Binding and regulation of the transcription factor NFAT by the peptidyl prolyl *cis–trans* isomerase Pin1

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Abstract Nuclear factor of activated T cells (NFAT) plays a key role in T cell activation. The activation of NFAT involves calcium- and calcineurin-dependent dephosphorylation and nuclear translocation from the cytoplasm, a process that is opposed by protein kinases. We show here that the peptidyl prolyl *cis–trans* isomerase Pin1 interacts specifically with the phosphorylated form of NFAT. The NFAT–Pin1 interaction is mediated through the WW domain of Pin1 and the serine–proline-rich domains of NFAT. Furthermore, binding of Pin1 to NFAT inhibits the calcineurin-mediated dephosphorylation of NFAT in vitro, and overexpression of Pin1 in T cells inhibits calcium-dependent activation of NFAT in vivo. These results suggest a possible role for Pin1 in the regulation of NFAT in T cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Nuclear factor of activated T cells; Pin1; WW domain; Ser–Pro repeat; Calcineurin; Calcium signaling

1. Introduction

Nuclear factor of activated T cells (NFAT) comprises a family of transcription factors that play an important role in transmitting calcium signals from the cytosol to the nucleus [1–3]. A hallmark of this family of transcription factors is their phosphorylation-dependent shuttling between the cytosol and the nucleus [4–6]. Thus, in quiescent T cells, NFAT is hyperphosphorylated and localized to the cytoplasm. Upon T cell activation and the accompanying increase in intracellular calcium, NFAT undergoes dephosphorylation by the calcium- and calmodulin (CaM)-dependent protein phosphatase calcineurin, subsequently translocating into the nucleus where it binds to the promoter region of a number of cytokines and initiates their transcription. NFAT consists of several distinct domains, including a highly conserved regulatory domain towards the N-terminus, a DNA binding domain, and a C-terminal transactivation domain [1]. The major sites of dynamic phosphorylation have been identified recently and are found to be concentrated at the N-terminal regulatory domain [7]. Among the sites for dephosphorylation by calcineurin, nearly half are phosphoserine residues preceding a proline.

Proline-containing peptide bonds exist in two alternative conformational states, i.e. *cis* and *trans*. Three families of enzymes are known that catalyze the interconversion between the two conformational states, the prototypes of which are cyclophilin A [8], FKBP [9,10] and Pin1/Ess1 [11]. These three families of peptidyl prolyl isomerases display distinct substrate specificity, with the Pin1 family of enzymes the only one that is capable of using phosphoserine/phosphothreonine–proline-containing polypeptides as substrates [12]. Pin1 was initially identified as an interacting protein for the critical mitotic kinase NIMA in a yeast two-hybrid screen [11]. It is highly conserved and essential in yeast and mammalian cells [11,13,14]. Specifically, depletion, mutations or disruption of Pin1 or its budding yeast homologue lead to mitotic block, disruption of DNA replication checkpoints and apoptosis, making Pin1 the only known peptidyl prolyl isomerase that is essential for cell division [11,13–15]. In addition to its unique substrate specificity, Pin1 differs from cyclophilins and FKBPs in that it has an N-terminal WW domain, which binds to phosphoserine/threonine–proline motifs in other proteins [12,16–18].

As NFAT contains several serine–proline repeat motifs that are subject to regulation by calcium-dependent phosphorylation and dephosphorylation [1,7] and Pin1 is known to bind to such motifs, we hypothesized that Pin1 also binds to NFAT and regulates its function. Herein, we show that Pin1 forms a stable complex with the phosphorylated form of NFAT. Binding of Pin1 to NFAT inhibits its dephosphorylation by calcineurin in vitro. Overexpression of Pin1 in Jurkat T cells leads to selective inhibition of NFAT transcriptional activity in response to stimulation by phorbol myristate acetate (PMA) and ionomycin. These results suggest a novel function of Pin1 in the regulation of calcium signaling in T cells and a new mechanism of regulation of NFAT by its association with the peptidyl prolyl isomerase Pin1.

