Hierarchical Phosphorylation of the TNF-α Receptor, TNF-R1, by p42$^{mapk/erk}$ at Basic Pro-Directed Kinase Sites†

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ABSTRACT: Phosphorylation of the TNF-α receptor TNF-R1 has been shown to differentially regulate receptor signaling and function and promote changes in its subcellular localization. Previous studies have shown that p42$^{mapk/erk2}$ phosphorylates Ser and Thr residues (T236, S240, S244, and S270) in the membrane proximal region of TNF-R1 and that mutation of these residues to Glu and Asp residues (TNF-R1.4D/E) mimics the effect of phosphorylation on receptor signaling and localization. In the present study, we investigated whether the initial phosphorylation of these residues by p42$^{mapk/erk2}$ promotes hierarchical phosphorylation of additional sites within the cytoplasmic domain of TNF-R1. This question was addressed by investigating the ability of the TNF-R1.4D/E mutant receptor to be phosphorylated in in vitro kinase assays using GST-mutant cytoplasmic domain fusion proteins as substrates and in intact cells following mutant receptor expression. In addition, we determined the location of the additional phophorylation sites. Incubation of Sepharose bead-bound GST-TNF-R1$_{207-425}$.4D/E fusion protein with lysates containing activated p42$^{mapk/erk2}$ led to the phosphorylation of Ser and Thr residues in addition to the previously defined sites at T236, S240, S244, and S270. Deletional mutagenesis localized these residues to a stretch of 14 amino acids that encompasses three basic Pro-directed ([S/T]P) kinase consensus sequences located between residues S256 and T267. Point mutagenesis of T257, S262, and T267 to Ala residues indicated that these sites are targets of phosphorylation by p42$^{mapk/erk2}$. These findings support the conclusion that p42$^{mapk/erk2}$ promotes extensive phosphorylation of the membrane proximal region in a hierarchical fashion at both consensus and nonconsensus ERK-phosphorylation sites.

The TNF-α[1] receptor, TNF-R1, (p55, CD120a) has been implicated in a wide range of functions including apoptosis, cell proliferation, innate immunity, and germinal center development. Ligand binding induces recruitment of the adaptor protein, TNF receptor-associated death domain protein (TRADD). This, in turn, promotes the recruitment of receptor-interacting protein (RIP) and TNF receptor-associated factor-2 (TRAF2) (*1, 2*) which are required for activation of the transcription factors, NF-κB and AP-1, respectively. RIP interacts with the γ-subunit (IKKγ, NEMO) of the I-κB kinase (IKK) complex (*3*), while TRAF2 activates MEKK1 and ASK-1 and couples to the activation of MKK7 and JNK leading to AP-1 activation (*4, 5*). TRAF2 also interacts with germinal center kinase (GCK), which in turn binds MEKK1 to activate JNK and AP-1 (*6*). Thus, the initial signaling events in TNF-R1 signaling involve protein−protein interactions and lead to the activation of protein Ser/Thr kinase cascades, which in turn promote transcription factor activation.

While transcription factors are important targets of TNF-R1-activated protein Ser/Thr kinases, several members of the TNF receptor superfamily, including TNF-R1, TNF-R2 (p75, CD120b), Fas (CD95), CD27, CD40, DR3, and the LT-β receptor, are also phosphorylated by protein Ser/Thr kinases (*7*−*14*). Members of this receptor superfamily lack intrinsic kinase activity, and for most of the receptors, the kinases involved are poorly characterized. On the basis of the usage of pharmacologic Ser/Thr kinase inhibitors, Beyaert

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[1] Abbreviations: TNF-α, tumor necrosis factor-α; TNF-R1, tumor necrosis factor receptor-1; GST, glutathione-*S*-transferase; MEKK, mitogen-activated protein kinase kinase kinase; JNK, c-Jun-NH_2-terminal kinase; ASK-1, apoptosis signaling kinase-1; TRAF-2, TNF receptor-associated factor-2; TRADD, TNF recepto-associated death domain protein; RIP, receptor-interacting protein, ERK, extracellular signal regulated-kinase.

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et al. (*8*) have shown that TNF-R2 is phosphorylated by casein kinase-1, an event that negatively regulates apoptosis induced by ligation of TNF-R2. The LT-β receptor and Fas are also targets of phosphorylation, but the Ser/Thr kinases that mediate these events have not been identified (*9*, *11*). With respect to TNF-R1, initial studies showed that the receptor is phosphorylated on Ser and Thr residues by p60-TRAK (*15*, *16*), a staurosporine-sensitive Ser/Thr kinase (*17*), and also by PKC-δ (*18*). Studies reported by us have shown that the cytoplasmic domain of TNF-R1 is phosphorylated by p42$^{mapk/erk2}$ at consensus ERK-phosphorylation motifs located within the membrane proximal region (*7*, *10*). These studies revealed that mutation of T236 and S270 to Ala residues abolished the inducible phosphorylation of TNF-R1 observed in response to TNF-α stimulation of bone marrow macrophages (*10*). A consequence of phosphorylation of TNF-R1 by p42$^{mapk/erk2}$ is that the ability of the receptor to induce apoptosis is lost (*19*), and it becomes redistributed from the Golgi complex to peri-nuclear elements of the endoplasmic reticulum (*7*). In contrast, phosphorylation of TNF-R1 by p42$^{mapk/erk2}$ has no effect on the ability of the receptor to promote the activation of NF-κB (*19*). Thus, the phosphorylation of TNF-R1 by p42$^{mapk/erk2}$ has important implications for the regulation of receptor signaling and the activation of downstream effector functions.

The primary sequence of the cytoplasmic domain of TNF-R1 is rich in Ser, Thr, and Pro residues and, in addition to the previously studied consensus ERK-phosphorylation sites, contains a number of potential basic Pro-directed Ser/Thr kinase phosphorylation sites conforming to the minimal sequence [S/T]P (*20*, *21*). Previous studies have shown heterogeneity in the degree of phosphorylation of TNF-R1 (*7*, *10*), raising the possibility that the initial phosphorylation of the cluster of Ser/Thr residues located at T236, S240, S244, and S270 may promote hierarchical phosphorylation at additional sites. Hierarchy and synergy in phosphorylation have been observed in other proteins and lead to changes in secondary or tertiary structure thereby rendering additional sites available for phosphorylation. Moreover, hierarchical phosphorylation has been shown to alter protein function. For example, Kollewe et al. (*22*) have shown that the phosphorylation of the interleukin-1 receptor-associated kinase 1 (IRAK-1) at T209 induces a conformational change of the kinase domain, leading to phosphorylation of T387. This enables further phosphorylation of IRAK-1 in the [S/T]P rich region between the N-terminal death domain and kinase domain leading to the dissociation of IRAK-1 from the upstream adapters MyD88 and Tollip. Similarly, upon activation by light, rhodopsin becomes phosphorylated by rhodopsin kinase on seven Ser and Thr residues located in the C-terminal region (*23*). Nuclear magnetic resonance (NMR) studies of the 19 amino acid C-terminal peptide have shown that phosphorylation at a single site induces a major conformational change leading to the unmasking of the remaining residues. These conformational changes are believed to be necessary and sufficient to promote β-arrestin binding, which is thought to interfere with heterotrimeric G-protein binding, leading to receptor desensitization. Thus, it is possible that the initial phosphorylation of TNF-R1 at T236 and S270 induces conformational changes in the membrane proximal region of TNF-R1 leading to the unmasking of other phosphorylation sites.

