

Pim-1 translocates sorting nexin 6/TRAF4-associated factor 2 from cytoplasm to nucleus¹

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Abstract Pim-1, an oncogene product of serine/threonine kinase, has been found to play roles in apoptosis induction/suppression, cell-cycle progression and transcriptional regulation by phosphorylating the target proteins involved in these processes. The target proteins phosphorylated by Pim-1, including p100, Cdc25A, PAP-1 and heterochromatin protein 1, have been identified. The precise functions of Pim-1, however, are still poorly understood. In this study, we identified tumor necrosis factor receptor-associated factor 4-associated factor 2/sorting nexin 6 (TFAF2/SNX6) as a Pim-1-binding protein, and we found that TFAF2/SNX6 was phosphorylated and translocated from the cytoplasm to nucleus by Pim-1. This translocation of the protein was not affected by Pim-1-dependent phosphorylation. Since sorting nexins, including TFAF2/SNX6, have been reported to be located in the cytoplasm or membrane by association with several receptors of tyrosine- or serine/threonine-kinase, this is the first report of TFAF2/SNX6 being located in the nucleus after binding to Pim-1. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Tumor necrosis factor receptor-associated factor 4-associated factor 2; Sorting nexin 6; Pim-1

1. Introduction

Pim-1 is a protooncogene product that possesses a serine/threonine protein kinase activity and has been found to cooperate with c-Myc, another protooncogene product, in lymphomagenesis [1–4]. Recently, Pim-2 and Pim-3/Kid1, other Pim-family proteins, have been identified [5–8], and the functions of these proteins appear to complement each other in mice in which the gene for one of the Pim-family proteins was disrupted, thereby giving no severe defect [9]. Pim-1 is located both in the cytoplasm and nucleus, but the distinct

roles of Pim-1 in these two locations have not been elucidated. To analyze the functions of Pim-1, several binding- and phosphorylation-target proteins of Pim-1 have been identified. These proteins include p100, which is an activator of c-Myb transcription factor [10]; cdc25A of protein phosphatase, which activates Cdk2, leading to progression of the cell cycle [11]; HP-1, which is a heterochromatin-binding protein and works in gene silencing [12]; and PAP-1, which is a novel protein functioning in transcription repression and splicing regulation [13]. All of these proteins are located in the nucleus, where Pim-1 is colocalized with these proteins. Pim-1 has been known to be involved in the cytokine signaling pathway [14–17]. In interleukin 6 (IL-6)-drive signaling in lymphocyte-lineage cells, STAT3 targets both *c-myc* and *pim-1* genes and functions in cell-cycle progression and cell survival, anti-apoptotic functions [18,19]. In fibroblasts, on the other hand, a proapoptotic function of Pim-1 dependent on *c-myc* has been reported [11,20,21]. Despite these recent findings regarding Pim-1, the precise functions of Pim-1 are still poorly understood.

In order to try to determine the functions of Pim-1, we have carried out a yeast two-hybrid screening to identify Pim-1-binding proteins. In this study, in addition to PAP-1 and HP-1, which we previously identified [12,13], we identified TFAF2/SNX6 (tumor necrosis factor receptor-associated factor 4 (TRAF4)-associated factor 2/sorting nexin 6) as a Pim-1-binding protein. The cDNAs of TFAF2 and SNX6 were independently cloned by us and others as cDNAs encoding TRAF4-binding protein and Smad1, epidermal growth factor (EGF)- or transforming growth factor (TGF)- β receptors [22,23], respectively. In these combinations, TFAF2/SNX6 is located in the cytoplasm targeting the Golgi network [23]. In this study, we found that TFAF2/SNX6 is translocated from the cytoplasm to nucleus by phosphorylation function-independent Pim-1.

2. Materials and methods

2.1. Cells

Human 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% calf serum.