2. Materials and methods

2.1. Cell culture and reagents

Jurkat T cells were cultured in minimal essential medium, RPMI 1640, supplemented with 10% fetal calf serum, 2 mM glutamate, penicillin (100 U/ml) and streptomycin (100 μg/ml). Ionomycin, PMA, and CaM were from Sigma (St. Louis, MO, USA) or Calbiochem (San Diego, CA, USA). Monoclonal antibodies anti-HA 12CA5 and M2 were obtained from Babco (Richmond, CA, USA) and Sigma. Anti-Pin1 antibodies have been described previously [19].

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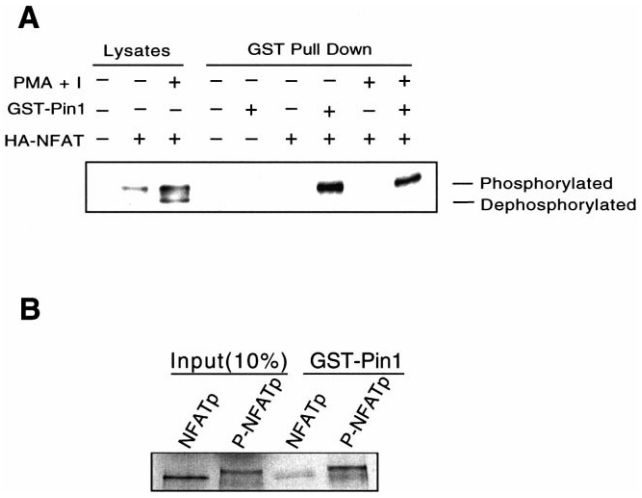

Fig. 1. Pin1 binds to phosphorylated NFATp. A: GST-Pin1 fusion protein binds to the phosphorylated NFATp. HA-tagged NFATp(1–460)-GFP was expressed in Jurkat T cells and cell extracts were incubated with immobilized GST and GST-Pin1. Bound NFATp(1–460)-GFP was detected by immunoblot. B: Phosphorylation-dependent binding of NFAT to Pin1. [^{35}S]Methionine-labelled full-length NFATp prepared by in vitro transcription and translation was incubated without or with PKA and GSK3. The binding of kinase-untreated and -treated NFATp to immobilized GST-Pin1 was determined by autoradiography.

2.2. Plasmid construction

Flag-tagged full-length NFATp and various N-terminal truncations of NFATp were amplified by PCR and cloned into pSG5 (Stratagene). PEGFP-NFATp(1–460) containing the HA-tagged N-terminal fragment (residues 1–460) of NFATp was kindly supplied by A. Rao (Harvard Medical School). The expression vector pETCN has been described previously [20]. Wide type and mutant Pin1 constructs were prepared by PCR, sequenced and cloned into pGEX-KG (Amersham Pharmacia Biotech). Pin1/P2, which directs the doxycycline-inducible expression of Pin1 in mammalian cells, and pUHD15-1 have been described previously [11,21].

2.3. In vitro transcription/translation

In vitro transcription/translation was performed using the STP3 (T7) kit (Novagen). Phosphorylation of in vitro translated NFATp was performed by incubating in vitro translated NFATp with protein kinase A (PKA) (2 U) and glycogen synthase kinase 3 (GSK3) (1 U) at 30°C for 1 h.

2.4. GST pull-down assay

Jurkat T cells were transfected with various NFAT expression constructs by electroporation (960 μF, 250 V). After recovery for 24 h, cell extracts were prepared with lysis buffer (20 mM Tris–HCl pH 7.4, 100 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml each of leupeptin and apoptinin). Binding assays were initiated by incubating cell extracts in lysis buffer with 5 μg of recombinant GST fusion protein bound to 40 μl glutathione *S*-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. After three washes with lysis buffer, the bound proteins were subjected to SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with 12CA5 or M2 antibodies. The protein bands were visualized with ECL as per the manufacturer's instructions.