In the present study, we addressed the question of whether the initial phosphorylation of TNF-R1 by p42$^{mapk/erk2}$ at residues T236, S240, S244, and S270 was required to initiate phosphorylation at additional sites within the cytoplasmic domain. As we will show, three Ser/Thr-rich clusters containing the core [S/T]P motif located within the membrane proximal region of TNF-R1 are phosphorylated by p42$^{mapk/erk2}$ in a hierarchical fashion following the initial phosphorylation at nearby consensus ERK-phosphorylation sites.

EXPERIMENTAL PROCEDURES

Materials. C3H/HeJ mice were used throughout the study and were bred at the Biological Resource Center at National Jewish. Recombinant mouse TNF-α and hamster monoclonal anti-TNF-R1 antagonist antibody were purchased from R&D Systems (Minneapolis, MN). The mitogen-activated protein kinase kinase (MEK) inhibitor, U0126, was obtained from Calbiochem (La Jolla, CA). The pGEX vectors and glutathione-Sepharose beads were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ). The pFLAG.CMV1 vector was purchased from Sigma (St. Louis, MO). ABI Prism Ready Reaction Dye Deoxy Terminator Sequencing Kits were obtained from Perkin-Elmer/ABI (Foster City, CA). Lipofectamine reagent and Opti-MEM were purchased from Life Technologies (Gaithersburg, MD). Gelcode blue was purchased from Pierce Scientific (Rockford, IL). The expression vectors for HA-tagged-*Xenopus* p42$^{mapk/erk2}$ (HA.ERK2.WT) and a constitutively active mutated form of *Xenopus* MEK-1 (MEK1.CA) in the pcDNA3 vector (Invitrogen, Carlsbad, CA) were a generous gift from Dr. Lynn Heasley (Department of Renal Medicine, University of Colorado Health Sciences Center, Denver, CO). Constitutively active MEK-1-activated purified rat p42$^{mapk/erk2}$ was purchased from Calbiochem (La Jolla, CA).

Macrophage Isolation and Culture. Bone marrow-derived macrophages were cultured from mouse femoral and tibial bone marrow as previously described (*24*). Briefly, bone marrow-derived progenitor cells were plated at a density of 1×10^6 cells per well in 6-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100 units/mL), streptomycin (100 μg/mL), 10% (v/v) heat-inactivated fetal calf-serum, and 10% (v/v) L929 conditioned medium (as a source of CSF-1). The cells were incubated at 37 °C in a 10% (v/v) CO_2 atmosphere. After 5 days, they were given additional media, which was replaced on day 6.

Construction and Expression of Deletional and Site-Directed Mutants of TNF-R1 (p55). The construction of the vectors encoding the GST-TNF-R1 cytoplasmic domain fusion protein (GST-TNF-R1$_{207-425}$) and the GST-TNF-R1$_{207-425}$ point and deletion mutants has been previously described (*10*). The full-length plasmids encoding FLAG-tagged wild-type and phosphorylation mimic TNF-R1 have also been described previously (*7*). The deletion mutants that are presented in Results were created by PCR using primers in which *Eco*RI and *Sal*I restriction sites were incorporated in the sense and antisense oligonucleotide primers, respectively. Single and multiple point mutants were constructed by site-directed mutagenesis using overlapping PCR as previously described (*10*) and also incorporating *Eco*RI and

*Sal*I sites as described (*10*). All PCR products were gel-purified, digested with *Eco*RI and *Sal*I, and ligated into *Eco*RI and *Sal*I-digested pGEX-5X-1. The fidelity of all constructs was verified by nucleotide sequencing using flanking and internal primers and was conducted in conjunction with the sequencing facility at National Jewish Medical and Research Center (Denver, CO).

Kinase Activity Assays and Two-Dimensional Phosphoamino Acid Analysis. The phosphorylation of solid-phase GST-TNF-R1 cytoplasmic domain fusion proteins was measured using in vitro kinase assays as previously described (*7*). Briefly, GST-TNF-R1 cytoplasmic domain fusion proteins were adsorbed to glutathione-Sepharose 4B beads. Lysates from stimulated or unstimulated bone marrow-derived macrophages were precleared with 20 μL GST-Sepharose beads for 20 min at 4 °C, followed by incubation with GST-TNF-R1-coated beads for 2 h at 4 °C. The beads were washed twice with NP-40 lysis buffer (50 mM Tris/HCl buffer, pH 8.0, containing 1% (v/v) NP-40, 137 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, 5 μg/mL aprotinin and 1 mM Na_3VO_4), twice in PAN buffer (10 mM Pipes buffer, pH 7.0, containing 100 mM NaCl and 10 μg/mL aprotinin), and were then resuspended in 35 μL of PAN buffer. Kinase buffer (20 mM Pipes buffer, pH 7.2, containing 10 mM $MnCl_2$ and 20 μg/mL aprotinin), together with 10 μCi [^{32}P]-γ-ATP, was added to the beads to a final volume of 75 μL, incubated at 30 °C for 20 min, and terminated by the addition of 5× Laemmli buffer containing 100 mM dithiothreitol. The samples were boiled for 5 min, separated on a 12% SDS−polyacrylamide gel, and then transferred to PVDF or nitrocellulose membranes for autoradiography. Two-dimensional phosphoamino acid analysis of phosphorylated fusion proteins was carried out as described by Hunter and Sefton (*25*) with previously reported modifications (*10*). Binding of p42$^{mapk/erk2}$ to GST-TNF-R1 cytoplasmic domain fusion proteins was determined by an in vitro pull-down assay using the same approach as described above for the in vitro phosphorylation assays. The beads were washed eight times with NP-40 lysis buffer, boiled in Laemlli sample buffer, separated by SDS−PAGE, and transferred to nitrocellulose membranes. Bound p42$^{mapk/erk}$ was detected by immunoblotting with an anti-ERK antibody.

Transfection and [^{32}P]-Labeling of Intact Cells. Transfection and [^{32}P]-labeling of COS-7 cells has been previously described (*10*). COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 U/mL of penicillin G, 100 μg/mL of streptomycin, and 2 mM glutamine. Approximately 2 × 10^5 COS-7 cells were seeded in 6-well (35 mm) dishes and transfected the following day with 0.5 μg of DNA and 10 μL of Lipofectamine reagent in a total volume of 1 mL of Opti-MEM medium. Six hours after transfection, 1 mL of Dulbecco's modified Eagle's medium containing 20% (v/v) fetal bovine serum, 100 U/mL of penicillin G, 100 μg/mL of streptomycin, and 2 mM glutamine was added to each well. Eighteen hours after transfection, the cells were washed with phosphate-free medium and metabolically labeled by incubation in phosphate-free Eagle's minimal essential medium containing 1 mCi of [^{32}P]-orthophosphate for 6 h. The cells were then lysed in 250 μL of NP-40 buffer (as described above in the kinase assays), and postnuclear supernatants were precleared with 25 μL of protein A-Sepharose beads. TNF-R1 was immunoprecipitated overnight at 4 °C with 10 μg of antagonist hamster anti-TNF-R1 monoclonal antibody and 50 μL of protein A-Sepharose beads. The beads were then washed 12 times with NP-40 lysis buffer and resuspended in 50 μL of Laemmli sample buffer, and the released proteins were resolved by SDS−PAGE, transferred to nitrocellulose, and subjected to autoradiography.

