Phosphorylation of 130- and 95-kDa Substrates Associated with Tumor Necrosis Factor-α Receptor CD120a (p55)*

(Received for publication, August 31, 1999)

Soo-taek Uh**, Annemie Van Linden§§, and David W. H. Riches‡‡**

From the §Division of Basic Sciences, Department of Pediatrics, National Jewish Medical and Research Center, Denver, Colorado 80206 and the ¶Department of Biochemistry and Molecular Genetics, Department of Medicine, Division of Pulmonary Sciences and Critical Care Medicine, and Departments of Pharmacology and Immunology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Cross-linking of CD120a (p55), a receptor for tumor necrosis factor α (TNFα), initiates downstream events, including the activation of protein Ser/Thr kinases. In this report, we have characterized two protein Ser/Thr kinase substrates that are intrinsically associated with CD120a (p55) in mouse macrophages, and we have investigated the mechanism involved in their phosphorylation. pp130 and pp95 were detected by co-immunoprecipitation with CD120a (p55) from lysates of mouse bone marrow-derived macrophages and were phosphorylated on Ser and Thr residues during in vitro kinase assays in the presence of [γ-32P]ATP. The level of phosphorylation of pp130 and pp95 was rapidly and transiently increased in response to TNFα in [32P]orthophosphate-labeled macrophages, although the level of pp130 protein associated with CD120a (p55) remained unchanged as detected by [32S]methionine labeling. In contrast, pp130 and pp95 were efficiently phosphorylated in in vitro kinase assays of CD120a (p55) immunoprecipitates from unstimulated cells, and the level of phosphorylation was rapidly and transiently reduced in response to TNFα. Both pp130 and pp95 were sensitive to dephosphorylation with purified protein phosphatase 2A, and okadaic acid, a PP1/PP2A inhibitor, mimicked the ability of TNFα to stimulate the phosphorylation of pp130 and pp95 in intact 32P-labeled macrophages. Collectively, these findings suggest that pp130 and pp95 are constitutively associated with CD120a (p55) and become inducibly phosphorylated in macrophages in response to TNFα. We propose that the underlying mechanism of their phosphorylation may involve the inactivation of a cytoplasmic pp130/pp95 Ser/Thr phosphatase.

Tumor necrosis factor-α (TNFα)† is a pleiotropic cytokine that regulates many aspects of the inflammatory response, host defense, and fibrogenesis (1–3). Cellular responses to TNFα are mediated by two cell surface receptors, designated CD120a (p55) and CD120b (p75), which initiate distinct signaling responses, initially through protein-protein interactions between the cytoplasmic domains of the receptors and a number of adaptor and other signaling proteins. Aggregation of CD120a (p55) facilitates the binding of TRADD to CD120a (p55) via the death domains of both proteins (4). The binding of TRADD enables the assembly of TNF-receptor associated factor-2, RIP, and Fas-associated death domain protein (5, 6), which then provide connections to the activation of c-Jun NH2-terminal kinase, IκB-kinases (IKKα and β), and caspase 8, respectively (7–10). However, whereas molecules such as TRADD, TNF-receptor associated factor-2, RIP, and Fas-associated death domain protein play indisputably important roles in signal transduction following ligation of CD120a (p55), other signaling proteins, including additional Ser/Thr kinases and their appropriate substrates, have been found to be present in CD120a (p55) immunoprecipitates (11) or bound by glutathione S-transferase (GST) fusion proteins containing the cytoplasmic domain of CD120a (p55) (12). For the most part, the identity and functions of these poorly defined proteins have remained elusive.