2.2. Plasmids

pGLex-Pim-1 Δ 2, pcDNA3-FLAG-Pim-1 and pcDNA3-Pim-1-deletion mutants were described previously [12]. pcDNA3-hemagglutinin-tagged (HA)-TFAF2 and pcDNA3-FLAG-TFAF2: TFAF2 cDNA

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¹ The nucleotide sequence reported in this paper has been submitted to the EMBL/DDBJ/GenBank data bank with accession number U83194.

Abbreviations: TFAF2, tumor necrosis factor receptor-associated factor 4-associated factor 2; SNX6, sorting nexin 6; PX, phox homolog; HP-1, heterochromatin protein 1

was inserted into *EcoRI*–*XhoI* sites of pcDNA-HA and pcDNA3-FLAG, respectively [12]. pACT-TFAF2 and pACT-TFAF2-deletion mutants: cDNAs of TFAF2 starting from the first ATG and of TFAF2-deletion mutants synthesized by PCR were inserted into *EcoRI*–*XhoI* sites of pACT2.

2.3. In vivo-binding assay

1 µg of pCMV-FLAG-Pim-1 together with 1 µg of pCMV-HA-TFAF2 was transfected to human 293T cells 60% confluent in a 10 cm dish using Lipofect Amine plus (Gibco BRL). Forty-eight hours after transfection, the whole cell extract was prepared by the published procedure [24]. Approximately 500 µg of the 293T cell proteins was first immunoprecipitated with a mouse anti-FLAG antibody (M2, Sigma) in a buffer containing 50 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.5), 1 mg/ml bovine serum albumin, 150 µg/ml phenylmethylsulfonyl fluoride and 0.25% NP-40. After washing with the same buffer except for 0.05% instead of 0.25% NP-40, the precipitates were separated in a 12% polyacrylamide gel containing SDS, blotted onto a nitrocellulose filter, and reacted with a mouse anti-HA antibody 12CA5 [25,26] or with the mouse anti-FLAG antibody.

2.4. Kinase assay

Glutathione *S*-transferase (GST) or GST-TFAF2 was expressed in *Escherichia coli* and purified as described above. In vitro-kinase reactions were carried out at 22°C for 20 min in a mixture containing 250 ng of either GST or GST-TFAF2, 2 µg of GST-free Pim-1, and 10 µCi (3000 Ci/mmol) of [γ -³²P]ATP in 200 µl of a kinase buffer (25 mM HEPES–KOH (pH 7.5), 30 mM NaCl, 10 mM MgCl₂, and 0.5 mM dithiothreitol). The reaction mixture was boiled in Laemmli buffer, and phosphorylated proteins were separated in a 12% polyacrylamide gel containing SDS, followed by autoradiography.

2.5. Indirect immunofluorescence

Human HeLa cells were cotransfected with pcDNA3-FLAG-Pim-1 and pcDNA3-HA-TFAF2 by the calcium phosphate precipitation

technique [27]. Forty-eight hours after transfection, the cells were fixed with a solution containing 4% paraformaldehyde and reacted with a mouse anti-FLAG monoclonal antibody (M2, Sigma) or anti-HA polyclonal antibody (Y-11, Santa Cruz). The cells were then reacted with an FITC-conjugated anti-mouse IgG or rhodamine-conjugated anti-rabbit IgG and observed under a confocal laser fluorescent microscope.

3. Results and discussion

3.1. Identification of TFAF2/SNX6 as a Pim-1-binding protein and determination of the Pim-1-binding region

To screen cDNAs encoding Pim-1-associating proteins, two-hybrid screening was carried out with Pim-1Δ2, an N-terminal fragment containing amino acids 1–93, as bait using a human cDNA library as described previously [12]. In addition to heterochromatin protein 1γ (HP-1γ), a partial cDNA encoding a region spanning amino acids 1–179 of TFAF2/SNX6 was obtained. We and others have already cloned cDNAs of TFAF2 or SNX6 by screening of the cDNA of a protein binding to TRAF4 (data not shown, accession number of TFAF2 cDNA sequence is U83194) or a Smad1-binding protein [22]. TFAF2/SNX6 was comprised of at least two domains, a phox-homology (PX) domain and a coiled coil region, which were thought to be important domains for its function [22,23]. To determine the Pim-1-binding region of TFAF2/SNX6, various deletion constructs fused to the GAL4-activation domain were used for a two-hybrid assay with Pim-1Δ2 as bait and the results of filter and liquid assays were shown (Fig. 1). Except for TFAF2-N, containing amino

