Functional interaction of Fas-associated phosphatase-1 (FAP-1) with p75^{NTR} and their effect on NF-κB activation

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Abstract The common neurotrophin receptor p75^{NTR}, a member of the tumor necrosis factor (TNF) receptor superfamily, plays an important role in several cellular signaling cascades, including that leading to apoptosis. FAP-1 (Fas-associated phosphatase-1), which binds to the cytoplasmic tail of Fas, was originally identified as a negative regulator of Fas-mediated apoptosis. Here we have shown by co-immunoprecipitation that FAP-1 also binds to the p75^{NTR} cytoplasmic domain in vivo through the interaction between the third PDZ domain of FAP-1 and C-terminal Ser-Pro-Val residues of p75^{NTR}. Furthermore, cells expressing a FAP-1/green fluorescent protein showed intracellular co-localization of FAP-1 and p75^{NTR} at the plasma membrane. To elucidate the functional role of this physical interaction, we examined TRAF6 (TNF receptor-associated factor 6)-mediated NF-κB activation and tamoxifen-induced apoptosis in 293T cells expressing p75^{NTR}. The results revealed that TRAF6-mediated NF-κB activation was suppressed by p75^{NTR} and that the p75^{NTR}-mediated NF-κB suppression was reduced by FAP-1 expression. Interestingly, a mutant of the p75^{NTR} intracellular domain with a single substitution of a Met for Val in its C-terminus, which cannot interact with FAP-1, displayed enhanced pro-apoptotic activity in 293T transfected cells. Thus, similar to Fas, FAP-1 may be involved in suppressing p75^{NTR}-mediated pro-apoptotic signaling through its interaction with three C-terminal amino acids (*t*SPV). Thus, FAP-1 may regulate p75^{NTR}-mediated signal transduction by physiological interaction through its third PDZ domain.

Key words: Fas-associated phosphatase-1; Protein-tyrosine phosphatase; PDZ; p75^{NTR}; Nerve growth factor; Nuclear factor κB

1. Introduction

p75^{NTR} is a neurotrophin receptor, a member of the tumor necrosis factor (TNF) receptor superfamily, which includes TNF receptor (TNFR) 1, TNFR2, CD40, and Fas [1]. Upon ligation with nerve growth factor (NGF), p75^{NTR} transduces a variety of signal cascades, including NF-κB activation [2], sphingomyelin hydrolysis [3], and apoptosis [4–8], independent of TrkA receptors. p75^{NTR} also binds with similar affinity to brain-derived neurotrophic factor, neurotrophin (NT) 3, and NT-4/5 [9,10]. Structural analysis by nuclear magnetic resonance reveals that the cytoplasmic region of p75^{NTR} has six α-helical folds, similar to the subtype 1 'death domain' of the TNF receptor superfamily [11]. Recent analysis indicated that TNF receptor-associated factor 6 (TRAF6) can physically associate with p75^{NTR} and can function as a signal transducer for NGF action [12]. However, the molecular mechanisms by which NGF/p75^{NTR} regulates those events remain unclear.

Fas-associated phosphatase-1 (FAP-1), also known as protein-tyrosine phosphatase (PTP)-BAS [13], PTP-L1 [14], hPTP1E [15], and PTP-BL [16], was previously identified by yeast two-hybrid screening as a negative regulator that binds to the cytoplasmic tail of Fas (APO-1/CD95) [17]. FAP-1 has a putative membrane binding domain similar to those found in ezrin family proteins and six PDZ domains. PDZ domains (also called GLGF repeats or DHR domains) consisting of 80–90 amino acids have been found in a number of intracellular proteins (reviewed in [18–20]). Recently, proteins with PDZ domains have been reported to bind to the C-terminal motif *t*(S/T)-X-V, including the PSD-95/*N*-methyl-D-aspartate (NMDA) receptor, the PSD-95/Shaker-type K^+ channel, and Dlg-APC interactions [19,20]. Thus, the PDZ domain is a novel motif for protein-protein interactions that may participate in signal transduction or cell-cell interactions. Recent work of ours clearly showed that the third PDZ domain of FAP-1 specifically recognizes the three C-terminal amino acid residues SLV of Fas [21]. Using combinatorial peptide libraries, we and others have determined that the consensus motif for the interaction with the third PDZ domain of Fas is *t*(S/T)-X-(V/L/I) [21,22]. The highest affinity was found in the C-terminal amino acid residue V. This binding characteristic provides the hypothesis that the third PDZ domain of FAP-1 may also bind to the C-termini of other receptors with the *t*(S/T)-X-V motif. As the last three amino acids of p75^{NTR}, SPV, are conserved among chicken, rat, and human, we examined whether FAP-1 interacts with the C-terminal cytoplasmic region of p75^{NTR}. Our results indicate that FAP-1 is capable of binding to the C-terminal SPV of p75^{NTR} both in vitro and in vivo. Moreover, we provide evidence that the

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Abbreviations: NGF, nerve growth factor; TNF, tumor necrosis factor; FAP-1, Fas-associated phosphatase-1; PTP, protein-tyrosine phosphatase; ICD, intercellular domain; PCR, polymerase chain reaction; GST, glutathione S-transferase; RT-PCR, reverse transcription polymerase chain reaction; GFP, green fluorescent protein; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis

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interaction between FAP-1 and $p75^{NTR}$ may be functionally involved in $p75^{NTR}$-mediated signal transduction.