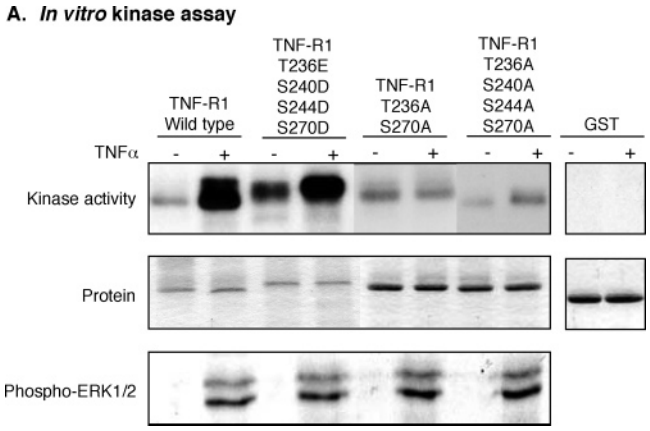

FIGURE 1: Phosphorylation of wild-type GST-TNF-R1$_{207-425}$ and consensus ERK-phosphorylation site mutants (GST-TNF-R1$_{207-425}$.4D/E, GST-TNF-R1$_{207-425}$.2A, and GST-TNF-R1$_{207-425}$.4A) by lysates from unstimulated and TNF-α-stimulated (10 ng/mL, 10 min) macrophages. Upper panel, in vitro kinase assays using the indicated GST-TNF-R1 cytoplasmic domain fusion proteins show that phosphorylation occurs at additional nonconsensus ERK sites. Phosphorylation at these sites is dependent on initial phosphorylation at the consensus ERK-phophorylation sites located at T236, S240, S244, and S270. Middle panel, gelcode blue staining shows that equal amounts of fusion protein were used in each experimental pair. Lower panel, immunoblot for active phosphorylated ERK in whole cell lysates showing that p42$^{mapk/erk2}$ was activated following stimulation with TNF-α. These results are representative of at least three experiments.

RESULTS

Phosphorylation of TNF-R1 by p42$^{mapk/erk2}$ Is Not Limited to Consensus ERK Sites. To address the question of whether the initial phosphorylation of residues T236, S240, S244, and S270 is necessary and sufficient to promote phosphorylation at other sites, we constructed a TNF-R1 cytoplasmic domain fusion protein (GST-TNF-R1$_{207-425}$.4D/E) in which the four consensus ERK-phosphorylation sites (T236, S240, S244, and S270) were mutated to acidic residues (Asp for Ser and Glu for Thr) to (i) mimic the effects of phosphorylation and (ii) render these sites unavailable for phosphorylation. Previous studies have shown that these mutations qualitatively recapitulate the effect of phosphorylation of the full-length receptor with regard to functional activity and subcellular localization (*7*, *19*). The GST-TNF-R1$_{207-425}$.4D/E fusion protein was bound to glutathione-Sepharose beads and used as a substrate in in vitro kinase assays using lysates from TNF-α-stimulated (10 ng/mL, 10 min) mouse macrophages as a source of p42$^{mapk/erk2}$. As shown in Figure 1 (top panel), the GST-TNF-R1$_{207-425}$.4D/E fusion protein was heavily phosphorylated in the presence of lysates from TNF-α-stimulated macrophages compared to unstimulated cells. Since the four consensus ERK-phosphorylation sites are not available for phosphorylation in this TNF-R1 cytoplasmic domain fusion protein, these findings suggest that phospho-

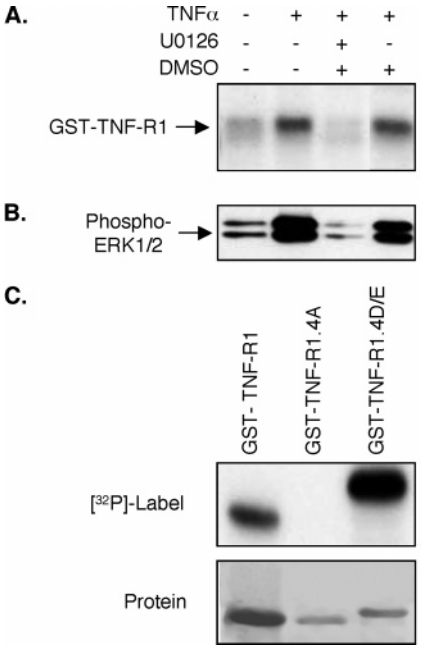

FIGURE 2: Phosphorylation of GST-TNF-R1$_{207-425}$.4D/E at nonconsensus ERK sites is mediated by p42$^{mapk/erk2}$. (A) Phosphorylation of GST-TNF-R1$_{207-425}$.4D/E by TNF-α-stimulated macrophage lysates is inhibited in the presence of the MEK1/2 inhibitor, U0126. (B) Inhibition of p42$^{mapk/erk2}$ activation in TNF-α-stimulated mouse macrophages in the presence of U0126. (C) In vitro phosphorylation of GST-TNF-R1$_{207-425}$.4D/E by recombinant MEK-1-activated p42$^{mapk/erk2}$. Upper panel, autoradiograph of in vitro kinase assay using wild-type full-length TNF-R1$_{207-425}$ (GST-TNF-R1), GST-TNF-R1$_{207-425}$.4A (GST-TNF-R1.4A), and GST-TNF-R1$_{207-425}$.4D/E (GST-TNF-R1.4D/E) as substrates in the presence of [^{32}P]-γ-ATP. Lower panel, gelcode blue staining showing that similar amounts of the TNF-R1.4A and TNF-R1-4D/E mutants were used. Results shown are representative of three different experiments.

rylation occurred at nonconsensus ERK sites. Mutagenesis of both the primary (T236 and S270) and primary and secondary ERK-consensus sites (T236, S240, S244, and S270) to Ala prevented the GST-TNF-R1 cytoplasmic domain fusion proteins from being phosphorylated by lysates from TNF-α-stimulated macrophages (Figure 1, top panel), indicating that phosphorylation at these sites is necessary to support the subsequent phosphorylation at non-ERK-consensus sites.