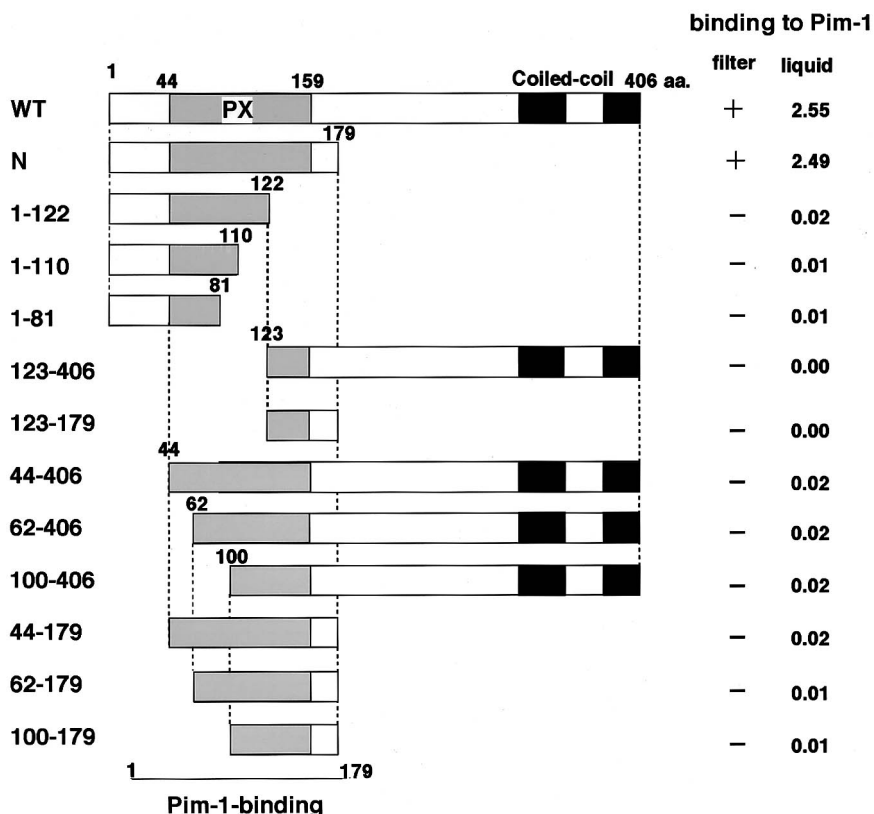


Fig. 1. Identification of the Pim-1-binding region in TFAF2. *Saccharomyces cerevisiae* L40 cells were cotransformed with pGLex-Pim-1Δ2 and a series of deletion constructs fused with the GAL4-activation domain, and the β-galactosidase activity of each colony was tested. After incubation in liquid medium or on filters, the β-galactosidase activity was assayed, and relative intensities of β-galactosidase activity are shown on the left. PX indicates the domain in TFAF2.