2. Materials and methods

2.1. Construction of mammalian expression vectors

Rat $p75^{NTR}$ expression plasmid constructs pcDNA3-$p75^{NTR}$ intercellular domain (ICD) (V396M) and pcDNA3-$p75^{NTR}$-IC were made by polymerase chain reaction (PCR). The common forward primer (5′-CGGGATCCATGAAGAGGTGGAACAGC-3′) for both constructs contains a *Bam*HI site (underlined). The reverse primer (5′-TGCGGTCGACTCACATTGGGGATGTGGCAGTGGA-3′) for mutant $p75^{NTR}$-ICD (V396M) contains a *Sal*I site (underlined), and substitutes valine with methionine at residue 396. The PCR products of these primers were digested with *Bam*HI and *Sal*I and ligated into the *Bam*HI and *Xho*I sites of pcDNA3 (Invitrogen) to generate pcDNA3-$p75^{NTR}$-ICD (V396M). The reverse primer (5′-GCTCTAGAACATCAGTCGTCGGA-3′) for the wild-type $p75^{NTR}$ contains an *Xba*I site (underlined). The products of the PCR using this reverse primer and the above forward primer were ligated into the *Bam*HI and *Xba*I sites of pcDNA3 to generate pcDNA3-$p75^{NTR}$-ICD. pcDNA3-$p75^{NTR}$ (V396M, ΔSPV) was constructed by inserting a full-length $p75^{NTR}$ cDNA fragment amplified with a forward primer (5′-CCCAAGCTTCCACCATGAGGAGGGCAGGTGCTGCCT-3′) and a reverse primer (5′-CCGCTCGAGTCACATTGGGGATGTGGCAGTGGA-3′ for V396M, 5′-CCGCTCGAGTCATGTGGCAGTGGACTCGCTGCAT-3′ for ΔSPV) into the *Hin*dIII and *Xho*I sites of pcDNA3. pcDNA3-hp75^{NTR} was constructed by inserting a full-length human $p75^{NTR}$ cDNA into the *Eco*RI site of the pcDNA3 vector. Human FAP-1 expression plasmid pRc/CMV-hFAP-1 was constructed by inserting the *Not*I cassette of the human full-length FAP-1 cDNA into the *Not*I site of pRc/CMV (Invitrogen). Human FAP-1 expression plasmid pEGFP-hFAP-1 was constructed by subcloning a complete coding sequence in-frame at the *Sac*I-*Sma*I sites of the pEGFP-N2 vector (Clontech). FAP-1 tagged with FLAG was prepared by using pFLAG-CMV2 and a PCR-amplified cDNA fragment encompassing the protein-coding region. pCMV-myc-mouse TRAF6 was a gift of MBL Co., Ltd.

2.2. Preparation of glutathione S-transferase (GST) fusion proteins and in vitro binding assays

DNA fragments encoding truncated variants of rat $p75^{NTR}$ – amino acids 245–396 (a whole cytoplasmic region), 245–337, 302–396, 302–337, 338–396, and 245–393 (Δ Ser-Pro-Val) – were amplified from rat $p75^{NTR}$ cDNA by PCR and subcloned at the *Eco*RI-*Sal*I sites of the pGEX-4T-1 vector (Pharmacia). Sense and antisense oligonucleotides connected with *Eco*RI and *Sal*I sites, respectively, which encode Ser-Pro-Val (corresponding to amino acids 394–396 of rat $p75^{NTR}$) or Ser-Pro-Met, were synthesized. After annealing of both strands, they were subcloned at the *Eco*RI-*Sal*I sites of the pGEX-4T-1 vector. The $p75^{NTR}$ deletion mutants and Ser-Pro-Met were expressed as GST fusion proteins by using these constructs in INVαF′ (Invitrogen) by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C. GST fusion proteins were affinity-purified from bacterial lysates with glutathione Sepharose 4B beads (Pharmacia Biotech). [^{35}S]Methionine-labeled proteins were generated with the TNT-coupled reticulocyte lysate system (Promega), and HFAP-10 cDNA (human FAP-1/PTP-BAS type 1 nucleotides 3897–5710) was subcloned into the Bluescript vector pSK-II (Stratagene), or HFAP-11 cDNA (human FAP-1/PTP-BAS type 1 nucleotides 4030–5525, encoding amino acids 1323–1821) was subcloned into the pcDNA 3.1-Myc-His vector (Invitrogen). ^{35}S-labeled product from pSK-II-HFAP-10 was in vitro translated from an internal methionine codon to give the peptides corresponding to amino acids 1323–1882 of human FAP-1. Following translation, 5 μl of total ^{35}S-labeled reticulocyte lysate was diluted into 200 μl of NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.2% NP40) and incubated with 10 μl of a 50% slurry of GST- or GST-fusion-protein-Sepharose beads for 4–16 h at 4°C. The beads were then pelleted, washed six times in NETN buffer, boiled in SDS sample buffer, and resolved on SDS-polyacrylamide gel. Bound proteins were made visible following autoradiography after enhancing the signal by Amplify (Amersham).

For coprecipitation experiments on native FAP-1 with the GST-$p75^{NTR}$ C-terminus, 293T cells growing in a 10 cm dish were transiently transfected with pRc/CMV-hFAP-1 by the calcium/phosphate procedure [23]. At 48–60 h after transfection, the cells were lysed in 1 ml of NETN buffer containing protease inhibitors (1 mM PMSF, 0.28 TIU/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzamidine, 0.7 μg/ml pepstatin) for 30 min on ice, and the insoluble fraction was removed by centrifugation. The resulting supernatant was incubated with GST-$p75^{NTR}$ (338–396) or GST alone, each being immobilized on 20 μl of glutathione Sepharose beads for ∼12 h at 4°C, then washed with NETN buffer and boiled in SDS sample buffer. The retained FAP-1 on beads was resolved on SDS-polyacrylamide gel (5%) and analyzed by Western blotting using anti-FAP-1 polyclonal antibody at 2 μg/ml.

2.3. Cell culture, transfection, and in vivo binding assays

293T cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. These cells were seeded in 10 cm plates at 2×10^6 cells per plate and transiently transfected with 20 μg of pFLAG-CMV-2-hFAP-1, pcDNA3-$p75^{NTR}$, or both plasmids. Transfected cells were cultured for 24 h and lysed in 1 ml of NETN buffer containing protease inhibitors. The supernatant was immunoprecipitated with 2 μg of anti-FLAG M2 monoclonal antibody (Sigma) coupled with protein G Sepharose 4B (Pharmacia) at 4°C for 2 h. They were washed five times with the same buffer and subjected to SDS-PAGE (7.5%). The samples were then transferred to nitrocellulose membrane and immunoblotted by rabbit polyclonal anti-$p75^{NTR}$ antibody.

