14-3-3τ associates with a translational control factor FKBP12-rapamycin-associated protein in T-cells after stimulation by pervanadate

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Abstract Proteins of the 14-3-3 family can associate with and/or modulate the activities of a variety of proteins, such as protooncogene and oncogene products, Cdc25 phosphatases and phosphatidylinositol 3-kinase, and thus are implicated in regulation of signaling pathways and the cell cycle. We report here that treatment of Jurkat T-cells with an inhibitor of protein tyrosine phosphatase, pervanadate, induces the association of 14-3-3τ with a translational control factor, FKBP12-rapamycin-associated protein (FRAP), with significant latter's autophosphorylation. Coimmunoprecipitation of various mutants of FRAP coexpressed with 14-3-3τ in COS-7 cells revealed that 14-3-3τ binds to the C-terminal side of FRAP at unknown site(s) different from the predicted binding motifs to date.

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Key words: 14-3-3; FKBP12-rapamycin-associated protein; Pervanadate; Autophosphorylation; Translational control

1. Introduction

The signal transduction pathways involved in translational control have remained uncharacterized, although work on the regulation of gene expression through transcription has made steady progress. Recently, the immunosuppressor rapamycin, an inhibitor of G1 cell cycle progression, was used to illuminate a growth factor-regulated signaling pathway in T-cells that leads to the enhanced translation of a specific subset of mRNAs which encode ribosomal proteins, translation elongation factors (eEF1A and eEF2), and an insulin-like growth factor II [1–3]. The inhibitory effects of rapamycin on cell cycle progression and translation are based first upon an association of rapamycin with the intracellular protein FKBP12 [4]. This FKBP12-rapamycin complex is a high affinity inhibitor of a 289 kDa protein termed FKBP12-rapamycin-associated protein (FRAP; also called RAFT1/mTOR), a member of a family of phosphatidylinositol (PI) kinase-related kinases [5]. The kinase domain of FRAP exhibits homology to the PI3-kinase p110 subunit and phosphorylates the eukaryotic translation initiation factor, 4E binding protein 1 (4E-BP1), and 70 kDa S6 kinase ($p70^{S6k}$) [6–13]. Although the biological regulation and function of FRAP are not completely understood, FRAP may be a key enzyme in the translational control of a growth factor-regulated signaling pathway.

Proteins of the 14-3-3 family have been implicated in regulation of the signaling pathway and cell cycle via their interaction with and/or modulation of the activity of Raf [14–17], Cdc25 phosphatase [18], protein kinase Cθ [19], PI3-kinase [20], Cbl [21], Bcr and Bcr-Abl [22,23], insulin-like growth factor I receptor and insulin receptor substrate I [24], BAD [25], and A20 [26]. In this study, we first demonstrate that 14-3-3τ, a predominant isoform among the 14-3-3 proteins in T-cells [19,27], associates with the autophosphorylated FRAP in the stimulated T-cells.

2. Materials and methods

2.1. Materials

Phorbol-12-myristate-13-acetate (PMA) and sodium orthovanadate were purchased from Sigma. Polyclonal antibodies against 14-3-3τ (also called 14-3-3θ) (C-17) and anti-FRAP (N-19) were from Santa Cruz Biotechnology, and the monoclonal anti-FLAG M2 antibodies and anti-FLAG M2 affinity gel were from Kodak. The horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fragment of goat anti-rabbit IgG and anti-mouse IgG were from Zymed, and the HRP-conjugated $F(ab')_2$ fragment of rabbit anti-goat IgG was from Cappel. All other reagents were commercial products of the highest grade available.

2.2. Plasmids

pBJ5-FLAG epitope-tagged wild-type FRAP [11], mutant D2357E FRAP and mutant S2035T FRAP, which has no binding activity as to the FKBP12-rapamycin complex, were kindly donated by Dr. S.L. Schreiber (Harvard University, Cambridge, MA, USA). Deletion mutants Δ44–1336 FRAP and Δ1703–1996 FRAP were generated by digestion with *Sac*I and *Afl*III, respectively. 14-3-3τ cDNA was cloned from a human T-cell cDNA library constructed using pcDL-Sra296 vector [28]. Glutathione *S*-transferase (GST)-14-3-3τ fusion protein was made as follows: *Bam*HI and *Eco*RI restriction sites were created at the 5′ and 3′ end, respectively, of human 14-3-3τ cDNA by PCR. The PCR product was then subcloned into pGEX-2T (Amersham Pharmacia Biotech).