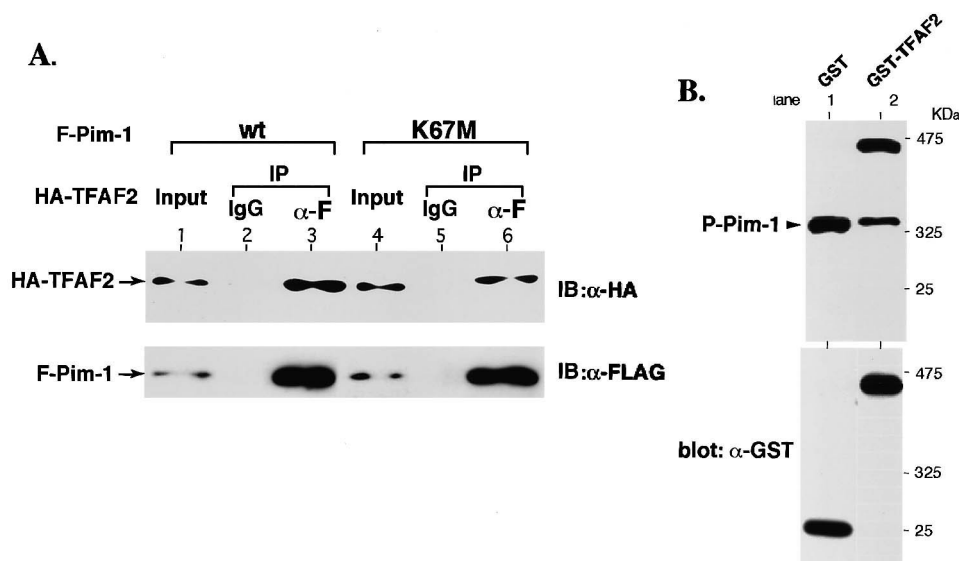


Fig. 2. Association of Pim-1 with TFAF2 in human 293T cells and phosphorylation of TFAF2 by Pim-1. A: Pim-1-wt, Pim-1-KM and TFAF2 were tagged with either FLAG or HA, and their expression vectors were co-introduced into human 293T cells. Two days after transfection, cell extracts were prepared, and the proteins in the extracts were first immunoprecipitated (IP) with an anti-FLAG antibody or non-specific IgG. The proteins in the precipitates were separated in a 12% polyacrylamide gel and blotted with the anti-FLAG or an anti-HA antibody (12CA5). 1/50 volumes of the extract used for the binding reaction were applied to the same gel (input, lanes 1 and 4). B: GST by itself and GST fusion proteins of TFAF2 were expressed in *E. coli*, purified, and incubated in the presence of [γ - 32 P]ATP and GST-free Pim-1. Labeled proteins bound to the resin were then separated in a 12% polyacrylamide gel and autoradiographed.

acids 1–179, no clones bound to Pim-1, indicating that Pim-1 binds to the N-terminal-half region of TFAF2, including the PX domain (Fig. 1). To examine a complex formation of TFAF2 with Pim-1 in vivo, expression vectors for FLAG-tagged wild-type (wt) Pim-1 or kinase-negative Pim-1, in which lysine at amino acid 67 had been changed to methionine (KM), was cotransfected together with HA-TFAF2 to human 293T cells. Forty-eight hours after transfection, the cell extract was prepared and the proteins in the extract were first immunoprecipitated with an anti-FLAG antibody or non-specific IgG. The precipitates were divided to two aliquots, and each of the precipitates was immunoblotted against either the anti-FLAG antibody or an anti-HA antibody (Fig. 2A). The anti-HA antibody was first confirmed not to react FLAG-Pim-1 in cell extract by Western blotting (data not shown). The anti-FLAG antibody precipitated FLAG-Pim-1-wt and FLAG-Pim-1-KM (Fig. 2A, F-Pim-1 in lanes 3 and 6), but the non-specific IgG did not (Fig. 2A, F-Pim-1 in lanes 2 and 5). HA-TFAF2, on the other hand, was detected in the immunoprecipitate from cells transfected with both FLAG-Pim-1-wt and FLAG-Pim-1-KM (Fig. 2A, HA-TFAF2 in lanes 3 and 6), indicating that TFAF2 was associated with the wild-type and mutant form of Pim-1 in ectopic-expressed 293T cells.

