The p80 TNF receptor-associated kinase (p80TRAK) associates with residues 354–397 of the p80 cytoplasmic domain: similarity to casein kinase

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Abstract **The cytoplasmic domain of the p80 TNF receptor associates with a protein kinase, termed p80TRAK, that phosphorylates both the p60 and p80 TNF receptors. To determine the region of the cytoplasmic domain that is necessary for binding of p80TRAK and the region that it phosphorylates, a series of deletions of the p80 cytoplasmic domain were constructed and expressed as glutathione-S-transferase fusion proteins. These fusions were then used to examine the binding of p80TRAK derived from cellular extracts. We found that out of 174 residues (266–439) in the cytoplasmic domain of p80 receptor, 44 residues (354–397) were sufficient for binding of p80TRAK. Interestingly, this was also the region that contained the phosphorylation site for p80TRAK. Phosphoamino acid analysis of this region revealed phosphorylation primarily on serine residues. Furthermore, we found that, like p80TRAK, purified casein kinase 1 (CK1) also binds to residues 354–397 of the p80 TNF receptor and causes its phosphorylation. Additionally, the activity of p80TRAK was inhibited by CK1-7, the CK1-specific inhibitor. Thus, our results indicate that p80TRAK associates with a short stretch of ~44 residues located in the cytoplasmic domain of the p80 TNF receptor and that this kinase is similar to CK1.**

Key words: Tumor necrosis factor; Kinase; p80 TNF receptor; Phosphorylation

1. Introduction

Human tumor necrosis factor (TNF)[1], a pleiotropic cytokine, interacts with two distinct receptors designated p60 (p55, Type I) and p80 (p75, Type II) [1]. Although most of the early response elicited by TNF are transmitted through the p60 TNF receptor [2], direct signaling via the p80 receptor has been demonstrated on cells of lymphoid origin such as proliferation of the CT6 cell line [3–5] and induction of granulocyte/macrophage colony stimulatory factor by a T-cell hybridoma [6]. Additionally, an independent signaling role for the p80 receptor has been demonstrated for TNF-mediated cytotoxicity in some specific cell lines [7–9] and for NF-kB activation [5,10]. Although TNF induces the phosphorylation of various proteins [[1], references therein], the role of protein phosphorylation mediated by the TNF receptors remains unclear. Recent reports have indicated that the p80 receptor itself is constitutively phosphorylated in p80-responsive and nonresponsive cell lines [11,12]. In addition, a casein kinase 1-like activity (CK1) coprecipitates with the p80 receptor only in p80-responsive cell lines [11].

Results from our laboratory using a glutathione-S-transferase (GST) fusion protein containing the cytoplasmic domain of the p80 receptor indicated a serine/threonine kinase activity termed p80TRAK physically associated with the p80 cytoplasmic domain [13]. Unlike the p60 TNF receptor-associated kinase (p60TRAK) [14], p80TRAK phosphorylated both the p60 and p80 receptor [13]. The intracellular domain of the p80 receptor contains 174 amino acids (residues 266–439), in which there are 43 potential phosphorylation sites (32 serine and 11 threonine residues). In the present report, we sought to identify the minimal region of the cytoplasmic domain necessary for interaction with p80TRAK and also the region that undergoes phosphorylation. In addition, we examined the relationship of p80TRAK to a recombinant, purified form of CK1.

2. Materials and methods

2.1. Materials

The histiocytic lymphoma cell line U-937 (CRL 1593) was obtained from the American Type Culture Collection (Rockville, MD) and maintained in a 5% CO_2 atmosphere at 37°C in RPMI-1640 medium supplemented with 10% fetal bovine serum and penicillin–streptomycin, all obtained from Life Technologies, Inc. (Grand Island, NY). Purified CK1 (4×10^6 U/mg;) was purchased from New England Biolabs, Beverly, MA. The CK1-specific inhibitor CK1-7 was purchased from Seikagaku America Inc. (Rockville, MD).