2.3. Cell culture and cell stimulation

The human leukemic T-cell line, Jurkat, was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G and 60 μg/ml kanamycin). Cells were stimulated with 100 μM pervanadate for 10 min [20] and stimulation was terminated by washing two times with RPMI 1640 medium. Then the cells were immediately lysed in 1.0 ml/10^7 cells of ice-cold lysis buffer (40 mM Tris–HCl buffer, pH 7.5, containing 300 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 10 mM NaF, 4 mM Na_3VO_4, 20 μg/ml aprotinin and 20 μg/ml leupeptin). Cells were sonicated for 10 s and then insoluble materials were removed by centrifugation at 15 000×*g* at 4°C for 20 min. COS-7 cells, cultured in Dulbecco's

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Abbreviations: PI, phosphatidylinositol; PMA, phorbol-12-myristate-13-acetate; PVDF, polyvinylidene difluoride; $p70^{s6k}$, 70 kDa S6 kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; GST, glutathione *S*-transferase; 4E-BP1, 4E binding protein 1

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modified Eagle's medium containing 10% fetal bovine serum and antibiotics, were transiently transfected with plasmid DNAs by electroporation using a Bio-Rad Gene Pulser (230 V, 960 μF). After transfection for 48 h, cells were stimulated with 100 μM pervanadate or 500 ng/ml PMA for 10 min. Then the cells were lysed in 0.5 ml/10 cm dish of ice-cold lysis buffer for 15 min and then centrifuged (15 000 × *g*, for 15 min at 4°C). The supernatant materials were then used for analyses.

2.4. Coimmunoprecipitation of FRAP with 14-3-3τ and Western immunoblotting

Cell lysates (1 ml) were mixed with 1 μg of antibodies for 3 h at 4°C, and then incubated with 20 μl of 50% protein G Sepharose beads for an additional 2 h. The immunoprecipitates were washed four times with lysis buffer, and then eluted with 3×Laemmli's sodium dodecyl sulfate (SDS) sample buffer with boiling for 10 min. In some experiments, the cell lysates (0.5 ml) were immunoprecipitated with 20 μl of anti-FLAG M2 affinity gel with incubation for 3 h at 4°C. The immunoprecipitates were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (4–20% gradient) under reducing conditions and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were then incubated with the indicated primary antibodies, followed by HRP-conjugated secondary antibodies. Bound antibodies were visualized with an ECL detection system (Amersham) according to the manufacturer's instructions.

2.5. Kinase assay

Autophosphorylation of the immunoprecipitates of FRAP was carried out for 20 min at 30°C in 25 mM HEPES buffer, pH 7.7, containing 50 mM KCl, 10 mM $MgCl_2$, 0.1% NP-40, 20% glycerol, 1 mM DTT, 100 μM ATP and 1 μCi of [γ-^{32}P]ATP, and the reactions were terminated by adding 3×SDS sample buffer. The reaction products were then separated by SDS–PAGE and transferred to a PVDF membrane, and then ^{32}P incorporation into the FRAP and various mutants was quantitated with a Bio Imaging Analyzer BAS1500 (Fuji Photo Film).

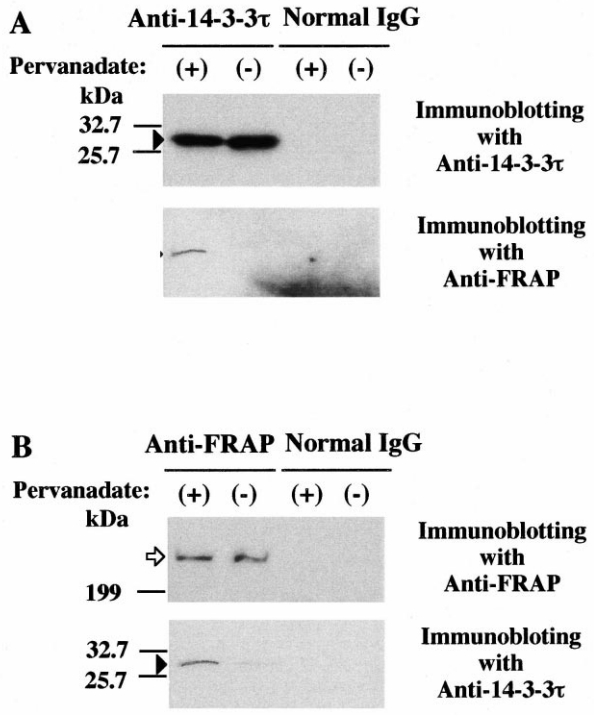

Fig. 1. Association of FRAP with 14-3-3τ in human T-cells. Jurkat cell lysates of unstimulated cells (−) or cells after stimulation by pervanadate (+) were immunoprecipitated with anti-14-3-3τ or normal control rabbit IgG (A), and anti-FRAP or normal goat IgG (B). The immunoprecipitates were resolved by 4–20% gradient SDS–PAGE and then transferred to PVDF membranes. The blots were probed with anti-FRAP and anti-14-3-3τ antibodies, and visualized with ECL. Arrows and arrowheads indicate FRAP and 14-3-3τ, respectively.