2.4. Reporter gene assays

293T cells were seeded in six well plates at 1.2×10^5 cells per well and transiently transfected with 5 μg of plasmids containing 1.4 μg of pELAM-luc reporter plasmid (a gift of MBL Co., Ltd.) and 0.1 μg of pRL-TK as an internal control (Promega), using the calcium/phosphate method. Twenty-four hours after transfection, the cells were washed twice with phosphate-buffered saline (PBS) and lysed in 0.5 ml of lysis buffer (Dual-Luciferase Reporter Assay System, Promega). Lysate (10 μl) was mixed with 50 μl of luciferase assay reagent. Luciferase activity was measured in a model LB9507 luminometer (EG and G Berthold, Germany).

2.5. Cell viability assays

293T cells were plated at a density of 5×10^5 in six well plates and transfected as described above. Eighteen hours after transfection, the cells were washed with serum-free medium and placed on DMEM with 3% FBS. After a further 18–24 h incubation, the cells were placed on 35 μM tamoxifen (Sigma) to induce apoptosis and incubated for 4–12 h. Viability was assessed by propidium iodide fluorescence [24] and trypan blue exclusion [4].

2.6. Fluorescence analysis

293T cells were plated on a poly-L-Lys-coated cover glass and transiently transfected with pEGFP-hFAP-1 or pcDNA3-$p75^{NTR}$. Cells on the cover glass were fixed with 2.5% paraformaldehyde/PBS for 30 min at room temperature. For the detection of $p75^{NTR}$, fixed cells were incubated with 5 μg/ml monoclonal anti-rat NGF receptor antibody (which reacts with low-affinity rat NGFR; Boehringer Mannheim) for 2 h at 4°C. Cells were rinsed with PBS twice and subsequently incubated for 2 h at 4°C with 5 μg/ml of goat anti-mouse IgG antibody conjugated with Cy5 (Jackson ImmunoResearch Laboratories). Cells on the cover glass were then mounted on a glass slide in glycerin jelly for microscopic measurement with a Zeiss LSM 510 confocal laser scanning microscope, equipped with a ×40 objective (Zeiss). An excitation line of 488 nm Ar laser was used for green fluorescent protein (GFP) detection and 647 nm Kr line was used for Cy5 ($p75^{NTR}$) detection.

3. Results

3.1. FAP-1 interacts with the C-terminal SPV of $p75^{NTR}$ in vitro

To investigate the interaction of FAP-1 with the cytoplasmic tSPV of $p75^{NTR}$, we used an in vitro binding assay. The GST fusion proteins including the cytoplasmic regions of