Since previous studies have shown that p42$^{mapk/erk2}$ is both necessary and sufficient to support the TNF-α-dependent phosphorylation of TNF-R1 (*7*), we next determined if the TNF-α-dependent phosphorylation of GST-TNF-R1$_{207-425}$.4D/E was also mediated by p42$^{mapk/erk2}$. Macrophage monolayers were pretreated with the MEK1 inhibitor, U0126, prior to stimulation with TNF-α (10 ng/mL, 10 min), lysis, and determination of the ability of the lysates to phosphorylate the GST-TNF-R1$_{207-425}$.4D/E fusion protein in an in vitro kinase assay. As can be seen in Figure 2A, pretreatment with U0126 completely inhibited the phosphorylation of the GST-TNF-R1$_{207-425}$.4D/E under conditions in which the inhibitor also blocked the activation of p42$^{mapk/erk2}$ (Figure 2B). In addition, incubation of GST-TNF-R1$_{207-425}$.4D/E-coated beads with purified MEK1-activated recombinant rat p42$^{mapk/erk2}$ in an in vitro kinase assay also resulted in substantial phosphorylation of the GST-TNF-R1$_{207-425}$.4D/E fusion protein (Figure 2C, lane 3). When the in vitro kinase assays were repeated with a GST-fusion protein in which the T236, S240, S244, and S270 ERK-consensus sites had been mutated to Ala (TNF-R1.4A), the fusion protein was not phosphorylated (Figure 2C, lane 2). These findings suggest that (i) the cytoplasmic domain of TNF-R1 is subject to further phosphorylation at additional sites to the four consensus ERK phosphate acceptor sites, (ii) phosphorylation is both dependent upon, and mediated by, activated p42$^{mapk/erk2}$, and (iii) the initial phosphorylation of the consensus ERK-phosphorylation sites (T236, S240, S244, and S270) is necessary to enable phosphorylation at nonconsensus ERK sites.

Additional Phosphorylation Sites Are Located between N250 and S300. Previous studies have shown that phosphorylation of the cytoplasmic domain of TNF-R1 by p42$^{mapk/erk2}$ occurs in the membrane proximal region (residues 207−300) but not in the C-terminal region containing the death domain (residues 294−425) (*10*). These findings suggest that the Ser/Thr phosphorylation sites, including those at nonconsensus ERK sites, are located between residues 207−300. To begin to map the additional nonconsensus ERK-phosphorylation sites, we created two TNF-R1 membrane proximal region deletion mutants, comprising residues 207−254 and 250−300 in which the consensus ERK-phosphorylation sites were mutated to acidic residues to mimic the effects of phosphorylation (constructs 8 and 9 shown schematically in Figure 3A). These regions were then expressed as GST-fusion proteins as described above and were tested, together with the full-length membrane proximal region spanning residues 207−300, in in vitro kinase assays for their ability to be phosphorylated by lysates from TNF-α-stimulated macrophages. As can be seen in Figure 3B (upper panel), the full-length membrane proximal region (TNF-R1$_{207-300}$.4D/E) and its distal half (TNF-R1$_{250-300}$.D) were heavily phosphorylated, while the proximal half comprising residues 207−254 (TNF-R1$_{207-254}$.3D/E) was not phosphorylated. These data suggest that the additional non-ERK consensus phosphorylation sites are located between residues N250 and S300. We therefore created a further series of deletion mutants, based on TNF-R1.4D/E, in which regions from the distal end of the full-length membrane proximal region were sequentially removed (GST-TNF-R1$_{207-265}$.3D/E), GST-TNF-R1$_{207-275}$.4D/E), and GST-TNF-R1$_{207-285}$.4D/E). Figure 3B (upper panel) shows that each of these deletion mutants was phosphorylated in a TNF-α responsive manner narrowing the region of additional phosphorylation to residues 250−285.

Examination of the amino acid sequence of TNF-R1$_{207-285}$ identified three areas which each encompasses a basic Pro-directed kinase motif ([S/T]P) and one or more additional Ser and/or Thr residues. The clusters are located at amino acids 256−257, 261−262, and 265−267 (Figures 3A and 6A). We therefore made further deletion mutants (TNF-R1$_{207-269}$.3D/E, TNF-R1$_{207-264}$.3D/E, and TNF-R1$_{207-259}$.3D/E) in which the Ser/Thr-rich clusters containing a [S/T]P motif were successively deleted (Figure 3A). The fusion proteins were then tested for their ability to be phosphorylated in in vitro kinase assays. As shown in Figure 3B (lower panel), TNF-α-stimulated phosphorylation of the fusion proteins decreased progressively as each of the clusters were sequentially eliminated. As noted earlier in Figure 3B, phosphorylation was completely lost when all three clusters were

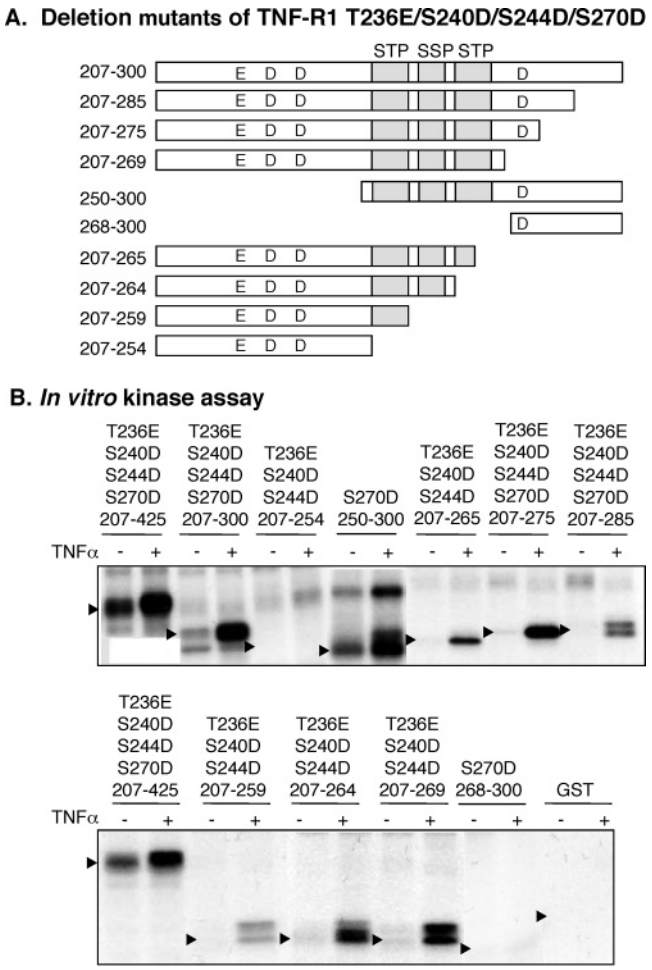

FIGURE 3: Phosphorylation of GST-TNF-R1$_{207-425}$.4D/E deletion mutants by lysates from TNF-α-stimulated macrophages. (A) Schematic representation of the deletion mutants tested showing the position of the consensus ERK (D and E) and basic [S/T]P kinase sites (dark shading). (B) In vitro kinase assays of the indicated deletion and point mutants. Lysates were obtained from unstimulated and TNF-α-stimulated (10 ng/mL, 10 min) mouse macrophages. The results are representative of three experiments. Filled arrowheads represent the position of the GST-fusion proteins as deduced by staining with Gelcode Blue.