2.2. Construction, expression, and purification of GST fusion protein

All subsequent DNA manipulations were carried out as described by Sambrook et al. [15]. Fig. 1 illustrates the fusion proteins that were made for this study. The 5′-primers

CTAAGAGGATCCAAAAAGAAGCCCTTGTCCTG	(WT,Δ1)
CTAAGAGGATCCGGGACCCAGGTCAATGTCACC	(Δ2, Δ5)
CTAAGAGGATCCCAGCACCTGCTGATCACAGCG	(Δ3, Δ4)
CTAAGAGGATCCACAATGGGAGACACAGATTCC	(Δ6)

and the 3′-primers

TCTTAGAAGCTTTTAACTTGGGCTTCATCCCAGC	(WT, Δ2, Δ6)
TCTTAGAAGCTTTTAATGGCCACCAGGGGAAGA	(Δ1, Δ4)
TCTTAGAAGCTTTTACTCGTCCTTCGGGGAACTC	(Δ3, Δ5)

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Abbreviations: CK1, casein kinase 1; PCR, polymerase chain reaction; GST, glutathione-S-transferase; CD, cytoplasmic domain; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

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were used to amplify fragments from pCMVXVBpL4-p80 with unique restriction sites PCR. The PCR products were digested with *Bam*HI/*Hin*dIII and inserted into digested pGEX-2TH to yield the appropriate expression vector. Expression and purification of the GST fusion proteins from BL21 cells were carried out as previously described [14,16].

2.3. Binding of p80 TRAK to TNF receptor–GST fusion proteins

For this U-937 cells (2×10^6) were lysed in 600 μl of lysis buffer (20 mM HEPES, pH 7.4, 0.1% NP-40, 250 mM NaCl, 10 mM NaF, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin) on ice for 30 min and then centrifuged for 10 min. The supernatant was diluted with lysis buffer without NaCl to yield a final concentration of 125 mM NaCl and precleared with 10 μg of GST bound to glutathione–agarose beads for 1 h at 4°C. The precleared supernatant was mixed with approximately 5 μg of GST or the appropriate fusion protein attached to glutathione–agarose beads for 1 h at 4°C. The beads were collected by centrifugation and washed extensively with the lysis buffer (4×500 μl) and with kinase buffer (2×500 μl: 20 mM HEPES, pH 7.4, 10 mM NaF, and 0.1% 2-mercaptoethanol).

2.4. In vitro kinase assays

Standard kinase assays were carried out for 15 min at 37°C in 30 μl containing 20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.2 mM ATP, 0.2 mM NaF, 0.1 mM sodium orthovanadate, and 10 μCi [γ-^{32}P]ATP. Reactions were stopped with 15 μl of SDS-sample buffer, boiled for 5 min, and subjected to SDS-PAGE. Protein bands were visualized by staining with Coomassie Blue, and the dried gels were analyzed by a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and quantitated by ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

For in vitro binding and kinase assays with purified CK1 and the GST fusion proteins, we used a binding buffer that contained 20 mM TRIS, pH 7.6, 125 mM NaCl, 0.1% Triton X-100, 0.1% bovine serum albumin, 1 mM EDTA, and 1 mM EGTA. Approximately 5 μg of GST or the appropriate fusion protein attached to glutathione–agarose beads was mixed with 10 U (2.5 ng) of purified CK1 in 600 μl of binding buffer for 1 h at 4°C. Proteins were collected by centrifugation and washed as described above (3× binding buffer and 2× kinase buffer). In vitro kinase assays were performed as above indicated.