2.5. In vitro dephosphorylation assay

Jurkat T cells were transfected with pEGFP-NFATp(1–460) by electroporation. Cell extracts were prepared as above 24 h after transfection. HA-NFATp(1–460)-GFP was immunoprecipitated by incubating the lysates with anti-HA antibody coupled to A/G protein beads for 2 h at 4°C. The beads were washed three times with lysis buffer, followed by incubation with activated calcineurin (CN+600 nM CaM+2 mM Ca^{2+}) in the absence or presence of varying concentrations of GST-Pin1 or GST-Pin1Y23A for 10 min at 30°C. The reaction was stopped by boiling in SDS sample buffer and NFATp protein was detected by Western blot as described above.

2.6. Luciferase reporter gene assay

Jurkat cells were cotransfected with 3 μg of 3×NFAT-luc reporter plasmid together with 10 μg Pin1/p2 and 5 μg pUHD15-1. Cells were cultured in the absence or presence of 1 μM of doxycycline for 18 h before stimulation with 1 μM ionomycin and 40 nM PMA for another 8 h. Luciferase activity was measured and luciferase units were normalized by the activity of β-galactosidase that was coexpressed.

3. Results and discussion

3.1. Phosphorylation-dependent interaction of NFAT with Pin1

There are three Ser–Pro repeat motifs [SPXXSPXXSPXX-XXX(D/E)(D/E)] in the N-terminal regulatory region of NFAT and several serine residues are phosphorylated in quiescent T cells, which are dephosphorylated by calcineurin upon T cell activation [1]. As the N-terminal WW domain of Pin1 has been shown to bind specifically to phosphoserine/threonine–proline motifs [18], we speculated that Pin1 might bind to NFAT. Using a GST-Pin1 fusion protein, we were able to pull down HA epitope-tagged NFATp (residues 1–460) from lysates prepared from non-activated Jurkat T cells transiently expressing NFAT as visualized by an anti-HA antibody (Fig. 1A). When Jurkat T cells were stimulated with PMA and ionomycin, both dephosphorylated and the phosphorylated forms of NFAT are present in the cell lysates. Only the phosphorylated form, but not the dephosphorylated form, of NFAT was found to associate with GST-Pin1, whereas the dephosphorylated form does not (Fig. 1A), consistent with the previous observation that the Pin1 WW domain specifically binds to phosphorylated Ser–Pro motifs.

To determine whether the interaction between NFATp and Pin1 is direct, we turned to an in vitro transcription/translation system to generate NFATp from reticulocyte lysates. Due to the lack of relevant kinase in the reticulocyte lysates, the recombinant NFATp thus produced is not phosphorylated. Among the various putative NFAT kinases reported [22–25], it has been shown that GSK3 is capable of phosphorylating NFAT upon priming by PKA in vitro [22]. We thus treated the recombinant NFATp with PKA and GSK3. As expected, treatment with those two kinases led to phosphorylation of NFATp, as judged by the slower gel mobility of the NFATp band upon treatment with the kinases in the presence of ATP. Using the GST pull-down assay, we were able to observe binding of GST-Pin1 with only the phosphorylated, but not the unphosphorylated, form of NFATp (Fig. 1B). Thus, Pin1 binds directly to the phosphorylated form of NFAT.

3.2. WW domain of Pin1 mediates its interaction with the Ser–Pro repeat region of NFAT

We next determined which domains in Pin1 and NFAT are involved in mediating their interaction. To determine whether the WW domain of Pin1 is responsible for its binding to the phosphorylated form of NFATp, we created two Pin1 mutants in its WW domain and its prolyl isomerase domain, respectively. Thus, we mutated Tyr23 to Ala and Lys63 to Ala. The Tyr23Ala mutant is known to be defective in binding phosphoserine motifs and the Lys63Ala mutant has been shown to be inactive as a prolyl isomerase [12,19]. Each Pin1 mutant was expressed and purified as a GST fusion protein. As shown in Fig. 2A, mutation of Tyr23 in the WW domain abrogated the interaction between Pin1 and NFATp. In contrast, inactivation of the prolyl isomerase ac-

tivity in the mutant Lys63Ala did not affect the binding of Pin1 to NFATp. Thus, the WW domain of Pin1 is necessary for the interaction between Pin1 and NFAT.

