formation upon activation of Txk, poly(ADP-ribose) polymerase 1 (PARP1) and elongation factor 1α (EF-1α). Cos7 cells were electrotransfected with the combinations of Txk-DsRed monomer, PARP1–GFP and EF-1α-CFP. To achieve activation and phosphorylation of the Txk protein, active Fyn and, as a negative control, inactive Fyn, were transfected simultaneously, and 24 h later the Cos7 cells were analysed. (a) DsRed-Txk was transfected with active and inactive Fyn. DsRed-labelled Txk was present in cytoplasm in the inactivated condition, and the Txk accumulated in the nucleus when active Fyn was co-transfected. (b) GFP-labelled PARP1 and DsRed-Txk were transfected. GFP-labelled PARP1 was located in the nucleus constitutively. In the activated condition, PARP and Txk co-localized in the nucleus. (c) CFP-labelled EF-1α and DsRed-Txk were transfected. A vast majority of CFP-labelled EF-1α and Txk were located in the cytoplasm in the inactive condition. Some of the EF-1α and Txk translocated into nucleus upon activation. EF-1α and Txk co-localized in the nucleus when activated. (d) When CFP-labelled EF-1α, GFP-labelled PARP1 and Ds-Red-labelled Txk were co-transfected with inactive Fyn, Txk and EF-1α located in the cytoplasm, whereas PARP1 located in the nucleus. When active Fyn was used, co-localization of the three molecules was detected in the nucleus. (e, f) Immunoblotting analysis of the IFN-γ promoter (–53/–39) oligoDNA-binding proteins of human T cells. To confirm that Txk binding proteins were PARP1 and EF-1α in the human T cells, Jurkat cells were stimulated with phytohaemagglutinin (PHA) and the nuclear proteins were recovered. The nuclear proteins were allowed to bind to the Txk binding element, and the binding proteins were purified and were analysed using the immunoblotting method. The blots were probed with anti-PARP1 antibody and anti-EF-1α antibody. (e) Nuclear proteins of the PHA-stimulated Jurkat cells for 3 h were probed with the DNA and the binding proteins were analysed. As a control, whole-cell lysate of unstimulated Jurkat cells was included. We confirmed that the 110 kDa protein in the nuclear protein complex from Jurkat cells was PARP1 protein. (f) Nuclear proteins of Jurkat cells with or without PHA stimulation were analysed similarly. The 50 kDa protein in the nuclear protein complex from Jurkat cells were EF-1α.

complex. *In vitro* experiments revealed that Txk and EF-1α formed a trimolecular complex with GST–PARP1N. These results suggest that Txk and EF-1α interact with the N-terminal region of PARP1, including the DNA binding domain.

Role of Txk kinase activity for complex formation

After TCR engagement, Tec family kinases were phosphorylated and activated by Src family kinases. For example, Itk is transphosphorylated at Y511 by Lck, a member of the Src family kinases, which is activated by signals delivered from TCR engagement [17]. Tec, another Tec family kinase, is also activated by Lck through TCR engagement [19]. Transphosphorylation of Itk induces the autophosphorylation of Itk at Y180. Autophosphorylated Itk causes PLCγ1 phosphorylation and activation [17]. Like Itk, Txk is transphosphorylated by Fyn at Y420 after the TCR engagement [20] and phosphorylated Txk subsequently enhances PLCγ1 phosphorylation and activation [52]. Here, we have demonstrated that recombinant Txk induced autophosphorylation [26] which, in turn, phosphorylated PARP1 and EF-1α. It has not been reported that functions of PARP1 and EF-1α are regulated by tyrosine phosphorylation.

However, Txk catalytic activity was essential for trimolecular complex formation because the complex was not formed without ATP. This result was confirmed by using catalytically inactive forms of Txk and suggests that the tyrosine kinase activity of Txk triggers the formation of the protein complex.

We have demonstrated that Y91 and K299 of Txk were important for autophosphorylation and that the mutated Txk reduced IFN-γ, but not IL-2 production from Jurkat cells [26]. When Txk-Y91A and Txk-K299E were used, a trimolecular complex was not formed. It is possible that the phosphorylation of Txk triggers and/or enhances trimolecular complex formation.