Since Pim-1 is a serine/threonine protein kinase, it is possible that TFAF2 is phosphorylated by Pim-1. To investigate this possibility, GST, GST-TFAF2 and GST-Pim-1 were purified from the expressed *E. coli*, and then GST-free Pim-1 was prepared after the digestion of GST-Pim-1 with PreScission protease. Both GST-TFAF2 and Pim-1 were incubated with [γ - 32 P]ATP, and the labeled proteins were separated on the gel (Fig. 2B). Pim-1 was autophosphorylated irrespective of the kinds of substrate proteins, and GST-TFAF2, but not GST, was phosphorylated with Pim-1 (Fig. 2B, lanes 2 and 1, re-

spectively). A kinase-negative mutant of Pim-1 did not phosphorylate GST-TFAF2 (data not shown).

3.2. Translocation of TFAF2/SNX6 from the cytoplasm to nucleus by Pim-1 in cells

Previous studies have shown that SNX6 is localized in the cytoplasm to target proteins to the Golgi network in the cells [23], and we have also found that TFAF2 is associated with TRAF4 in the Golgi body (data not shown). To determine the cellular localization of TFAF2/SNX6 and Pim-1, expression vectors for FLAG-Pim-1-wt or FLAG-Pim-1-KM Pim-1 and HA-TFAF2 were transfected alone or together into human HeLa cells. Two days after transfection, the cells were stained with anti-FLAG and anti-HA antibodies, and the proteins were detected by FITC- and rhodamine-conjugated second antibodies, respectively, and then visualized under a confocal laser microscope (Fig. 3). The anti-HA antibody was first confirmed not to stain FLAG-Pim-1 in transfected cells (data not shown). Both wild-type and kinase-negative Pim-1 (green) were localized in nucleus, while HA-TFAF2 (red) was localized in the cytoplasm (Fig. 3A). When both FLAG-Pim-1 and HA-TFAF2 were cotransfected into HeLa cells, 50–70% of HA-TFAF2 was translocated to the nucleus and 25–30% of it was present both in the cytoplasm and nuclei, irrespective of the presence or absence of phosphorylation activity of Pim-1. Pim-1 and TFAF2 were colocalized in nuclei as shown by the yellow color (Fig. 3B, merge), and distributions of HA-TFAF2 in cells transfected with or without FLAG-Pim-1 were calculated (Fig. 3C). EGFP (promoter)-tagged TFAF2 was also used and found to be in the same localization in cells as that of HA-TFAF2 (data not shown). These results indicate that Pim-1 determines the location of TFAF2/SNX6 in cells. In order to confirm the above results, the effects of the Pim-1-deletion mutants on the localization of TFAF2/SNX6