To locate the Pin1 binding site on NFATp, we generated a series of deletion mutants of NFAT by removing parts of the domain encompassing the three Ser–Pro repeat regions and determining the association between these mutants and GST-Pin1. While deletion of the N-terminal 147 amino acid fragment had no effect on the binding of NFATp to Pin1, further deletion of the fragment containing two of the three Ser–Pro regions abolished the binding (Fig. 2B,C). Moreover, the deletion of the C-terminal fragment containing the third Ser–Pro region also abrogated the binding, suggesting that the Ser–Pro repeat motifs are required for NFATp to bind Pin1 (Fig. 2B,C). The requirement of the Ser–Pro repeats for interaction between NFAT and Pin1 is consistent with the aforementioned results demonstrating that NFATp binds to the WW domain of Pin1.

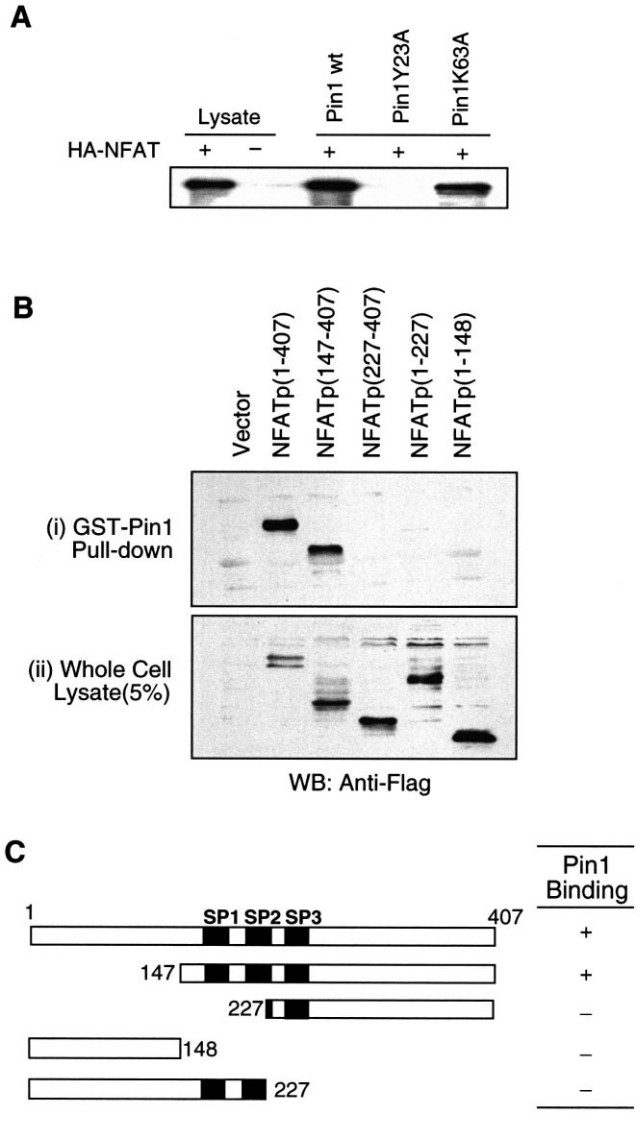

Fig. 2. Mapping of the domains involved in Pin1–NFATp interaction. A: The WW domain of Pin1 mediates its interaction with NFAT. Immobilized GST-Pin1, GST-Pin1Y23A, and GST-Pin1-K63A fusion proteins were incubated with extracts prepared from Jurkat T cells expressing HA-NFATp(1–460)-GFP. Bound NFATp was detected by immunoblot with anti-HA antibody. B,C: Identification of the Pin1 binding site on NFAT. Flag-tagged NFATp N-terminal fragments corresponding to residues 1–407, 1–227, 149–407, and 227–407 were expressed in Jurkat T cells and detected by immunoblot analysis with monoclonal antibody M2. The binding of different NFATp fragments to immobilized GST-Pin1 was examined (B) and the results are schematically summarized in (C).