Role of PARP1 and PARP1 catalytic activity

PARP1 is an enzyme involved in DNA repair. PARP1 is activated in response to nicks and strand breaks in DNA and catalyses the covalent attachment of ADP-ribose units from NAD^+ molecules onto nuclear target proteins [27].

PARP1 is also required for specific NFκB-dependent gene expression [28,29]. Although IκB was normally degraded and NFκB p65 was translocated into nuclei in response to stimulation, even in the PARP1-deficient cells, binding of NFκB to its responsive element DNA [28] and NFκB-mediated transcriptional activation were reduced in the PARP1-deficient cells [29]. These reports suggest that PARP1 participates in NFκB-dependent gene expression by acting as a co-activator. In addition to NFκB, PARP1 acts as a transcriptional co-factor in response to a variety of transcription factors. PARP1 up-regulates the transcriptional activities mediated by B-MYB [30], AP-2α [31] and Oct-1 [32]. In contrast, PARP1 suppresses transcription mediated by nuclear receptor, such as retinoid X receptors [33].

We investigated whether pharmacological PARP1 inhibitor affects IFN-γ production by human PBL [34]. PARP1 inhibitor, PJ34, suppressed IFN-γ production in PHA-stimulated human PBL. IL-4 production was marginally affected. Another PARP1 inhibitor, benzamide, inhibited IFN-γ protein production by PBL (data not shown). This result suggests that catalytic activity of PARP1 is essential for Th1 cell function and, at least in part, this effect is dependent upon the Txk complex-mediated mechanism.

Previously, several reports have indicated that catalytic activity of PARP1 is important for transcriptional regulation. Chiarugi *et al*. showed that the PARP1 inhibitor, 6(5H)-phenanthridinone, reduced IFN-γ, IL-2 and TNFα mRNA levels in PMA/ionomycin-stimulated lymphocytes and TNFα mRNA level in LPS-stimulated glial cells [35,36]. The PARP1 inhibitor reduced NFκB activation induced by oxidative stress in Hela cells [37]. NFκB has been shown to be poly(ADP-ribosyl)ated by PARP1 [38]. However, another report has shown that enzymatic activity of PARP1 is not essential for transcriptional regulation by NFκB [28]. Implication of PARP1 enzymatic activity in transcriptional regulation may vary dependent upon an individual gene, cell type and experimental model. Our results suggest that enzymatic activity of PARP1 is necessary for the Txk-mediated transcriptional regulation of IFN-γ gene.

PARP1 affects the activity of other enzymes. DNA-dependent kinase (DNA-PK), which functions in the DNA repair pathway, phosphorylated PARP1 and in turn DNA-PK was poly(ADP-ribosyl)ated by phosphorylated PARP, resulting in enhanced kinase activity of DNA-PK [39]. Recently, it has been shown that activation of the p38 MAP kinase and subsequent phosphorylation of ATF-2 by LPS stimulation were impaired in PARP1-deficient glial cells [40]. Although these impairments were signalling pathway- and cell/tissue type-specific, it was suggested that PARP1 participated in the specific signalling pathway that leads to phosphorylation of transcription factors.

In this study, we observed the poly(ADP-ribosyl)ation of the transcriptional complex, including Txk. In addition a PARP1 inhibitor, PJ34, suppressed IFN-γ production by human PBL. Accordingly, PARP1 catalytic activity may modify Txk kinase activity, binding the Txk complex to the Txk-responsive element region, and/or initiation (and maintenance) of Txk-mediated transcription. We hypothesize that Txk complex-mediated signal transduction is regulated by multi-step mechanisms. The first step is Txk autophosphorylation, the second step is Txk–PARP1–EF-1α interaction and the final step is binding of the trimolecular complex to the Txk-responsive element region. Recombinant PARP1 by itself bound to the double-stranded DNA in the gel shift assay (two arrows in Fig. 5b) [41]. A specific band detected in the reaction consisted of PARP1N, EF-1α and Txk, which was sensitive to the excess of the relevant DNA but was resistant to the irrelevant DNA in the cold inhibition study. A supershift assay using specific antibodies was unsuccessful in our hands for reasons unknown. We speculate that PARP1 in the reaction affected the antigen-binding ability of the antibody in the gel shift assay.