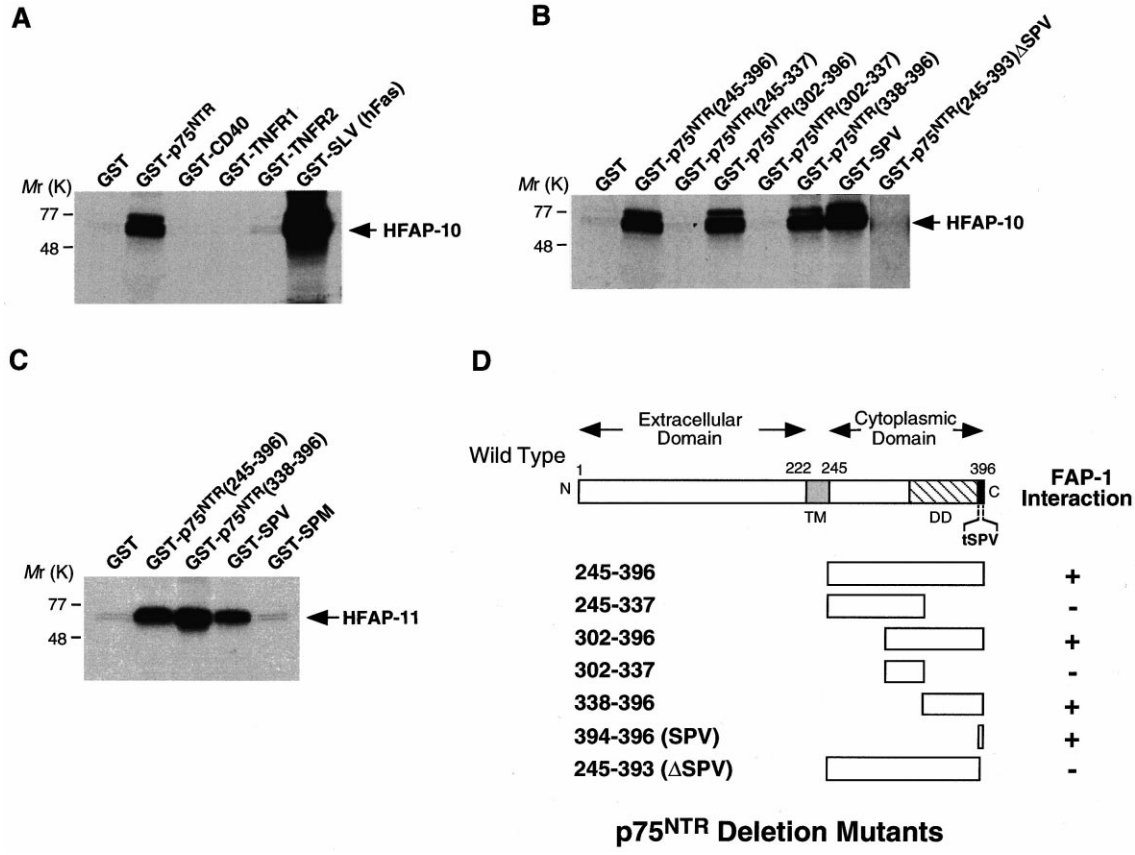

Fig. 1. Interaction of FAP-1 with $p75^{NTR}$ cytoplasmic region in vitro. A: In vitro binding of HFAP-10 with various receptors of the TNF receptor superfamily. The cytoplasmic region of several receptors-rat $p75^{NTR}$, CD40, TNFR1, TNFR2, and the C-terminal amino acid residues SLV of human Fas-were prepared as GST fusion proteins. The GST fusion proteins, immobilized on glutathione-Sepharose beads, were incubated with ^{35}S-labeled in vitro translated HFAP-10 peptide containing the third through fifth PDZ domains of human FAP-1 protein. After the beads were washed, retained HFAP-10 peptide was analyzed by 10% SDS-PAGE and autoradiography. B: Interaction of FAP-1 (HFAP-10) with various $p75^{NTR}$ deletion mutants in vitro. ^{35}S-labeled HFAP-10 polypeptide, corresponding to amino acids 1323–1882 of human FAP-1, was incubated with the indicated GST fusion proteins of $p75^{NTR}$-truncated peptides. C: FAP-1 is incapable of binding to C-terminal SPM. ^{35}S-labeled HFAP-11 was incubated with the indicated GST fusion proteins immobilized on glutathione Sepharose beads. After the beads were washed, retained HFAP-11 protein was analyzed by SDS-PAGE and autoradiography. Molecular size markers are shown at the left in kDa. D: Schematic representation of rat $p75^{NTR}$ deletion mutants and interaction with HFAP-10. The mature rat $p75^{NTR}$ is a 396 amino acid protein with a single transmembrane domain. The cytoplasmic region consisting of 152 amino acids possesses a death domain (amino acids 325–390 of mature rat $p75^{NTR}$) and a C-terminal *t*(S/T)-X-V motif. The results of the interactions of HFAP-10 with $p75^{NTR}$ deletion mutants shown in B are indicated here by + or − signs. N = NH_2-terminal. C = COOH-terminal. TM = transmembrane domain, amino acids 223–244. DD = death domain (hatched), amino acids 325–390. *t*SPV = C-terminal SPV (solid), amino acids 394–396.

$p75^{NTR}$, CD40, TNFR1, and TNFR2 were expressed in *Escherichia coli* and affinity-purified on glutathione Sepharose. HFAP-10 polypeptide containing the third PDZ domain (amino acids 1323–1882 of human FAP-1) was in vitro translated and tested for binding to these GST fusion proteins in vitro. HFAP-10 polypeptide was coprecipitated with the GST fusion protein containing the cytoplasmic domain of $p75^{NTR}$ as well as that containing the human Fas C-terminal amino acids SLV; it did not interact with the GST fusion proteins containing the cytoplasmic domains of CD40, TNFR1, and TNFR2, which do not possess the C-terminal *t*(S/T)-X-V motif (Fig. 1A). To determine the minimum region of $p75^{NTR}$ necessary for interaction with FAP-1, a series of $p75^{NTR}$ cytoplasmic truncated mutants were prepared as GST fusion proteins and were tested for binding to the in vitro translated HFAP-10 polypeptide. The results indicate that ^{35}S-labeled HFAP-10 polypeptide was associated with $p75^{NTR}$ truncation mutants containing the intact C-terminus and was bound to the C-terminal amino acid residues SPV of $p75^{NTR}$ (Fig. 1B–D). To further confirm this result, we also used a C-terminal truncation mutant of $p75^{NTR}$ (245–393), ΔSPV, in which the last three amino acids were deleted. In an in vitro binding assay, this mutant, lacking tSPV, did not retain FAP-1 binding activity (Fig. 1B,D), leading to the conclusion that the C-terminal amino acids SPV of $p75^{NTR}$ are necessary and sufficient for the binding to FAP-1.

We also did an in vitro binding assay using in vitro translated FAP-1 polypeptide (HFAP-11) and GST fusion proteins containing the three amino acids SPM or SPV at their C-termini. The results indicate that the FAP-1 polypeptide interacted with GST-SPV as well as with other GST fusion proteins retaining the cytoplasmic tSPV motif of $p75^{NTR}$, whereas GST-SPM failed to associate with FAP-1 (Fig. 1C,D). These results strongly support previous findings that the third PDZ domain of FAP-1 specifically recognizes the C-terminal sequence motif *t*(S/T)-X-V [28,29].

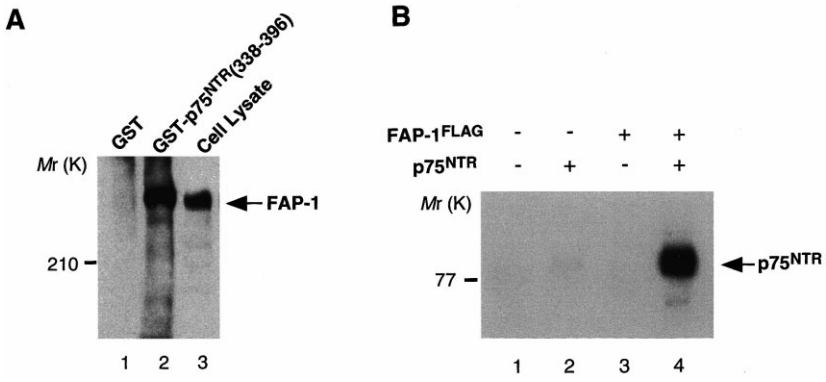

Fig. 2. FAP-1 interacts with $p75^{NTR}$ in vivo. A: Cell lysate prepared from 293T cells transiently transfected with an expression vector encoding human FAP-1 (pRc/CMV-hFAP-1) was incubated with GST or GST-$p75^{NTR}$ (338–396) (59 C-terminal amino acids) immobilized on glutathione-Sepharose beads. After incubation, the beads were washed, and retained FAP-1 protein was analyzed by Western blotting with anti-FAP-1 polyclonal antibody (lanes 1 and 2). Cell lysate before precipitation with GST fusion proteins was also analyzed (lane 3). B: Cell lysates prepared from 293T cells transiently transfected with control vectors pcDNA3 and pFLAG-CMV-2 (lane 1), pcDNA3-rat $p75^{NTR}$ (lane 2), pFLAG-CMV-2-hFAP-1 (lane 3), and pFLAG-CMV-2-hFAP-1 and pcDNA3-rat $p75^{NTR}$ (lane 4) were immunoprecipitated with anti-FLAG M2 monoclonal-antibody-coupled protein G Sepharose 4B. After incubation, the beads were washed, and retained $p75^{NTR}$ protein was analyzed by Western blotting with anti-$p75^{NTR}$ polyclonal antibody.