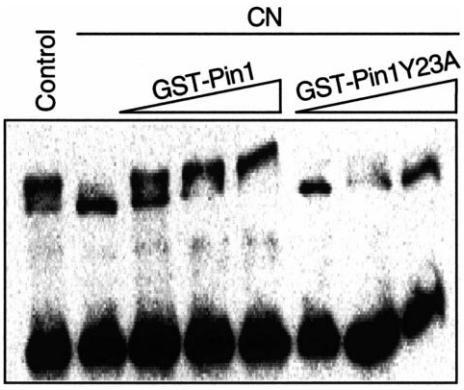

Fig. 3. Pin1 inhibits dephosphorylation of NFAT by calcineurin in vitro. HA-NFATp(1–460)-GFP was expressed in Jurkat T cells and immunoprecipitated with anti-HA antibody. The immunoprecipitates were incubated without or with activated calcineurin (CN+CaM+Ca^{2+}) in the absence or presence of 0.1, 0.5 and 1 μM GST-Pin1. The phosphorylation status of NFATp proteins was evaluated by immunoblot with anti-HA antibody.

3.3. *Pin1 inhibits the dephosphorylation of NFATp by calcineurin in vitro*

If Pin1 forms a stable complex with NFATp through the Ser–Pro region, we predicted that such a complex might resist dephosphorylation by calcineurin. To test this prediction, we carried out an in vitro immunoprecipitation phosphatase assay in which NFATp(1–460)-GFP fusion protein was immunoprecipitated from Jurkat cell extracts and incubated with recombinant calcineurin in the absence or presence of Pin1. The phosphorylation status of NFAT proteins was followed from the gel mobility of NFATp using Western blot. As shown in Fig. 3, pre-incubation of immunoprecipitated NFATp with Pin1 inhibited the dephosphorylation of NFATp by calcineurin in a dose-dependent manner. With the calcineurin concentration fixed at 50 nM, appreciable inhibition was already observed when the same concentration of GST-Pin1 was used (Fig. 3). A concentration of 1 μM GST-Pin1 almost completely inhibited dephosphorylation by calcineurin. As a control, we also determined the effect of the GST-Pin1 Tyr23Ala mutant on the dephosphorylation of NFAT by calcineurin. As expected, the GST-Pin1Y23A mutant did not affect the dephosphorylation of NFAT by calcineurin, in agreement with the observation that this mutant is incapable of binding to NFATp (Fig. 2A). This result also indicated that the inhibitory effect observed with GST-Pin1 was not due to the GST portion of the fusion protein. Thus, the binding of Pin1 to NFAT appears to have a masking effect on the phosphoserine residues in NFAT to prevent their dephosphorylation by calcineurin.

3.4. *Overexpression of Pin1 selectively inhibits activation of NFAT in T cells*

To determine whether the interaction between NFAT and calcineurin we observed in vitro is physiologically relevant in