Recently, it has been shown that PARP1 inhibitor, 6(5H)-phenanthridinone (PHE), benzamide and PJ34 ameliorate experimental allergic encephalomyelitis (EAE) in rats [35] and mice [42]. These reports have suggested that the Th1-mediated immune response may be reduced by treatment of PARP1 inhibitors. Here, we have demonstrated that PJ34 reduced IFN-γ production, but not IL-4 production, in PHA-stimulated human PBL. Taken together, these results suggest the possibility that the catalytic activity of PARP1 participates in the Th1-specific immune response by enhancing Txk-mediated transcriptional activation.

Role of EF-1α

It is well known that EF-1α mediates peptide elongation by catalysing transfer of aminoacyl-tRNA to ribosome [43]. The majority of the EF-1α proteins are located in cytoplasm [44], while a subpopulation of EF-1α is observed in nuclei

[45]. Therefore, EF-1α in nuclei seem to have an alternative function.

It has been demonstrated that the C-terminal region of EF-1α binds to SH2 and SH3 domains of PLCγ using a yeast-two-hybrid system [46]. Because Txk has SH2 and SH3 domains, it is possible that SH2 and SH3 domains of Txk bind to the C-terminal region of EF-1α. Interaction between EF-1α and the zinc finger protein, ZPR1, was demonstrated in EGF-treated A431 cells from epidermoid carcinoma. EF-1α was translocated to the nuclei together with ZPR1 by activation of A431 cells with EGF treatment [47]. EF-1α/ZRP1 complex and its translocation to nuclei was essential for normal cell proliferation. This led us to consideration of the role of EF-1α in Th1 cell functions. Here we have demonstrated that EF-1α was phosphorylated at tyrosine residues by Txk. It may be possible that translocation of EF-1α from cytoplasm to nuclei is induced by the tyrosine phosphorylation of EF-1α. However, the hypothesis will need to be tested in the near future.

We have demonstrated that EF-1α formed a complex with Txk and PARP1 on the IFN-γ gene promoter region in PHA-stimulated Jurkat cells (Fig. 1a). Our results suggest that T lymphocyte activation may induce translocation of EF-1α to the nuclei together with Txk, and that EF-1α may be involved in the Txk-mediated transcription. Studies of EF-1α mutated mice suggest its importance for lymphocyte development. Wasted mice (wst/wst) show neurological abnormality, deficient DNA repair and immunodeficiency. The wasted mice have a spontaneous recessive mutation of EF-1α2 and deficiency of the mRNA expression, both of EF-1α1 as well as EF-1α2, leading to a total EF-1α shortage [48]. The immunological abnormalities of wasted mice include progressive atrophy of thymus and spleen, decrease in the level of circulating lymphocytes and the increased apoptosis of thymocytes [49–51]. These abnormalities imply the importance of EF-1α in lymphocyte function. Moreover, our results suggest that EF-1α participates in Txk-mediated transcriptional regulation of the IFN-γ gene. To clarify whether EF-1α is critical for the Txk complex-mediated transcriptional regulation, investigation of the T lymphocyte function in wasted mice will be required.

Molecular interactions of the trimolecular complex were analysed in the Cos7 cells transfected with the relevant plasmids. PARP1 was constitutively present in the nucleus. Txk was located in the cytoplasm in the resting condition, and upon activation with Fyn, Txk moved to the nucleus. EF-1α located in the cytoplasm, but considerable amounts were found in the nucleus. Multi-colour analysis of Txk, PARP1 and EF-1α confirmed that the three molecules accumulated in the nucleus upon activation. The merged panel showed that the overlapping areas of Txk, PARP1 and EF-1α were much smaller than the nucleus itself (cf. Figure 7d, panel PARP inactive *versus* active). Thus, a possibility exists that the three molecules accumulated somewhere in the nucleus upon activation.

These results suggest that the formation of the Txk complex participates in Th1-specific transcriptional regulation. Further studies are needed to develop a new therapeutic approach against diseases accompanying skewed Th1/Th2 balance targeting these molecules.

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