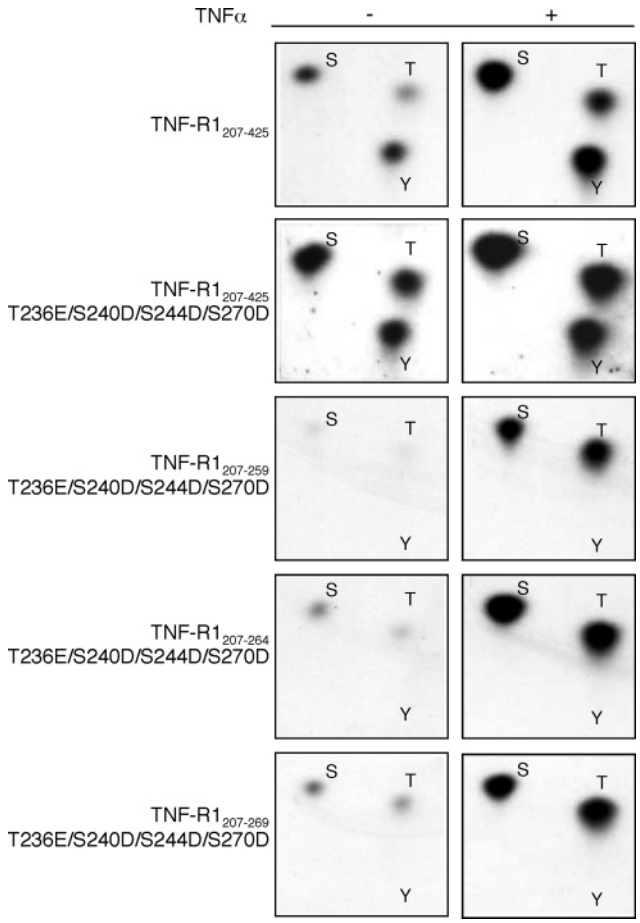

FIGURE 4: Phosphoamino acid analysis of the deletion mutants shown in Figure 3 following in vitro kinase assays with lysates from unstimulated and TNF-α-stimulated macrophages. Left upper spot = Ser, right upper spot = Thr, and bottom spot = Tyr. The results shown are representative of three different experiments.

removed in the TNF-R1$_{207-254}$.3D/E mutant. Two-dimensional phosphoamino acid analyses of the phosphorylated GST-TNF-R1$_{207-259}$.3D/E, GST-TNF-R1$_{207-264}$.3D/E, and GST-TNF-R1$_{207-269}$.3D/E fusion proteins revealed that all three fusion proteins were phosphorylated on both Ser and Thr residues (Figure 4). We also constructed a cytoplasmic domain fusion protein encompassing residues 268−300 to determine the relative contribution of Ser and Thr residues present in this region. In view of the importance of residue S270 in the phosphorylation of TNF-R1 (*10*), we designed the mutant to contain the S270D mutation. However, phosphorylation of the GST-TNF-R1$_{268-300}$.S270D fusion protein was barely detectable (Figure 3B, lower panel), suggesting that Ser/Thr residues within this region are not significant phosphate acceptor sites. We also noted basal and/or inducible tyrosine phosphorylation of TNF-R1$_{207-425}$ in some experiments, though this was not a consistent finding. However, previous studies by Darnay and Aggarwal (*26*) have shown that Y331, located in the death domain, can be phosphorylated by c-Src. Collectively, these findings suggest that each of the three Ser/Thr-rich clusters containing the [S/T]P motif is phosphoryated on both Ser and/or Thr residues within the region encompassed by residues 250−269.

To verify the role of p42$^{mapk/erk2}$ in the phosphorylation of the three Ser/Thr-rich clusters, we also examined the ability of MEK1-activated recombinant rat p42$^{mapk/erk2}$ to phosphorylate the deletion mutants in an in vitro kinase assay. As shown in Figure 5A, incubation of GST-TNF-R1$_{207-259}$.3D/E, GST-TNF-R1$_{207-264}$.3D/E, and GST-TNF-R1$_{207-269}$.3D/E fusion proteins with activated p42$^{mapk/erk2}$ resulted in a similar degree of phosphorylation to that seen with lysates from TNF-α-stimulated macrophages. In addition, two-dimensional phosphoamino acid analysis of the phosphorylated fusion proteins revealed an identical pattern of phosphorylation of Ser and Thr residues to that obtained using lysates from TNF-α-stimulated macrophages (Figure 5B). Collectively, these findings suggests that multiple Ser and Thr residues located between A250 and I269 are phosphorylated at nonconsensus ERK sites by p42$^{mapk/erk2}$.

Point Mutagenesis of the Pro-Directed MAPK Consensus Sites. To map the location of the nonconsensus ERK-phosphorylation sites, the TNF-R1$_{207-425}$.4D/E mutant was subjected to further point mutations to Ala at single or multiple combinations of the [S/T]P kinase motifs located at T257, S262, and T267. These mutants were also tested for their ability to be phosphorylated in in vitro kinase assays. A summary of the single, double, and triple mutants of T257,

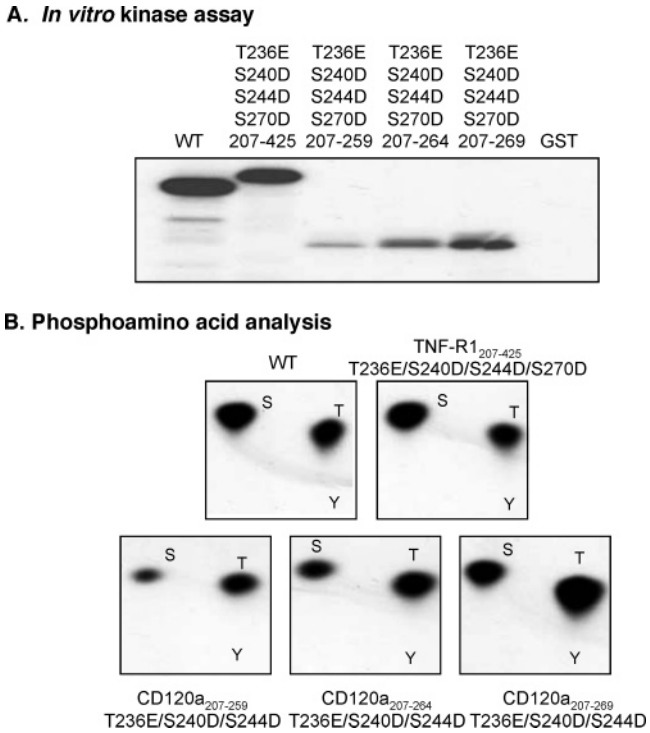

FIGURE 5: Phosphorylation of GST-TNF-R1$_{207-425}$.4D/E deletion mutants by constitutively active rat recombinant p42$^{mapk/erk2}$. (A) In vitro kinase assay with the indicated point and deletion mutants. (B) Phosphoamino acid analysis of the in vitro phosphorylated GST-TNF-R1$_{207-425}$.4D/E deletion mutants. The results shown are representative of three different experiments.