3.2. FAP-1 interacts with $p75^{NTR}$ in vivo

To confirm the interaction of full-length FAP-1 protein with $p75^{NTR}$, an in vitro pull-down assay was done using cell lysates extracted from human embryonic kidney cell line 293T, which overexpresses FAP-1. Overexpression of FAP-1 protein in 293T cells transiently transfected with pRc/CMV-hFAP-1 was confirmed by Western blot analysis to have an apparent molecular mass of ~250 kDa (Fig. 2A, lane 3). The results clearly indicate that GST-$p75^{NTR}$ (59 C-terminal amino acids, 338–396) pulled down the full length of the FAP-1 protein but that GST alone did not (Fig. 2A, lanes 1 and 2). To determine whether FAP-1 interacts with $p75^{NTR}$ in vivo, we coexpressed FLAG epitope-tagged FAP-1 with $p75^{NTR}$ in 293T cells, and immunoprecipitated it from cell extracts by using anti-FLAG M2 monoclonal antibody (Fig. 2B). Co-immunoprecipitate of $p75^{NTR}$ was clearly detected by Western blotting using anti-$p75^{NTR}$ antibody (Fig. 2B, lane 4), but $p75^{NTR}$ proteins were not detected in the anti-FLAG monoclonal antibody immunoprecipitate from cells transfected with a FLAG-tagged FAP-1 expression plasmid without $p75^{NTR}$ (Fig. 2B, lane 3), or with a $p75^{NTR}$ expression plasmid without a FLAG-tagged FAP-1 (Fig. 2B, lane 2).

3.3. Co-localization of FAP-1 and $p75^{NTR}$

To examine the subcellular localization of FAP-1 and $p75^{NTR}$, 293T cells were cotransfected with expression plasmids encoding $hp75^{NTR}$ and GFP-FAP-1 fusion protein. As expected from the presence of a putative membrane binding domain in FAP-1, FAP-1 fusion protein was localized mostly at the plasma membrane (Fig. 3A). $p75^{NTR}$ was also located at the plasma membrane (Fig. 3B). Superimposition of the expression patterns of GFP-FAP-1 and $p75^{NTR}$ indi-

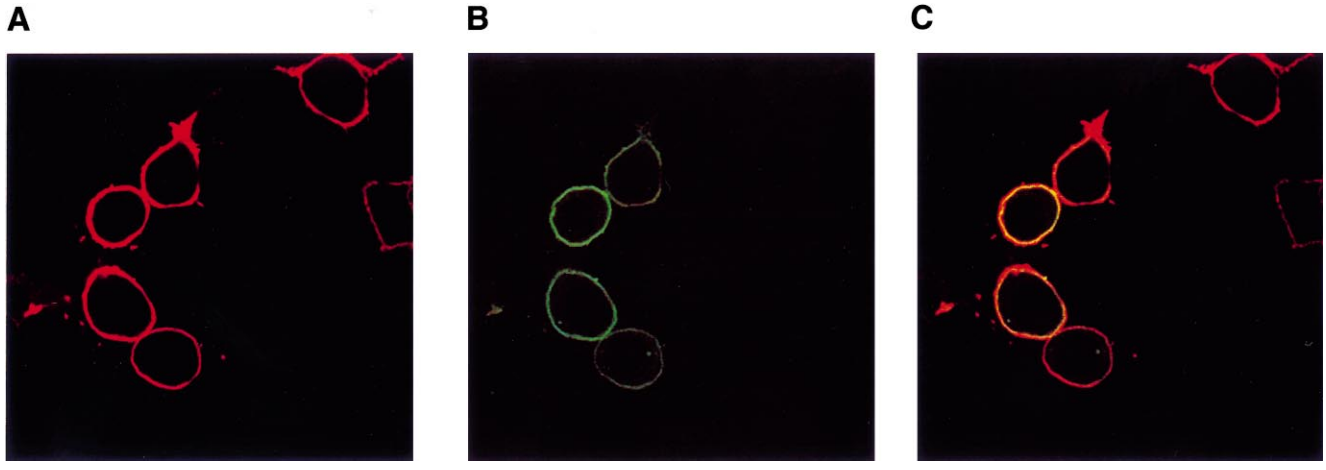

Fig. 3. Co-localization of FAP-1 and $p75^{NTR}$. Subcellular localization of FAP-1 and $p75^{NTR}$ in transfected 293T cells. 293T cells were cotransfected with pEGFP-hFAP-1 and pcDNA3-$hp75^{NTR}$. Twenty-four hours after transfection, cells were fixed. A: $p75^{NTR}$ was detected with an anti-$p75^{NTR}$ polyclonal antibody and a phycoerythrin-conjugated anti-rabbit IgG F(Ab′)2 (red). B: GFP-hFAP-1 was detected by its green fluorescence (green). C: Co-localization of $p75^{NTR}$ and GFP-hFAP-1 in the plasma membrane was detected by using combined fluorescence (yellow).