3. Results

We have previously reported that the cytoplasmic domain of the p80 TNF receptor physically associates with a serine/threonine kinase termed p80TRAK [13]. To define the p80TRAK binding region in the cytoplasmic domain of the p80 receptor, we constructed a series of deletions of the cytoplasmic domain as illustrated in Fig. 1A. p80 wild-type (WT) contained the entire cytoplasmic domain of the p80 receptor (residues 266–349), and p80Δ1 and p80Δ2 were the N- and C-terminal halves of the cytoplasmic domain, respectively. Deletion mutant p80Δ3 had truncations at both the N- and C-terminus, and p80Δ4 and p80Δ5 were the N- and C-terminal halves of p80Δ3, respectively. The deletion mutant p80Δ6 encompassed the C-terminal 58 residues. All deletion mutants were made as GST fusion proteins, expressed in *Escherichia coli*, and purified by affinity chromatography on glutathione–agarose beads [14]. A Coomassie Blue-stained gel of the fusion proteins used in this study appears in Fig. 1B.

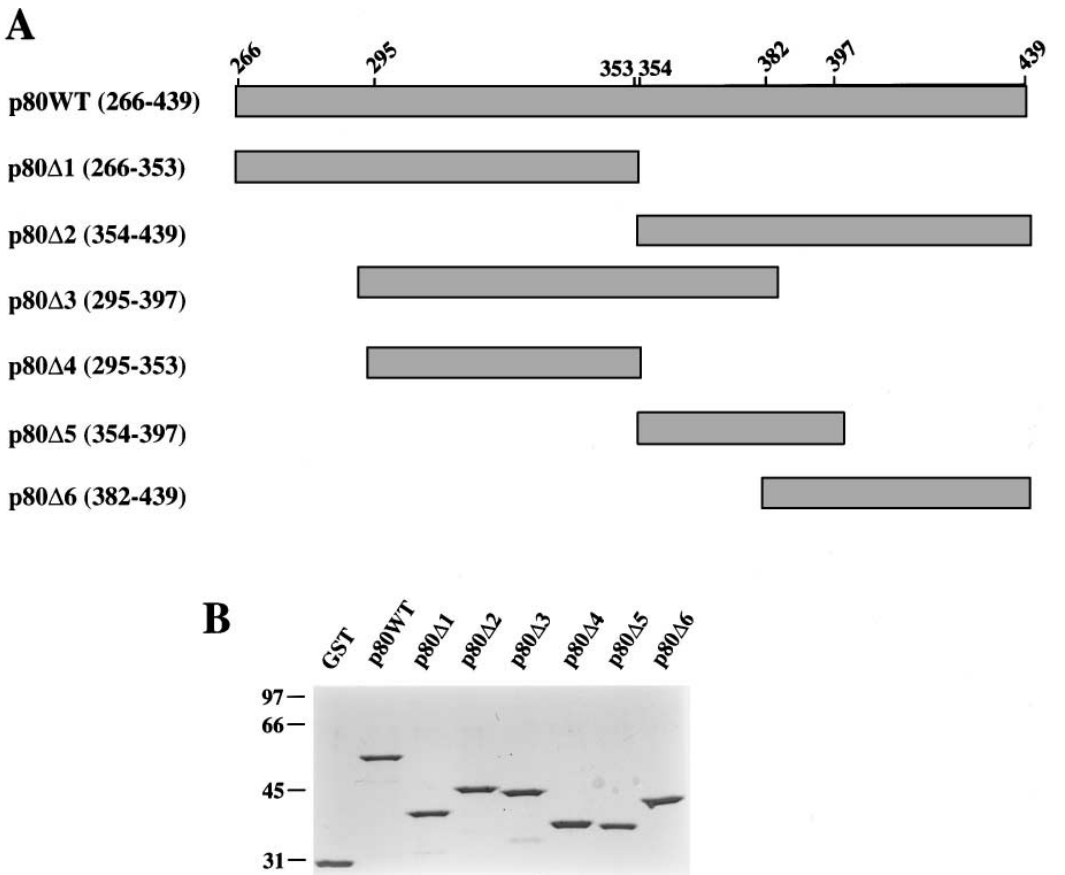

Fig. 1. Deletions of the cytoplasmic domain of the TNF receptor. A: Schematic of the cytoplasmic domain of the p80 TNF receptor (residues 266-439) with the deletion constructs used in this study. All deletions were expressed as GST fusion proteins. B: An SDS-polyacrylamide gel stained with Coomassie Blue of the GST fusion proteins with the indicated molecular mass standards.