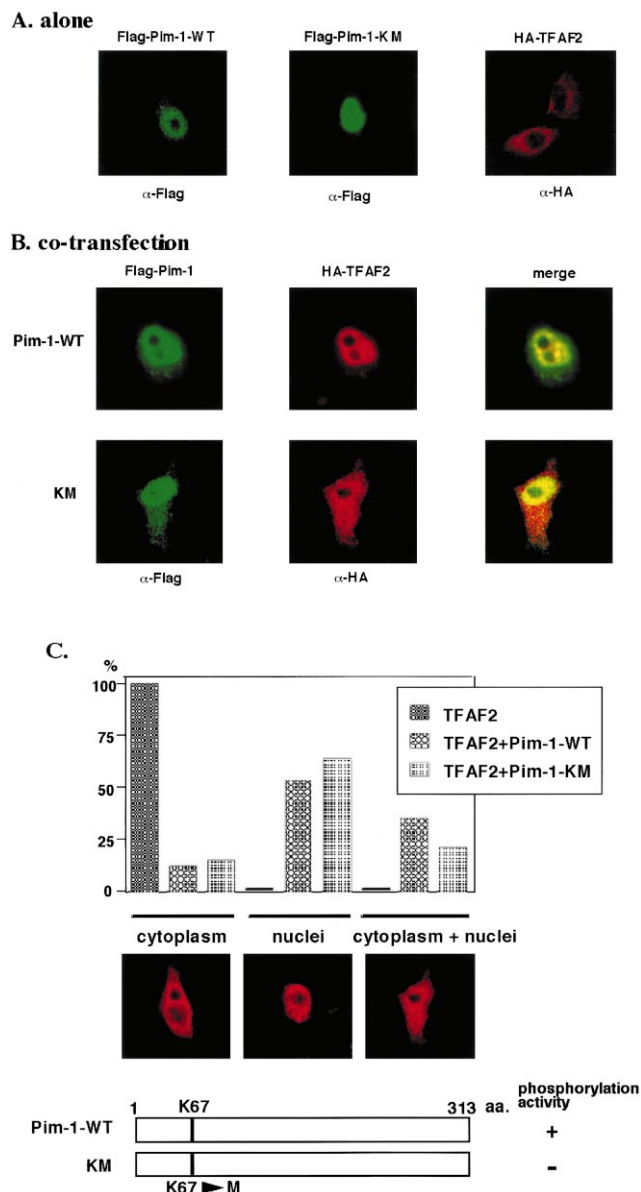


Fig. 3. Translocation of TFAF2 from cytoplasm to nucleus by Pim-1. A: HeLa cells were transfected with expression vectors for FLAG-Pim-1-wt, FLAG-Pim-1-KM and HA-TFAF2 by the calcium phosphate precipitation technique. Two days after transfection, the cells were fixed, reacted with an anti-FLAG monoclonal antibody (M2, Sigma) and an anti-HA polyclonal antibody (Y-11, Santa Cruz), and visualized with a fluorescein isothiocyanate-conjugated anti-mouse IgG and a rhodamine-conjugated anti-rabbit IgG, respectively. B: HeLa cells were cotransfected with expression vectors for FLAG-Pim-1-wt, FLAG-Pim-1-KM and HA-TFAF2 and then stained as described in (A). The two figures have been merged (merge). C: More than 200 cells cotransfected with FLAG-Pim-1-wt, FLAG-Pim-1-KM and HA-TFAF2 were stained as described in (A), and the number of cells which contain Pim-1 located in the cytoplasm, nuclei or cytoplasm+nuclei in each transfected cell was counted.

in cells were further examined. First, Pim-1-deletion mutants alone were transfected into HeLa cells and stained with the anti-FLAG antibody (Fig. 4A). Nuclei was stained with DAPI. Pim-1- Δ 1, containing amino acids 1–230, was found to be located as a speckle or a dot in nuclei, whereas Pim-1-