S262, and T267 are shown in Figure 6A. As shown in Figure 6D, mutation of all three [S/T]P kinase motifs to Ala prevented the TNF-R1.4D/E cytoplasmic domain fusion protein from being phosphorylated, indicating that one or more of the [S/T]P sites is subject to phosphorylation. In addition, mutation of these sites to Asp and Glu to mimic their phosphorylation did not lead to further phosphorylation, suggesting that there are no additional sites that become inducibly phosphorylated. Single and double mutations of the [S/T]P kinase motifs were also created (Figure 6A) and tested for their ability to be phosphorylated. Figure 6B shows that mutation of any one site to Ala did not substantially alter phosphorylation compared to other sites. Mutation of any two sites to Ala (Figure 6C) likewise did not lead to a striking loss of inducible phosphorylation. Collectively, these findings suggest that all three sites become inducibly phosphorylated regardless of the order in which they are phosphorylated.

The Kinase p42$^{mapk/erk2}$ Interacts with the Membrane Proximal Region of TNF-R1. The finding that p42$^{mapk/erk2}$ is responsible for the phosphorylation of multiple sites located in the membrane proximal region of TNF-R1 raised the question of whether p42$^{mapk/erk2}$ interacts with TNF-R1 and, if so, whether phosphorylation affects this interaction. To address these issues, we first determined if p42$^{mapk/erk2}$ interacted with the cytoplasmic domain of TNF-R1 using an in vitro pull-down assay with bead-bound GST-TNF-R1$_{207-425}$ and lysates from unstimulated and TNF-α-stimulated macrophages as a source of dephosphorylated and activated p42$^{mapk/erk2}$. Figure 7B (upper panel) shows that p42$^{mapk/erk2}$ bound to bead-bound GST-TNF-R1$_{207-425}$ but not to GST alone. No differences were noted in the level of

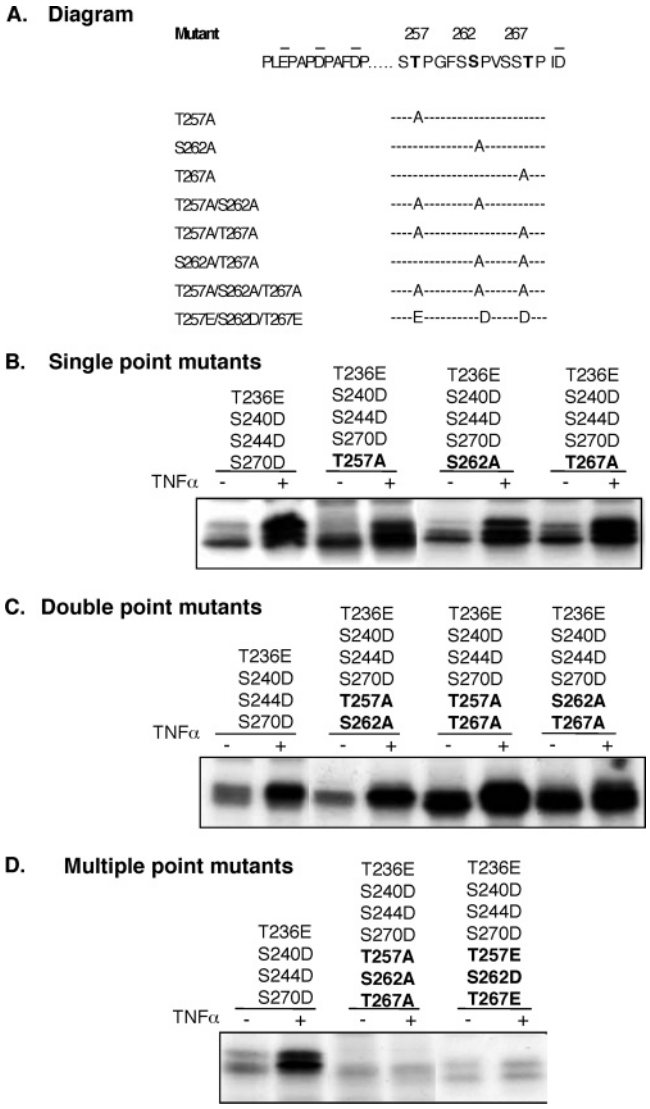

FIGURE 6: Phosphorylation of GST-TNF-R1$_{207-425}$.4D/E point and deletion mutants by lysates from TNF-α-stimulated (10 ng/mL, 10 min) and unstimulated macrophages. (A) Schematic representation of basic [S/T]P kinase motif point mutants tested. (B) In vitro kinase assays using GST-TNF-R1$_{207-425}$.4D/E single [S/T]P kinase point mutants. (C) In vitro kinase assays using GST-TNF-R1$_{207-425}$.4D/E double [S/T]P kinase point mutants. (D) In vitro kinase assays using GST-TNF-R1$_{207-425}$.4D/E triple [S/T]P kinase point mutants. The basic [S/T]P kinase sites that were subjected to mutagenesis are highlighted in boldface. The results shown are representative of three different experiments.

binding of p42$^{mapk/erk2}$ from unstimulated and TNF-α-stimulated macrophages, suggesting that binding to TNF-R1 was not affected by p42$^{mapk/erk}$ activation (data not shown). Next, we determined if p42$^{mapk/erk2}$ interacted with the membrane proximal region, the death domain or both regions, using bead-bound GST-TNF-R1$_{207-300}$ and GST-TNF-R1$_{294-425}$ fusion proteins (Figure 7A). As can be seen in Figure 7B (upper panel), p42$^{mapk/erk2}$ was bound by GST-TNF-R1$_{207-300}$, but not by GST-TNF-R1$_{294-425}$, indicating that the interaction between p42$^{mapk/erk2}$ and TNF-R1 occurs via the membrane proximal region. We also investigated the effect of phosphorylation of TNF-R1 at T236, S240, S244, and S270 on the interaction with p42$^{mapk/erk2}$ by conducting in vitro pull-down assays using the GST-TNF-R1 and GST-TNF-R1.4D/E fusion proteins. As can be seen in Figure 7B (lower panel), we observed a similar level of binding of