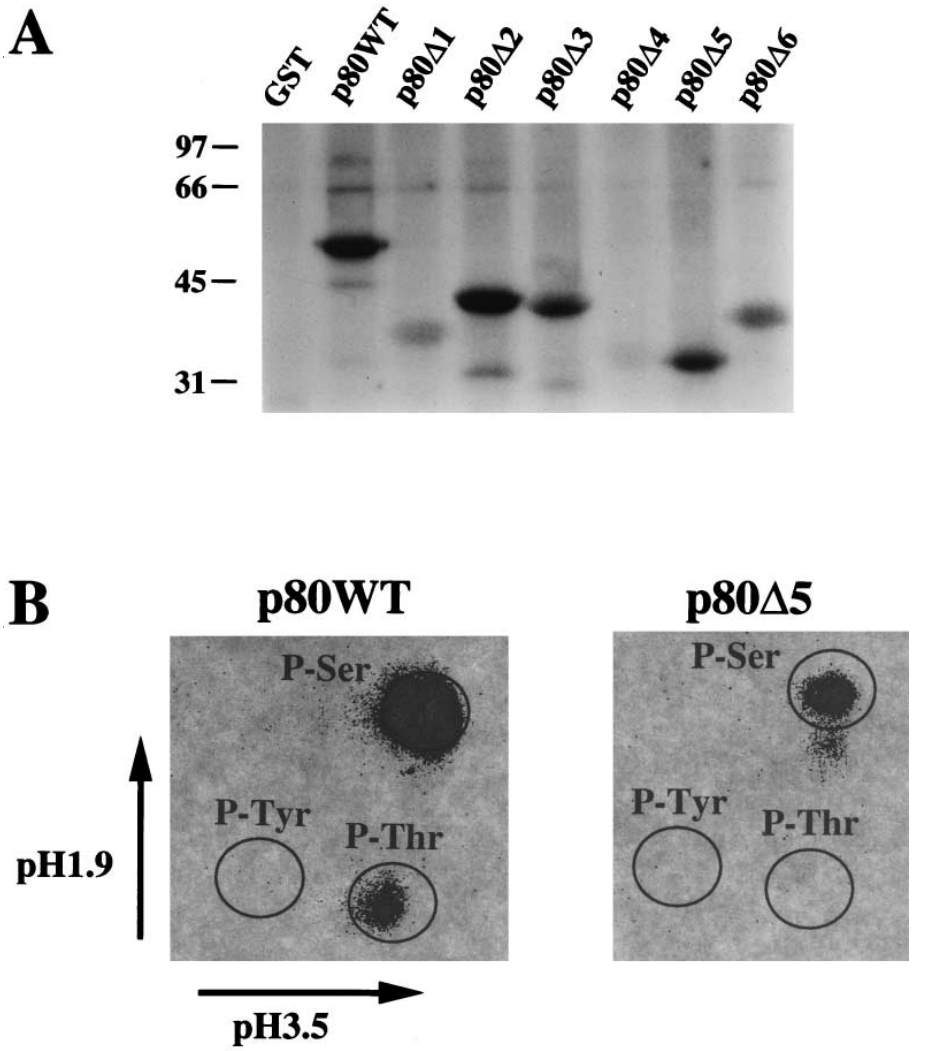

Fig. 2. p80TRAK binds to and phosphorylates a minimal region of 44 residues within the p80 cytoplasmic domain. A: U937 cell lysates from 2×10^6 per assay were prepared, and affinity precipitations were performed with the indicated GST fusion protein. In vitro kinase assays were performed as described under Section 2. The samples were analyzed by 9% SDS-PAGE, and the dried gel exposed to X-ray film for 3 h at -70°C. Relative mobilities for the various fusion proteins were identified by Coomassie Blue staining. B: Phosphoamino acid analysis of p80WT and p80Δ5 was performed as previously described [17]. The positions of phosphoserine, -threonine, and -tyrosine was indicated by staining the TLC plate with ninhydrin.