wt was located uniformly in the nucleus with some staining in the cytoplasm. Pim-1 Δ 2, spanning amino acids 1–97, was mainly located in the nucleus with much more cytoplasm localization than that of Pim-1-wt. Nuclear staining of Pim-1 Δ 2 was likely to be emphasized due to the sickness of the nucleus. Only nuclei of Pim-1 Δ 3, which lacks amino acids 1–97, and Pim-1 Δ 5 containing amino acids 98–230, were stained. Since the sizes of FLAG-tagged-deletion mutants of Pim-1 were small enough to diffuse to nucleus, EGFP-tagged Pim-1-deletion mutants were transfected into HeLa cells and their distribution in cells were tested. Since molecular weight of EGFP is 27 kDa, all the Pim-1-deletion mutants are not able to diffuse to the nucleus by themselves. Pim-1 Δ 2-wt and Pim-1 Δ 3-wt were found to have the same localization as that of the FLAG-tagged versions and the nuclear localization of Pim-1 Δ 3 was confirmed. Contrary to the location of the FLAG-tagged version, Pim-1 Δ 1 and Pim-1 Δ 5 were located as a speckle or a dot outside of nucleus, indicating that nuclear location of FLAG-Pim-1 Δ 1 and Pim-1 Δ 5 was due to the diffusion. These results also suggested that a nuclear localization signal is present in amino acids 230–313 in Pim-1, and this signal is necessary but not sufficient for nuclear location of Pim-1, since some parts of Pim-1 Δ 2 are still located in the nucleus. Furthermore, since the nucleotide sequences corresponding to the typical nuclear localization signal so far analyzed are not present in Pim-1, some proteins that bind to the nuclear localization signal present in amino acids 230–313 or to Pim-1 Δ 2 might catalyze the nuclear translocation of Pim-1. The expression vectors for FLAG-Pim-1-deletion mutants, Δ 1, Δ 2 and Δ 3, were then cotransfected with HA-TFAF2 into HeLa cells, and both proteins were stained (Fig. 4B). The main parts of TFAF2 were colocalized with Pim-1-wt uniformly in nuclei. In the case of Pim-1 Δ 1 and Pim-1 Δ 2, lacking nuclear localization signals, both proteins were colocalized as a speckle outside of the nucleus, which is the reported localization of TFAF2/TNX6 [22,23]. In the case of Pim-1 Δ 3 that contains the nuclear localization signal, on the other hand, the majority of TFAF2 were translocated to the nuclei and some parts of TFAF2 remained in the cytoplasm. These results suggest that TFAF2/SNX6 is translocated from the cytoplasm to nuclei by the nuclear localization signal present in Pim-1.

The sorting nexins have been found to be a family of cytoplasmic and membrane-associated proteins that are thought to function in the intracellular trafficking of plasma-membrane receptors. All the sorting nexin-family proteins contain a PX domain, and more than 10 sorting nexin-family proteins in mammals have been identified ([28], the references therein). SNX6 was first identified as a protein interacting with Smad1 [22]. Recently, SNX6 has been found to bind more strongly with the TGF- β receptors of serine/threonine kinase as well as EGF, insulin or platelet-derived growth factor receptors of tyrosine kinase and to be located in the cytoplasm and membrane [23]. We first identified TFAF2 as a protein binding to TRAF4, and TFAF2 was later found to be identical to TNX6. Furthermore, TFAF2 and TRAF4 were found to be colocalized in the Golgi body (data not shown). On the other hand, Pim-1 is thought to function in cell-cycle progression, induction or suppression of apoptosis, or transcription regulation by phosphorylating proteins contributing to these processes, and several nuclear proteins, including p100 [10], Cdc25A [11], PAP-1 [13] and HP-1 [12], have been identified as binding- and phosphorylation-target proteins. What is the