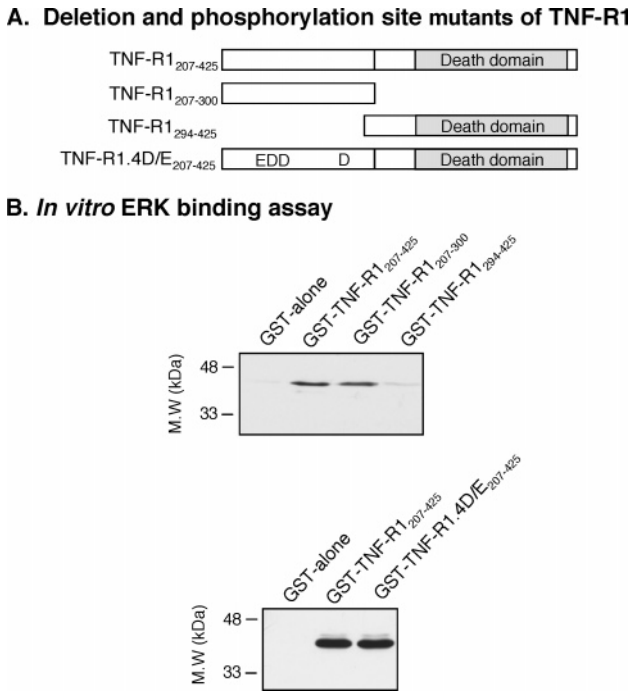

FIGURE 7: (A) Schematic representation of the delection and point mutants tested. (B) Upper panel, p42$^{mapk/erk2}$ interacts with the membrane proximal region of TNF-R1. Lysates of unstimulated mouse bone marrow-derived macrophages were incubated with Sepharose bead-bound GST alone, GST-TNF-R1$_{207-425}$, GST-TNF-R1$_{207-300}$, or GST-TNF-R1$_{294-425}$. Bound p42$^{mapk/erk2}$ was detected by Western blotting with anti-ERK antibody. Lower panel, p42$^{mapk/erk2}$ binding to TNF-R1$_{207-425}$ is unaffected by TNF-R1 phosphorylation. Binding was analyzed as described above using Sepharose bead-bound GST alone, GST-TNF-R1$_{207-425}$, or GST-TNF-R1.4D/E$_{207-425}$. The results shown are representative of three different experiments.

p42$^{mapk/erk2}$ to both wild-type TNF-R1 and TNF-R1.4D/E fusion proteins, indicating that the interaction was not affected by phosphorylation of TNF-R1.

Phosphorylation of TNF-R1 and the Phosphorylation Mimic TNF-R1 in Intact Cells. We next determined if the TNF-R1.4D/E mutant was also phosphorylated in intact cells. As with the GST-fusion protein, the full-length TNF-R1$_{1-425}$.4D/E receptor mimics the phosphorylated receptor in addition to being rendered incompetent for phosphorylation at any of the consensus ERK-phosphorylation sites. TNF-R1$_{1-425}$.4D/E was transiently transfected into COS-7 cells in the presence or absence of wild-type p42$^{mapk/erk2}$ and constitutively active MEK1. The cells were then labeled with [^{32}P]-orthophosphate (1 mCi/well, 5 h) prior to immunoprecipitation and analysis by SDS−PAGE and autoradiography. As can be seen in Figure 8, wild-type TNF-R1 was phosphorylated with ^{32}P in the presence, but not in the absence, of MEK1.CA and p42$^{mapk/erk2}$. In contrast, while there was a low level of phosphorylation of TNF-R1$_{1-425}$.4D/E in the absence of MEK1.CA and p42$^{mapk/erk2}$, the mutant receptor was heavily labeled with ^{32}P in the presence of active p42$^{mapk/erk2}$, indicating the presence of additional sites of phosphorylation. Western blotting with an anti-TNF-R1 antibody showed that both the wild-type and TNF-R1.4D/E receptors underwent an upward shift in mobility in the presence of MEK1.CA and p42$^{mapk/erk2}$, consistent with the interpretation that both receptors are subject to phosphorylation (Figure 8).

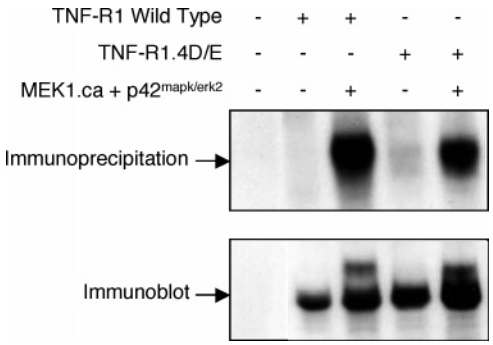

FIGURE 8: Phosphorylation of wild-type TNF-R1 and TNF-R1.4D/E in COS-7 cells. COS-7 cells were transfected as indicated with either plasmids that express constitutively active MEK1 (MEK1.CA) and wild-type p42$^{mapk/erk2}$ or empty vector as a control to induce phosphorylation of the transfected receptors. The cells were labeled with [^{32}P]-orthophosphate and lysed, and TNF-R1 was immunoprecipitated and analyzed by SDS−PAGE, blotting, and autoradiography. Upper panel, autoradiograph of [^{32}P]-labeled wild-type TNF-R1 and TNF-R1.4D/E. Lower panel, immunoblot with anti-TNF-R1 (p55) antibody. The results shown are representative of three different experiments.

DISCUSSION

The cytoplasmic domains of several members of the TNF receptor superfamily become inducibly phosphorylated on Ser and Thr residues in response to ligand binding. With regard to TNF-R1, recent work has established that the receptor is initially phosphorylated by p42$^{mapk/erk2}$ at consensus ERK-phosphorylation sites located at T236 and S270, with subsequent phosphorylation occurring at S240 and S244 (*7, 10*). Mutation of T236 and S270 to Ala residues prevents receptor phosphorylation, suggesting that phosphorylation of TNF-R1 occurs in a sequential fashion. These findings are consistent with the interpretation that the initial phosphorylation of T236 and S270, perhaps by altering the secondary structure of the membrane proximal region of the receptor, exposes S240 and S244 thereby enabling the kinase to catalyze their phosphorylation. In the present study, we have determined whether phosphorylation of T236, S240, S244, and S270 initiates additional hierarchical phosphorylation at other sites. This question was addressed by constructing a mutant receptor in which the T236, S240, S244, and S270 phosphorylation sites were mutated to acidic residues to (i) mimic the charge change associated with phosphorylation and (ii) prevent the phosphorylation of these residues. This approach thereby enabled us to distinguish between phosphorylation at both consensus ERK sites and nonconsensus ERK-phosphorylation sites. The major finding of the work presented herein is that p42$^{mapk/erk2}$ is capable of phosphorylating TNF-R1 within the membrane proximal region at three additional nonconsensus ERK-phosphorylation sites. These findings are consistent with earlier studies showing heterogeneity in phosphorylated species of TNF-R1 as detected by gel-shifting upon SDS−PAGE (*7, 10*) and support the conclusion that the Ser-, Thr-, and Pro-rich membrane proximal region of TNF-R1 undergoes an ordered hierarchical sequence of phosphorylation events in the presence of activated p42$^{mapk/erk2}$.

The phosphorylation of the TNF-R1$_{207-425}$.4D/E GST-fusion protein by lysates from TNF-α-stimulated macrophages raised the possibility that the responsible kinase(s) was either (i) an unidentified kinase(s) or (ii) p42$^{mapk/erk2}$ itself

acting through its ability to phosphorylate the molecule at sites other than the consensus ERK-phosphorylation motif, $PX_{(1,2)}$[S/T]P. Previous work has shown that p42$^{mapk/erk2}$ alone is capable of phosphorylating TNF-R1 both in vitro and intact cells (*7*). In addition, p42$^{mapk/erk2}$ can catalyze phosphorylation at nonconsensus sequences, though the degree of phosphorylation is reduced (*20*, *21*). To address the possibility that p42$^{mapk/erk2}$ was responsible for these additional phosphorylation events, we investigated the effect of (i) pharmacologic inhibition of MEK1/2 and (ii) purified activated p42$^{mapk/erk2}$, on the phosphorylation of the GST-TNF-R1$_{207-425}$.4D/E fusion in in vitro kinase assays. Both approaches supported the conclusion that p42$^{mapk/erk2}$ mediated the hierarchical phosphorylation of the cytoplasmic domain of TNF-R1 at nonconsensus ERK-phosphorylation sites. In addition, enforced expression of the phosphorylation mimic receptor, TNF-R1$_{1-425}$.4D/E, in [^{32}P]-orthophosphate-labeled COS-7 cells in the presence or absence of MEK1.CA and p42$^{mapk/erk2}$, further confirmed that p42$^{mapk/erk2}$ was required to permit the phosphorylation of TNF-R1 at additional nonconsensus ERK-phosphorylation sites.