3.1. p80TRAK binds to a minimal region of 44 residues of the p80 cytoplasmic domain

To ascertain which region of the cytoplasmic domain associates with p80TRAK, the GST fusion proteins were used to affinity precipitate p80TRAK from U937 cell lysates followed by in vitro kinase assays. p80TRAK activity bound to and phosphorylated p80WT (100%), p80Δ2 (103%), p80Δ3 (49%), p80Δ5 (52%), and p80Δ6 (30%), but not GST (6%), p80Δ1 (14%), and p80Δ4 (6%) (Fig. 2A). The variable phosphorylation was not due to lack of potential phosphorylation sites because when myelin basic protein was used as an exogenous substrate, the phosphorylation by the kinase which associates with p80WT, p80Δ1, p80Δ2, p80Δ3, p80Δ4, p80Δ5, p80Δ6 and GST remained unchanged (i.e., 100%, 36%, 72%, 49%, 32%, 78%, 34% and32%, respectively). The phosphorylation patterns of p80WT, p80Δ1, and p80Δ2 considered together imply that the C-terminal half (p80Δ2), but not the membrane proximal half (p80Δ1) of the p80 cytoplasmic domain is necessary for p80TRAK binding. Within the C-terminal half, a small region of 44 residues (p80Δ5, residues 354–397) was sufficient for binding p80TRAK (Fig. 2A).

Phosphoamino acid analysis of p80WT and p80Δ5 indicated predominantly serine phosphorylation (85% and 91%, respectively) with a small amount of threonine phosphorylation and (14% and 9%, respectively) (Fig. 2B). There was no incorporation on tyrosine in either case, since the cytoplasmic domain of the p80 receptor does not contain any tyrosine residues. Thus, the minimal region of the cytoplasmic domain of the p80 receptor that interacts with p80TRAK is contained in the C-terminus, residues 354–397 (p80Δ5).

3.2. Purified CK1 also associates with residues 354–397 (p80Δ5) in vitro

Previously, it has been shown that CK-1 coprecipitates with the p80 TNF receptor from PC60 cells transfected with both TNF receptors [11]. Since p80Δ5 is sufficient for binding and