3. Results and discussion

3.1. Association of FRAP with 14-3-3τ in T-cells after stimulation by pervanadate

An intracellular stimulatory regulator for FRAP has not been detected so far, although inhibitory regulators have been well characterized [4,6,11]. In the studies on T-cell stimulation by a tyrosine phosphatase inhibitor, pervanadate, which rapidly induces a series of proximal T-cell activation events closely resembling those induced by T-cell antigen [29,30], we found 14-3-3τ associates with PI3-kinase as reported [20,21] as well as FRAP as a novel binding protein, the C-terminal domain sequence of 660 amino acids of FRAP exhibiting a homology to mammalian PI3-kinase (about 40% identity), and several other known PI kinases, such as yeast PI3-kinase, *VPS34* and *PIKI* [12].

Jurkat cells were stimulated with pervanadate for 10 min, and then cell lysates were immunoprecipitated with anti-14-3-3τ or anti-FRAP antibodies. The precipitates were then subjected to SDS–PAGE followed by Western immunoblotting. As shown in Fig. 1A, a protein band immunoreactive with anti-FRAP IgG was detected for the immunoprecipitates of cell lysates with anti-14-3-3τ IgG, but not for those with non-immunized IgG. Conversely, a protein band immunoreactive with anti-14-3-3τ IgG was observed for the immunoprecipitates with anti-FRAP IgG (Fig. 1B). The association between FRAP and 14-3-3τ in T-cell lysates without activation by pervanadate, however, was very low.

To confirm the signal-dependent association of FRAP with 14-3-3τ in other cells, FRAG-tagged FRAP was coexpressed in COS-7 cells with 14-3-3τ, and after 48 h, the cells were treated with 100 μM pervanadate or 500 ng/ml of PMA for 10 min. Then cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel. After Western blotting of the immunoprecipitates, 14-3-3τ, which was coimmunoprecipitated with FRAP, was detected with the anti-14-3-3τ antibodies. Although the association between FRAP and 14-3-3τ was low in the unstimulated COS-7 cells, the FRAP/14-3-3τ association in the cells stimulated by pervanadate was significantly enhanced (Fig. 2). PMA, however, did not induce the FRAP/14-3-3τ association, although the mechanism underlying this finding has not been clarified yet.

To determine whether or not the association of FRAP with 14-3-3τ is direct, we treated nitrocellulose membranes containing the electrophoresed FRAG-tagged FRAP immunoprecipitates with recombinant GST-14-3-3τ protein or GST as a control for 3 h, and then detected the binding of 14-3-3τ with anti-GST monoclonal antibodies. GST-14-3-3τ but not the control GST protein bound to the FRAP (Fig. 3). This indicates that 14-3-3τ most likely binds directly to FRAP.

3.2. Association of 14-3-3τ with the autophosphorylated FRAP and the binding domain of FRAP for 14-3-3τ

In a mitogen-stimulated signaling pathway in T-cells, the autophosphorylation of FRAP accompanies the activation of FRAP kinase, which phosphorylates translation factors [12]. We analyzed the effect of the autophosphorylation of FRAP on the association of 14-3-3τ with FRAP. As a neg-

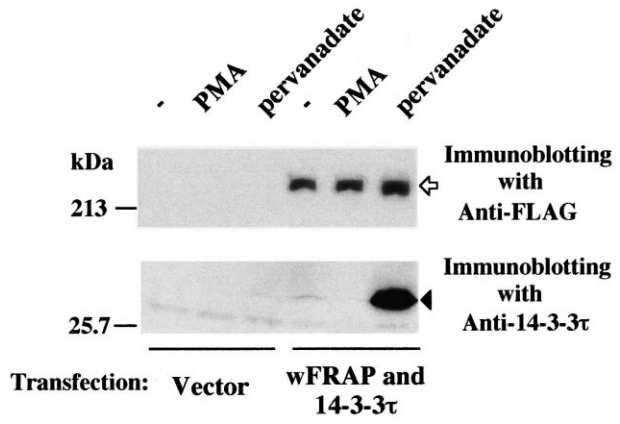

Fig. 2. Association of FRAP with 14-3-3τ in COS-7 cells. COS-7 cells were transiently co-transfected with pBJ5-FLAG-tagged wild-type FRAP (wFRAP) and pcDL-SRa296-14-3-3τ (5 and 7.5 μg of DNA, respectively), or the vectors. After transfection for 48 h, the cells were stimulated with 100 μM pervanadate or 500 ng/ml PMA for 10 min. Cell lysates of the stimulated or unstimulated (−) cells were immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitates were resolved by 4–20% gradient SDS–PAGE and then transferred to PVDF membranes. The blots were probed with anti-FLAG and anti-14-3-3τ antibodies, and visualized with ECL. Arrows and arrowheads indicate FRAP and 14-3-3τ, respectively.