Deletional mutagenesis of the cytoplasmic domain of TNF-R1 was used to obtain information about the position of the residues that were phosphorylated by p42$^{mapk/erk2}$. From earlier studies, we had concluded that the C-terminal region containing the death domain (residues 294−425) was not inducibly phosphorylated (*10*), while dissection of the two regions of the membrane proximal region, spanning residues 207−254 and 250−300, showed significant phosphorylation of residues located between 250 and 300 (*10*). Deletion mutants of TNF-R1$_{207-425}$.4D/E to encompass residues 207−300 or 207−254 indicated that the additional targets of phosphorylation by p42$^{mapk/erk2}$ were located between residues 250−300, while the region between residues 207−254 was not phosphorylated. Further in vitro kinase assays using deletion mutants encompassing residues 207−265, 207−275, and 207−285 indicated that multiple sites within these regions were targets for phosphorylation by p42$^{mapk/erk2}$, while a deletion mutant comprising residues 268−300 in which S270 was mutated to Glu showed no significant inducible phosphorylation. These findings suggest that the sites of additional phosphorylation by p42$^{mapk/erk2}$ are located between residues 254−268. The amino acid sequence of 250−300 contains three basic [S/T]P kinase motifs, each surrounded by one or more additional Ser or Thr residues. Although [S/T]P kinase motifs are supposedly neither optimal nor preferred targets of p42$^{mapk/erk2}$ phosphorylation (*21*), we nevertheless considered them to be candidate sites for phosphorylation, and we addressed their possible role as phosphate acceptor sites by point mutational analysis. Substitution of all three sites with either Ala or Glu/Asp completely inhibited their ability to be phosphorylated and substantiated the concept that one or more of these sites are the target of phosphorylation by p42$^{mapk/erk2}$. In addition, these studies provide conclusive evidence that there are no additional phosphate acceptor sites that are phosphorylated by p42$^{mapk/erk}$ (or by other kinases that are activated in response to stimulation of macrophages with TNF-α).

In view of the central requirement for p42$^{mapk/erk2}$ in promoting hierarchical phosphorylation of TNF-R1, we also investigated the regions of the cytoplasmic domain of TNF-R1 that were sufficient for p42$^{mapk/erk2}$ binding. Consistent with the data from the in vitro kinase assays, p42$^{mapk/erk}$ was found to interact with the membrane proximal region (residues 207−300) of TNF-R1, and this was not affected by phosphorylation at positions T236, S240, S244, and S270. Previous studies have defined at least two sequence motifs that promote ERK docking to upstream activating kinases and downstream substrates. The D-domain is characterized by an LxL sequence located 3−5 residues downstream of a cluster of basic residues and has been shown to promote the docking of ERK and JNK to acceptor proteins (*27*, *28*). In contrast, the FxFP motif exhibits specificity for ERK docking (*29*). Other docking sites comprising basic and hydrophobic residues are less well-defined but may also be important in ERK docking to different proteins (*30*). The membrane proximal region of TNF-R1 does not contain consensus ERK docking motifs, though the membrane proximal region is rich in hydrophobic and basic residues. Thus, the interaction between p42$^{mapk/erk}$ and TNF-R1 is presumed to occur via nonconsensus ERK docking sites or through an indirect mechanism.

The findings from the present study support the view that the cytoplasmic domain of TNF-R1 undergoes sequential phosphorylation at multiple sites in a hierarchical fashion that is initiated by phosphorylation at T236 and S270. Such synergy and hierarchy in phosphorylation has been observed with other inducibly phosphorylated proteins. For example, Chu et al. (*31*) have shown that the phosphorylation of heat shock factor-1 (HSF-1) at position S307 by p42$^{mapk/erk2}$ enables the secondary phosphorylation at position S303 by glycogen synthase kinase 3 (GSK3). Moreover, mutation of S307G prevented the secondary phosphorylation of HSF-1 by GSK3 (*31*). Although it is currently unclear how phosphorylation of TNF-R1 at T236 and S270 initiates subsequent phosphorylation at additional sites, other studies have suggested that the initial phosphorylation event may induce conformational changes in the secondary or tertiary structure to render additional sites available for phosphorylation. Conformational changes induced by phosphorylation have been observed in the MARCKS protein (*32*) and the T cell receptor ζ-chain ITAMs (*33*), both of which undergo a series of ordered phosphorylation events that are interdependent (*33*, *34*). Similarly, upon activation by light, rhodopsin becomes phosphorylated by rhodopsin kinase on seven Ser and Thr residues located in the C-terminal region (*23*). Nuclear magnetic resonance (NMR) studies of a 19 amino acid peptide of the C-terminal portion of rhodopsin phosphorylated at one (residue 343), three (residues 343, 338, and 334) and seven residues (residues 334, 335, 336, 338, 340, 342, and 343) have shown that phosphorylation at a single site induces a major conformational change leading to the unmasking of the remaining residues (*23*). Furthermore, mutation of T340E and S343D results in enhanced phosphorylation by rhodopsin kinase compared to the corresponding Ala mutants. These combined conformational changes are thought to be necessary and sufficient to promote β-arrestin binding, an event that is believed to sterically hinder the interaction with heterotrimeric G-proteins leading to receptor desensitization (*35*). Phosphorylation of other receptors has also been shown to mediate their desensitization, including the human CXCR4 receptor (*36*), the glucagon-like peptide-1 receptor (*37*), and the thrombin receptor (*38*). Thus, it is possible that the initial phosphorylation of

TNF-R1 at T236 and S270 induces conformational changes in the membrane proximal region of TNF-R1 leading to the unmasking of other targets of phosphorylation.

In conclusion, we have shown that the membrane proximal region of TNF-R1 is subject to phosphorylation by $p42^{mapk/erk2}$ at multiple sites, including nonconsensus ERK-phosphorylation sites containing three basic Pro-directed kinase motifs located between residues 254 and 268. Recent studies have shown that the phosphorylation of the cytoplasmic domain of TNF-R1 alters the signaling properties of the receptor by inhibiting its ability to stimulate apoptosis while preserving its capacity to activate NF-κB (*19*). Thus, defining the kinases responsible and the residues that are targeted for phosphorylation may allow us to better understand how TNF-R1 phosphorylation may contribute to the decisive events that regulate differential responses to TNF-α including cell death, survival and differentiation.

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