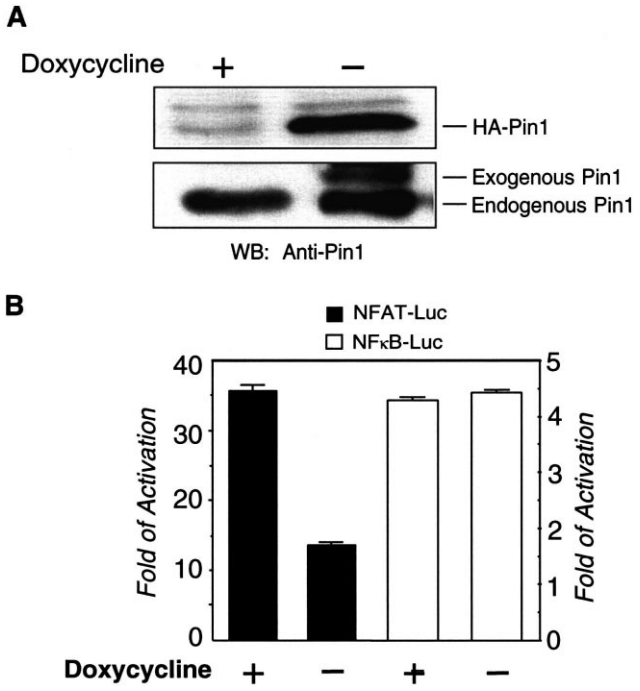

Fig. 4. Overexpression of Pin1 leads to selective inhibition of NFAT reporter gene activation. A: Overexpression of HA-Pin1 in Jurkat T cells by doxycycline. Cell lysates (normalized for equal amount of total proteins) prepared from Jurkat T cells with or without induction by doxycycline were subjected to SDS–PAGE followed by Western blot analysis using anti-HA antibodies or anti-Pin1 antibodies. B: Inhibition of NFAT reporter gene by Pin1 overexpression. Jurkat T cells transfected with plasmid for conditional expression of Pin1 along with NFAT-luc or NF-κB-luc reporter plasmid were cultured in the presence or absence of doxycycline for 18 h. The reporter genes were then activated by treatment with 1 μM ionomycin and 40 nM PMA for 8 h before cells were harvested for measurement of luciferase activity.

vivo, we determined the effect of overexpression of Pin1 on NFAT activation in response to PMA and ionomycin stimulation. As long-term overexpression of Pin1 inhibits entry into mitosis, we turned to a tetracycline-dependent expression system with Pin1 expression under the control of tetracycline. With this expression system, we were able to overexpress HA-Pin1 at a level slightly lower than endogenous Pin1 in Jurkat T cells, as determined by the Western blot analysis using anti-Pin1 antibodies (Fig. 4A). We then measured the NFAT and NF-κB reporter gene activity in the presence and absence of overexpressed Pin1. Overexpression of Pin1 led to significant inhibition of the NFAT reporter gene. In contrast, the activation of NF-κB, which is also dependent on calcineurin activation, was unaffected. These results strongly imply that Pin1 specifically binds to NFAT in vivo and has a negative effect on the activation of NFAT by calcineurin, most likely by preventing NFAT from dephosphorylation by calcineurin.

NFAT is regulated by calcium-dependent dephosphorylation in a highly dynamic process. In this process, kinases and the phosphatase calcineurin play opposing roles on the activation state and the subcellular localization of NFAT. Although the identify of the NFAT kinase(s) responsible for its inactivation has been a subject of extensive investigation and debate recently, the role of phosphorylation in the inactivation of NFAT has been unambiguously demonstrated. In this report, we show that phosphorylated NFAT may be subject to another type of regulation, i.e. sequestration by the peptidyl prolyl isomerase Pin1. Since Pin1 is a relatively abundant protein, the effect of Pin1 on NFAT is likely to be significant. It is possible that Pin1 may work in synergy with such kinases as GSK3, MEKK1, CK1 or MAPK/SAPK family members to maintain the phosphorylated state of NFAT in non-activated T cells, preventing its access to calcineurin. It is also possible that Pin1 may facilitate NFAT inactivation upon attenuation of T cell receptor signaling at the late phase of T cell activation when the cytoplasmic calcium level decreases. In summary, our results suggest that Pin1 may sequester phosphorylated NFAT and inhibit its dephosphorylation. Further studies will be needed to further elucidate the role of Pin1 in T cell activation.

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