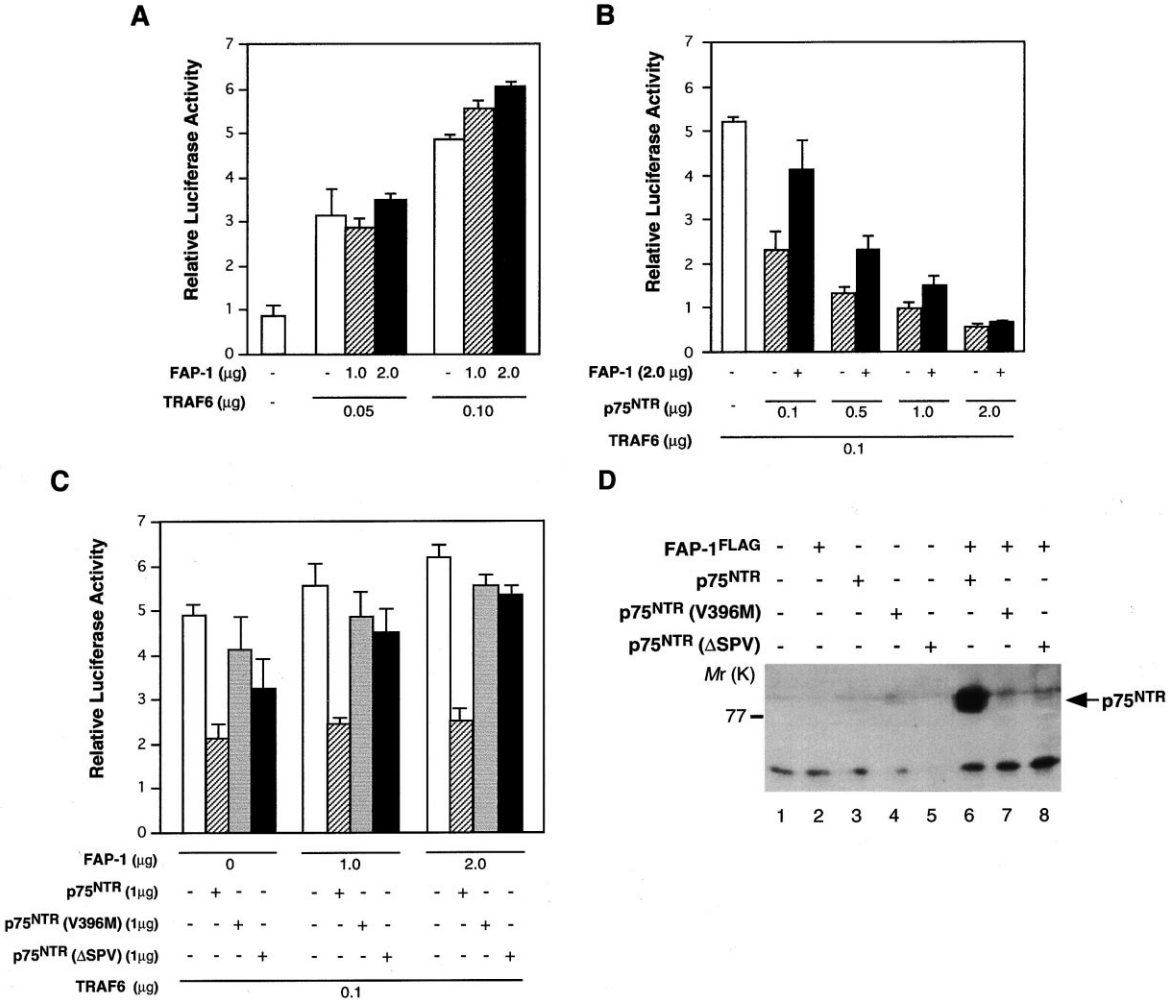

Fig. 4. Effects of interaction of FAP-1 with $p75^{NTR}$ on NF-κB activation in 293T cells. A: FAP-1 up-regulates TRAF6-mediated NF-κB activation. B: $p75^{NTR}$ down-regulates TRAF6-mediated NF-κB activation. C: Transfection of $p75^{NTR}$ (V396M) or pcDNA3-$p75^{NTR}$ (ΔSPV) up-regulates TRAF6-mediated NF-κB activation. 293T cells were cotransfected with 1.4 μg of pELAM-luc reporter plasmid, 0.1 μg of pRL-TK and indicated expression plasmids (pRc/CMV-FAP-1, pcDNA3-$p75^{NTR}$, pcDNA3-$p75^{NTR}$ [V396M], pcDNA3-$p75^{NTR}$ [ΔSPV], pCMV-myc-TRAF6), and control plasmid pcDNA3 to give 5 μg of total DNA. After 24 h, NF-κB activity was determined by an E-selectin promoter-luciferase assay. Luciferase activity was measured with a dual-luciferase assay system (Promega) and normalized to the level of pRK-TK expression. The results presented are averages of three individual experiments. D: Interaction of FAP-1 with a wild-type $p75^{NTR}$, but not with $p75^{NTR}$ (V396M) or $p75^{NTR}$ (ΔSPV) mutants. Cell lysates prepared from 293T cells transiently cotransfected with control vectors pcDNA3 and pFLAG-CMV-2 (lane 1), pFLAG-CMV-2-hFAP-1 (lane 2), pcDNA3-$p75^{NTR}$ (lane 3), pcDNA3-$p75^{NTR}$(V396M) (lane 4), pcDNA3-$p75^{NTR}$(ΔSPV) (lane 5) and their combinations were immunoprecipitated with anti-FLAG M2 monoclonal-antibody-coupled protein G Sepharose 4B. After incubation, the beads were washed, and retained $p75^{NTR}$ protein was analyzed by Western blotting with anti-$p75^{NTR}$ polyclonal antibody.

cates that the plasma membrane portions of their distributions are identical (Fig. 3C). These results are consistent with the evidence that FAP-1 physically binds $p75^{NTR}$ in vivo.