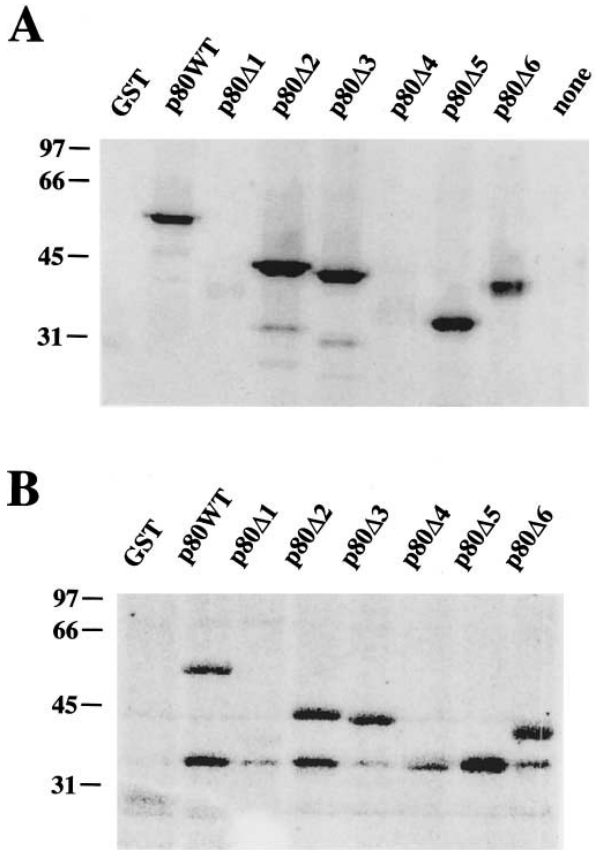

Fig. 3. Purified CK1 binds to and phosphorylates the p80 cytoplasmic domain. An in vitro kinase assay with 2.5 U of purified CK1 (A) or an in vitro binding and kinase assay with 10 U of purified CK1 (B) with the indicated fusion proteins was performed as described under Section 2. Reaction mixtures were analyzed by 9% SDS-PAGE, and the dried gel exposed to a PhosphorImager screen. ←

phosphorylation by p80TRAK and this deletion contains potential CK1 phosphorylation sites, we tested all the p80 deletion mutants to identify which region could potentially serve as substrate for purified CK1. Purified CK1 phosphorylated p80WT (100%), p80Δ2 (217%), p80Δ3 (152%), p80Δ5 (149%), and to a lesser extent p80Δ6 (75%), but not GST (7%), p80Δ1 (7%), and p80Δ4 (3%) (Fig. 3A). The phosphorylation was dose- and time-dependent (data not shown). Differences in the amount of phosphorylation of the GST fusion proteins by CK1 may reflect the number of different phosphorylation sites remaining in the deletion mutant. The phosphorylation pattern of the p80 deletion mutants observed for CK1 (Fig. 3A) was similar to that for p80TRAK precipitated from U937 cell extracts (Fig. 2A).

Since CK1 could phosphorylate specific regions of the p80 cytoplasmic domain, we next determined whether CK1 could bind to the p80 cytoplasmic domain. We first incubated purified CK1 with each of the GST fusion proteins for 1 h at 4°C, washed the mixtures extensively with binding buffer, and then performed an in vitro kinase assay (Fig. 3B). The results with purified CK1 (Fig. 3B) were indistinguishable from those obtained with p80TRAK from U937 cells (Fig. 2A). The phosphorylated band migrating at approximately 33 kDa may reflect autophosphorylation of the purified CK1 in the presence