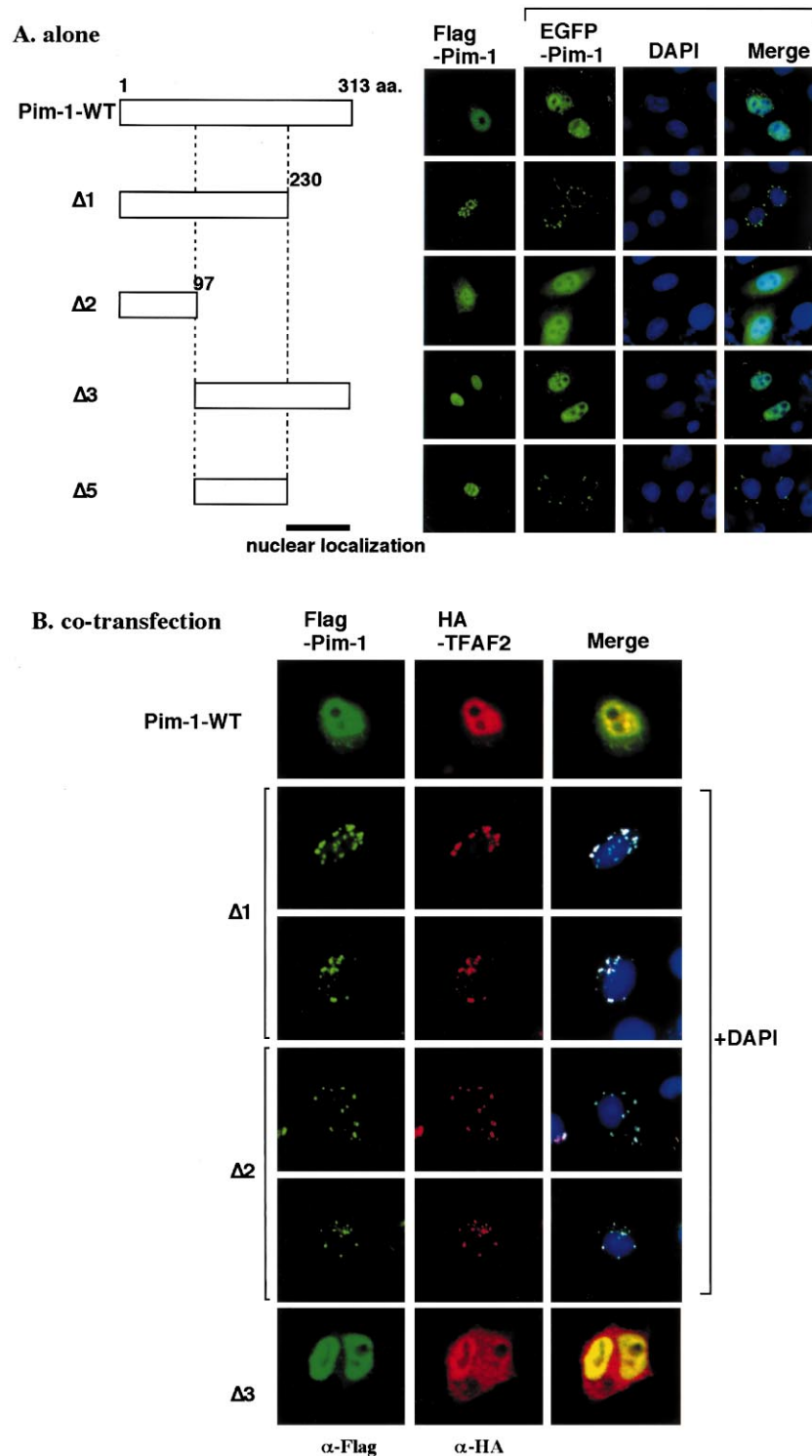


Fig. 4. Effect of Pim-1 deletion mutants on the localization of TFAF2. A: FLAG-Pim-1-wt or four deletion mutants of Pim-1, $\Delta 1$, $\Delta 2$, $\Delta 3$, $\Delta 5$, was transfected into HeLa cells and stained with an anti-FLAG antibody, followed by a fluorescein isothiocyanate-conjugated anti-mouse IgG as described in Fig. 3A. EGFP-tagged Pim-1-wt or three deletion mutants of Pim-1, $\Delta 1$ – $\Delta 3$, was also transfected into HeLa cells and the nuclei were stained with DAPI. These two figures have been merged (merge). B: HeLa cells were cotransfected with expression vectors for FLAG-Pim-1-wt, FLAG-Pim-1 deletion mutants and HA-TFAF2, and then stained as described in Fig. 3A. The two figures have been merged (merge).

function of nuclei-targeted SNX6/TFAF2? From the supported function of cytoplasm- or membrane-located SNX proteins, it is possible that Pim-1 is recruited to the regions in nucleus, in which Pim-1 functions or is degraded, by SNX6/

TFAF2. These processes, different from those of the phosphorylation-target proteins of Pim-1, may not need the phosphorylation function of Pim-1. We are now examining these possibilities.

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