3.4. Interaction of FAP-1 with $p75^{NTR}$ is involved in the regulation of TRAF6-mediated NF-κB activation

A recent report suggested that TRAF6 associates with $p75^{NTR}$, leading the localization of the p65 subunit of NF-κB in Schwann cells [12]. To assess whether the interaction of FAP-1 and $p75^{NTR}$ regulates the NF-κB activation mediated by TRAF6, we measured the NF-κB of 293T cells transfected with $p75^{NTR}$, $p75^{NTR}$ (V396M), $p75^{NTR}$ (ΔSPV), or FAP-1 by using a dual-luciferase assay system. Transfection of 293T cells with FAP-1 or $p75^{NTR}$ alone did not affect NF-κB activation in the absence of TRAF6 (data not shown). However, co-expression with TRAF6 showed regulation of its NF-κB activation. 293T cells were cotransfected with TRAF6, which used at least 0.1 μg per transfection, and FAP-1 enhanced NF-κB activation in proportion to dose (Fig. 4A). In contrast, $p75^{NTR}$ suppressed NF-κB activity mediated by TRAF6 in proportion to the concentration of $p75^{NTR}$ (Fig. 4B). The repression of NF-κB activation by $p75^{NTR}$ was significantly decreased by FAP-1 expression (Fig. 4B). The suppression of NF-κB activation was also decreased by FAP-1 when $p75^{NTR}$ (V396M) or $p75^{NTR}$ (ΔSPV) was transfected instead of wild-type $p75^{NTR}$ (Fig. 4C). Interactions of FAP-1 with $p75^{NTR}$ (V396M) or $p75^{NTR}$ (ΔSPV) were not detected in 293T cell transfectants (Fig. 4D). These results imply that the association of FAP-1 and $p75^{NTR}$ may be related to down-regulation of NF-κB activation, or that FAP-1, which does not bind to $p75^{NTR}$, may up-regulate NF-κB activation.

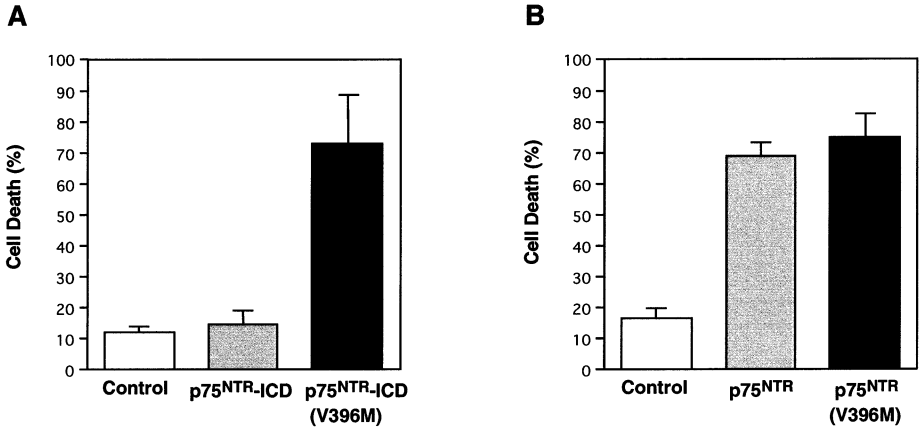

Fig. 5. Effect of mutation in the FAP-1 binding site (C-terminal SPV) of the intracellular domain of $p75^{NTR}$ to tamoxifen-induced apoptosis. A: 293T cells were transfected with a control plasmid (pcDNA3), pcDNA3-$p75^{NTR}$ICD, or pcDNA3-$p75^{NTR}$ICD (V396M). After the cells were washed with serum-free medium and placed on DMEM with 3% FBS, the cells were placed on 35 μM tamoxifen for 4–12 h. Viability was assessed by propidium iodide fluorescence and trypan blue exclusion. B: 293T cells were transfected with a control plasmid (pcDNA3), pcDNA3-$p75^{NTR}$, or pcDNA3-$p75^{NTR}$ (V396M). Methods as in A. The results are derived from 3–6 individual experiments.

3.5. $p75^{NTR}$ C-terminal mutation ($V \rightarrow M$) enhances its pro-apoptotic activity

To determine the biological significance of the *t*SPV sequence of $p75^{NTR}$, we examined the functional effects of a substitution mutation in the *t*SPV motif of $p75^{NTR}$ by using a cell line expressing FAP-1. We first confirmed that FAP-1 is endogenously expressed in 293T cells by RT-PCR and immunoblot analysis with a newly generated FAP-1 antibody (data not shown). Immunoblot analysis did not detect expression of endogenous $p75^{NTR}$ protein in 293T cells (data not shown). Using this cell line, we transfected a mammalian expression plasmid encoding the entire intracellular domain of wild-type rat $p75^{NTR}$ (pcDNA3-$p75^{NTR}$-ICD) or a mutant $p75^{NTR}$-ICD in which the C-terminal valine residue was replaced with a methionine (pcDNA3-$p75^{NTR}$-ICD [V396M]). It was shown previously that overexpression of $p75^{NTR}$ in some types of cells increases their sensitivity to various apoptotic stimuli [4]. Interestingly, transfection of pcDNA3-$p75^{NTR}$-ICD (V396M) enhanced pro-apoptotic activity induced by tamoxifen, an anti-estrogen, anti-proliferative reagent, whereas the wild-type $p75^{NTR}$ did not (Fig. 5A). The percentage of dead cells in the pcDNA3-$p75^{NTR}$-ICD (V396M) transfectants was $73 \pm 16\%$, in contrast to only 12–14% in the pcDNA3 (control) and pcDNA3-$p75^{NTR}$-ICD transfectants. Furthermore, a wild-type $p75^{NTR}$ with an extracellular domain also showed weak pro-apoptotic activity in the $p75^{NTR}$ (V396M) transfectants (Fig. 5B). These results reveal that the V396M mutation in the C-terminus of $p75^{NTR}$ increases the pro-apoptotic function of $p75^{NTR}$, and imply that potential negative regulators, such as FAP-1 sensitization, may be involved in suppressing $p75^{NTR}$-mediated signals that lead to apoptosis.

4. Discussion

We have shown clearly that FAP-1 physically interacts with the cytoplasmic region of $p75^{NTR}$ both in vitro and in vivo, and that this association is mediated through the interaction between the third PDZ domain of FAP-1 and the tSPV motif of $p75^{NTR}$. This binding characteristic of FAP-1 is consistent with previous findings that the third PDZ domain of FAP-1 specifically recognizes the C-terminal (S/T)-X-V motif, indicated by using random peptide libraries [21]. Because the PDZ domains have been thought to contribute to the formation of a protein complex to trigger a signal transduction [19,20], the interaction of the third PDZ domain of FAP-1 with the tSPV of $p75^{NTR}$ may also play an important role in $p75^{NTR}$-mediated signal transduction. Thus, FAP-1 is the first identified protein tyrosine phosphatase to interact with $p75^{NTR}$.