ative control in an investigation of the function of FRAP kinase and its autophosphorylation, mutant D2357E FRAP, which has no kinase and autophosphorylation activities but retains binding activity as to FKBP12-rapamycin, was used. FLAG-tagged wild-type FRAP, mutant D2357E FRAP and the N-terminal side-deletion mutant, Δ44–1336 FRAP, were coexpressed in COS-7 cells with 14-3-3τ, and after 48 h, the cells were treated with 100 μM pervanadate for 10 min. Immunoprecipitates of the cell lysates with anti-FLAG M2 affinity gel were then subjected to an in vitro kinase assay (Fig. 4). Pervanadate induced the association of 14-3-3τ with wild-type FRAP, together with a marked increase in the autophosphorylation of FRAP. Pervanadate also stimulated the 14-3-3τ/Δ44–1336 FRAP association and the latter's autophosphorylation, the association and autophosphorylation being almost equivalent to those observed for the cells expressing the wild-type FRAP and 14-3-3τ. On the other hand, the D2357E FRAP/14-3-3τ association induced by pervanadate was significantly lower than the wild-type FRAP/14-3-3τ association, and no autophosphorylation of D2357E FRAP was observed. Since autophosphorylation of FRAP is accompanied by the kinase function of FRAP that leads to activation of $p70^{S6k}$ and 4E-BP1 [12], autophosphorylation of FRAP accompanied by 14-3-3τ association may be an important process in a T-cell activation by pervanadate. The results suggest that 14-3-3τ predominantly binds to the autophosphorylation site(s) on the C-terminal side of FRAP, although the binding and autophosphorylation site(s) have not been clarified yet. This speculation is supported by the previous findings that the interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine [31–33]. On the other hand, our findings also indicate that 14-3-3τ also binds partly to the non-autophosphorylation site(s) of FRAP, because FRAP D2357E bound to 14-3-3τ to a limited extent (Fig. 4).

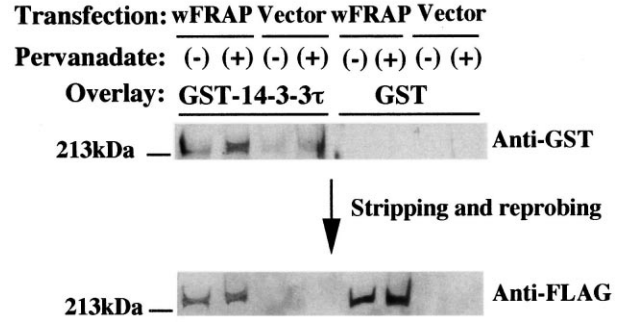

Fig. 3. Direct interaction of 14-3-3τ to FRAP. COS-7 cells were transiently transfected with pBJ5-FLAG-tagged wild-type FRAP (wFRAP) or the empty vector (5 μg of DNA) for 48 h, and then stimulated with 100 μM pervanadate for 10 min. Cell lysates of the stimulated (+) or unstimulated (−) cells were immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitates were subjected to 4–20% gradient SDS–PAGE and then transferred to a nitrocellulose membrane. The membrane was overlaid with 5 μg/ml of recombinant GST-14-3-3τ or GST, and the binding was detected with anti-GST antibodies. The membrane was then stripped and reprobed with anti-FLAG antibodies. The bound antibodies were visualized with ECL.