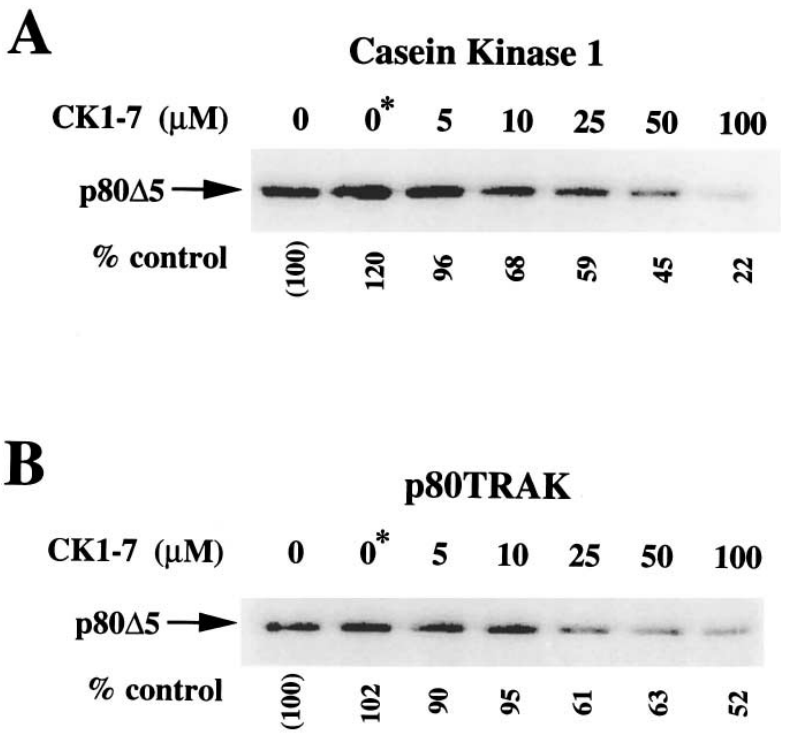

Fig. 4. A CK1-specific inhibitor blocks p80TRAK activity. An in vitro kinase assay with 10 U of purified CK1 (A) or p80TRAK precipitated from U937 cells with p80Δ5 (B) using p80Δ5 as a substrate was performed in the presence of the indicated concentration of CK1-7 dissolved in DMSO. The asterisk represents a control in which DMSO was added to a final concentration of 1.6%. Reaction mixtures were analyzed by 9% SDS-PAGE, and the dried gel was exposed to a PhosphorImager sreen. The phosphorylation of p80Δ5 was quantitated by ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

of bovine serum albumin in the binding assay[2]. Thus, it appears that p80TRAK which binds the cytoplasmic domain of the p80 receptor is closely related to CK1.

3.3. p80TRAK activity is inhibited by CK1-7, a CK1-specific inhibitor

To further support that p80TRAK activity is similar to CK1 activity, we compared the kinase activity of purified CK1 and p80TRAK with increasing concentrations of CK1-7, a specific inhibitor of CK1. As measured by phosphorylation of p80Δ5, CK1-7 inhibited the phosphorylation of the receptor by both CK1 and p80TRAK in a dose-dependent manner. A concentration of 100 μM CK1-7 was needed to inhibit by 80% and 50% of CK1 and p80TRAK, respectively (Fig. 4).

4. Discussion

This report provides evidence that a minimal region of 44 residues (p80Δ5, 354–397) residing near the C-terminus of the p80 cytoplasmic domain is necessary for binding of p80TRAK and also undergoes serine phosphorylation by p80TRAK. We further demonstrated using CK1, that CK1's binding site and its phosphorylation of the p80 cytoplasmic domain is indistinguishable from that of p80TRAK. Additionally, p80TRAK could be partially inhibited by a CK1-specific kinase inhibitor, suggesting that p80TRAK is similar to CK1.

The p80 cytoplasmic domain contains 43 potential residues for phosphorylation. While there are nine potential phosphorylation sites for CK1 within the N-terminus of the p80 cytoplasmic domain, p80Δ1 (residues 266–353) was not phosphorylated by CK1 nor p80TRAK. However, the minimal region (p80Δ5; residues 354–397) of the p80 cytoplasmic domain that was serine phosphorylated by p80TRAK contains 13 serines and 4 threonine residues. Within this region, a CK1 consensus site of the sequence Ser(P)–Xaa–Xaa–Ser/Thr [18] is repeated 4 times. The putative binding site of p80TRAK is also located within a region of the p80 cytoplasmic domain that is necessary for binding TRAF2 [5]. The constitutive presence of the phosphorylated form of the p80 TNF receptor [11,12] and its association with TRAF2 [5] may suggest that the phosphorylated form of the receptor binds to TRAF2. Whether phosphorylation of the p80 TNF receptor is critical for the recruitment of TRAF2 is not known. Interestingly, blocking the phosphorylation of the p80 receptor by a CK1 inhibitor, potentiated TNF-induced apoptosis mediated by the p80 receptor [11], suggesting an inhibitory role for phosphorylation of the receptor by CK1.

In summary, our data demonstrate that a small region of approximately 44 residues of the p80 cytoplasmic domain is sufficient for binding p80TRAK and purified, recombinant CK1. The observations that the p80 TNF receptor is phosphorylated in vivo and that p80TRAK binds within a region also necessary for TRAF2 interaction suggests that phosphorylation may play a role in TRAF2 association. Besides the receptor, TRAF2 itself contains potential phosphorylation sites for p80TRAK. It is possible that p80TRAK described here phosphorylates not only the receptor, but also TRAF2 and other related proteins that mediated signal transduction via the p80 TNF receptor.

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