Interestingly, FAP-1 possesses a putative membrane binding domain similar to that found in the cytoskeleton-associated proteins ezrin [25], protein 4.1, and the neurofibromatosis type II gene product [26], as well as in the PTPases PTPH1 [27] and PTP-MEG [28]. By fluorescence analysis of GFP/FAP-1-expressing cells, we showed that FAP-1 is localized in the plasma membrane, as speculated from the existence of a putative membrane binding domain (Fig. 3). Co-localization of FAP-1 and $p75^{NTR}$ at the cell membrane raises the possibility of their interaction in mammalian cells. The FAP-1 mRNA is widely expressed in various tissues in vivo and is present at particularly high levels in adult tissues such as kidney, testis, and ovary, and at moderate levels in brain, lung, prostate, and heart ([13,14]; and our data not shown). Interestingly, the expression of FAP-1 in fetal tissues was markedly higher in brain, kidney, and lung than in adult tissues [13]. $p75^{NTR}$ is also highly expressed in the fetal brain. It is possible that FAP-1 is involved in the regulation of neuronal cell death by $p75^{NTR}$-mediated signal transduction pathways. In this regard, studies of $p75^{NTR}$ knock-out mice have shown that the numbers of surviving neurons increased in brain tissues known to express this receptor [29], implying a pro-apoptotic role for $p75^{NTR}$ in the developing brain in vivo. $p75^{NTR}$ is also expressed in non-neuronal tissues in adults and fetuses ([30,31]; and our data not shown). Therefore, FAP-1 may potentially bind to $p75^{NTR}$ and transduce the signals mediated by $p75^{NTR}$ in those tissues expressing both of them.

The regulation of NF-κB activation through $p75^{NTR}$ is related to apoptosis in some cell lines [32,33]. NF-κB activity is induced by NGF in primary Schwann cells expressing $p75^{NTR}$

but no TrkA receptors [2] and $p75^{NTR}$ associates with TRAF6 in an NGF-dependent manner [12]. The result that FAP-1 is related to NF-κB activation by TRAF6 (Fig. 4) supports the idea that FAP-1 might affect the signaling of $p75^{NTR}$ to activate NF-κB. We did not test whether NF-κB activation stimulated by NGF was affected by FAP-1, but NF-κB activity might be repressed when FAP-1 binds $p75^{NTR}$ and be accelerated by dissociation of FAP-1 from $p75^{NTR}$ without extracellular signaling. Further studies are required to examine whether FAP-1 and $p75^{NTR}$ dissociate in response to the binding of NGF to $p75^{NTR}$ and how the phosphatase activity of FAP-1 relates to NF-κB activation.

We showed that the V→M substitution in the *t*SPV motif at the C-terminus of $p75^{NTR}$ did not significantly enhance tamoxifen-induced apoptosis caused by ectopic expression of $p75^{NTR}$ in 293T cells that endogenously express FAP-1 (Fig. 5B). This implies that the dissociation of FAP-1 from the C-terminus of $p75^{NTR}$ may be caused by signaling from an extracellular region of $p75^{NTR}$ necessary for tamoxifen-induced apoptosis. The results also imply that FAP-1 may be a modular protein that regulates apoptotic signaling by $p75^{NTR}$ through its dissociation from the tSPV motif of $p75^{NTR}$. Because FAP-1 is a PTPase, this implicates tyrosine phosphorylation in some aspect of the pro-apoptotic signaling mechanism by which $p75^{NTR}$ promotes cell death. It has previously been shown that the ectopic expression of FAP-1 partially inhibits Fas-mediated apoptosis and, moreover, that the expression level of the FAP-1 protein is correlated with resistance to Fas-induced apoptosis in a variety of human tumor cell lines [17]. FAP-1 therefore appears to be a negative regulator of Fas-mediated apoptosis. Among the members of the TNFR superfamily, only $p75^{NTR}$ and Fas possess a *t*(S/T)-X-V motif. Therefore, it can be speculated that $p75^{NTR}$ and Fas share some mechanisms for regulating apoptosis.

It has been found that the Src homology 2 (SH2)-domain-containing PTPases SHP-1 and SHP-2 are tyrosine-phosphorylated and -activated following NGF treatment in PC12 cells. SHP-1 associates with TrkA in vitro, and SHP-2 associates with TrkA following TrkA receptor activation [34,35]. Thus, PTPases appear to play an important role in regulation of TrkA-mediated signal transduction. Based on the binding reported here, it is also possible that FAP-1 may participate the level of tyrosine phosphorylation elicited by activation of TrkA in the $p75^{NTR}$-TrkA receptor complex. Further studies are required to establish whether any other molecules related to the signal transduction are dephosphorylated by FAP-1.

In this report, we showed that the third PDZ domain of FAP-1 interacts with the tSPV motif of $p75^{NTR}$. This is the first demonstration that a PDZ-domain-containing protein interacts with the C-terminus of $p75^{NTR}$. Moreover, we provide indirect evidence that FAP-1 might participate in regulation of NF-κB activation and $p75^{NTR}$-mediated pro-apoptotic signaling pathways. FAP-1 is the largest of the known intracellular protein tyrosine phosphatases, possessing 6 PDZ domains. Through its multiple PDZ domains, FAP-1 may aid in the assembly of multimeric complexes of proteins, including $p75^{NTR}$, NMDA receptors, iNOS, and Shaker-type K^+ channels. Likewise, the binding of an S/T-X-L/V/I motif at the C-terminus of $p75^{NTR}$ raises the possibility that other PDZ-domain-containing proteins besides FAP-1 may compete for binding to this region of the $p75^{NTR}$ protein. Though much remains to be determined concerning the role of FAP-1-$p75^{NTR}$ interactions, the findings reported here reveal a previously unrecognized aspect of a neurotrophin receptor signaling mechanism.

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