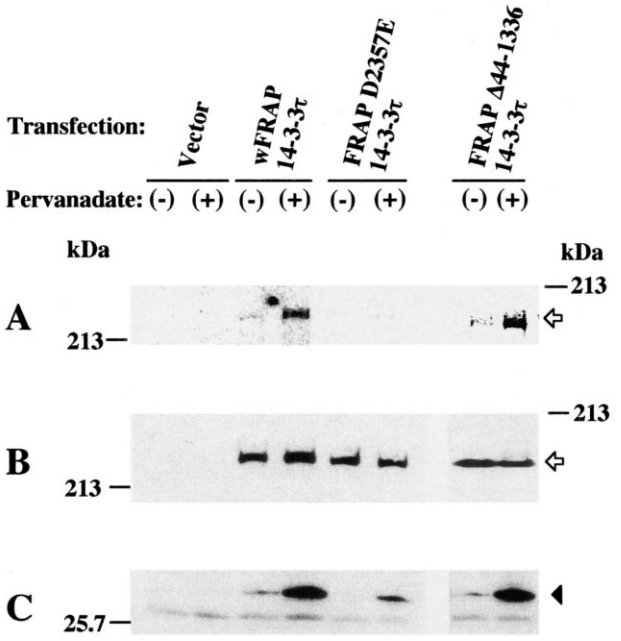

Fig. 4. Autophosphorylation of FRAP and its variants, and their association with 14-3-3τ after stimulation by pervanadate. COS-7 cells were transiently co-transfected with pBJ5-FLAG-tagged wild-type FRAG (wFRAP), FRAP variants D2357E and Δ44–1336, or the empty vector (each 7.5 μg of DNA), and pcDL-SRa296-14-3-3τ (5 μg of DNA), for 48 h. After transfection, the cells were stimulated with 100 μM pervanadate for 10 min. Cell lysates of the unstimulated (−) or pervanadated stimulated (+) cells were immunoprecipitated with anti-FLAG M2 affinity gel. The immunoprecipitates were incubated with [γ-^{32}P]ATP for 20 min at 30°C, and the reactions were terminated by adding 3×SDS sample buffer. The reaction products were resolved by 4–20% gradient SDS–PAGE and transferred to PVDF membranes, and then ^{32}P incorporation into wFRAP and its mutants, D2357E and Δ44–1336, was detected with a BAS1500 (A). FLAG-tagged wFRAP, D2357E and Δ44–1336, in the immunoprecipitates (B) and 14-3-3τ in the immunoprecipitates (C) were quantified by immunoblotting with anti-FLAG and 14-3-3τ antibodies, respectively. Arrows indicate wFRAP and FRAP variants, and arrowhead indicates 14-3-3τ.

Based on the results of analysis of the interaction of 14-3-3 with Raf-1 and Cdc25, the sequences, RSxpSxP and RxY/FxpSP (single-letter amino acid code, where x is any amino acid and pS is a phosphoserine residue), are proposed for 14-3-3 binding motifs [31–33]. Although FRAP does not contain the exact motifs, it has the sequence, R^{1890}SISLS^{1895}, which is similar to the putative binding motif, RSxpSxP, but the C-terminal proline is substituted by serine, on the N-terminal adjacent region to the FKBP12-rapamycin binding site of FRAP. In order to determine whether or not this motif in FRAP plays a role in the association with 14-3-3τ, truncation mutant, Δ1703–1996 FRAP, in which the area encoding this sequence was deleted, was constructed. As assessed by FRAP coexpression assaying of COS-7 cells with 14-3-3τ described in Fig. 2, the Δ1703–1996 FRAP/14-3-3τ association after stimulation by pervanadate was almost equivalent to the wild-type FRAP/14-3-3τ association (data not shown). We further examined the possibility of FRAP/14-3-3τ association at the FKBP12-rapamycin binding site, S2035, because both the protein kinase activity and autophosphorylation of FRAP are inhibited by FKBP12-rapamycin [11]. However, S2035T FRAP/14-3-3τ association in COS-7 cells after stimulation by pervanadate was also equivalent to the wild-type FRAP/14-3-3τ association (data not shown). Further definition through deletion and mutation of the C-terminal side domain of FRAP will facilitate investigations to identify the exact binding site(s) for 14-3-3τ, and to analyze the mechanism of stimulation of FRAP kinase.

In studies on immunosuppressor rapamycin, it was suggested that FRAP promotes the phosphorylation of S6 via $p70^{S6k}$ activation, and releases 4E-BP1 from eIF-4E through the phosphorylation of 4E-BP1, and that FRAP regulates the translation to proteins [34–36]. In addition, biological evidence that the 14-3-3 protein positively regulates rapamycin-sensitive signaling in budding yeast has recently been reported [37], although the target of the 14-3-3 protein has not been clarified yet. This biological evidence strongly supports our finding of an interaction between 14-3-3τ and FRAP and suggests the biological significance of the 14-3-3τ/FRAP association. In the present study, we demonstrated the 14-3-3τ/FRAP association. The other isoforms of 14-3-3 family proteins may also bind to FRAP in various cells because of high structural similarity among 14-3-3 family proteins. Further studies on the role of 14-3-3τ in FRAP-mediated translational regulation in T-cells are now in progress.

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