The ability of Ser/Thr kinases to associate with sequences present in the cytoplasmic domains of CD120a (p55) and CD120b (p75), as well as the related lymphotoxin-β receptor, has been known for several years. Darnay et al. (12) have characterized a Ser/Thr protein kinase activity that binds to sequences located in the death domain of human CD120a (p55) using fusion proteins of GST and various truncations and deletions of the cytoplasmic domain of the receptor. Designated p60 TRAK, the kinase exhibits a preference for CD120a (p55), histone H1, and casein, but not for CD120b (p75) or myelin basic protein. A distinct Ser/Thr kinase activity identified as casein kinase I has also been found to constitutively associate with the cytoplasmic region of CD120b (p75), and its activity promotes rescue from apoptosis (13). A similar approach employing GST fusion proteins has also been used to characterize a Ser/Thr kinase activity that interacts with sequences present within the cytoplasmic domain of the lymphotoxin-β receptor (14). This latter kinase also appears to be specific for the lymphotoxin-β receptor and does not trans-phosphorylate CD120a (p55). A Ser kinase activity has also been detected in immunoprecipitates of human CD120a (p55) from U937 cells and was found to phosphorylate substrates of 125, 97, 85, and 60 kDa that were co-immunoprecipitated with the receptor (11). RIP and RIP2 are 74- and 61-kDa death domain-containing proteins bearing Ser/Thr kinase domains (15, 16) and are the only CD120a (p55)-associated kinases to be cloned although their molecular weights are distinct from other receptor-assoc-
ciated kinase activities. Thus, in addition to the previously identified proteins that interact with CD120a (p55), a number of other proteins, including Ser/Thr kinase(s), appear to interact with the cytoplasmic domain of the receptor.

Immunoprecipitation of mouse CD120a (p55) from mouse bone marrow-derived macrophages co-immunoprecipitates two proteins, pp130 and pp95, that are phosphorylated on Ser and Thr residues in vitro by a receptor-associated kinase activity. The major goal of the work reported herein was to determine the mechanism of phosphorylation of pp130 and pp95 and their mode of interaction with CD120a (p55). As we will show, pp130 undergoes a rapid and transient TNFα-induced increase in phosphorylation. In addition, we provide evidence suggestive of an indirect interaction between pp130 and pp95 with the cytoplasmic domain of CD120a (p55).

**EXPERIMENTAL PROCEDURES**

**Materials**—C57Bl/6 mice were bred in the Biologic Resource Center at the National Jewish Medical and Research Center and were used throughout the study to avoid the possibility of stimulation by trace amounts of endotoxin contaminants (17). Dulbecco’s modified Eagle’s medium and phosphate-free minimum essential medium (MEM) were purchased from Whittaker Bioproducts (Walkersville, MD). Methionine-free MEM was purchased from Life Technologies, Inc. Fetal bovine serum was obtained from Irvine Scientific (Santa Ana, CA). Monoclonal anti-CD120a (p55) and anti-CD120b (p75) antibodies and recombinant mouse TNFα were purchased from Genzyme (Cambridge, MA). Nonimmune hamster IgG was obtained from Accurate Chemical and Scientific Co. (Westbury, NY). BCA protein assay kits were purchased from Pierce. [γ-32P]ATP (>3,000 Ci/mmol), [32P]orthophosphate (>8500 Ci/mmol), and [35S]methionine (1175 Ci/mmol) were purchased from NEN Life Science Products.

**Macrophage Isolation and Culture**—Bone marrow-derived macrophages were prepared from femoral and tibial bone marrow as described above for bone marrow-derived progenitor cells. Bone marrow monocyte cells were preincubated with a density of 2.4 × 10^6 cells/cm² at 37 °C before inducing with isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.1 mM. One thousand cpm of each sample were loaded on cellulose thin-layer plates (Merck, Whitehouse Station, NJ). The final dimension electrophoresis was performed at 1.5 kV for 30 min in pH 1.9 buffer, whereas the second dimension was electrophoresed at 1.3 kV for 20 min in pH 5.5 buffer (5% [v/v] acetic acid, 0.5% [v/v] glycerol, and 0.5 mM EDTA). The plates were then washed twice with 2.5% [v/v] ninyhydrin in acetone, and baked at 65 °C for 20 min. The 32P-labeled threonine, serine, and tyrosine were detected by autoradiography using high performance autoradiography film (Amersham Pharmacia Biotech) and compared with the localization of the authentic unlabeled standards.

**Phosphopeptide mapping** (20, 21), excised membranes were soaked in 0.5% [v/v] polyvinylpyrrolidone dissolved in 100 mM acetic acid for 30 min at 37 °C. The membrane segments were then digested with 40 μl of TPCK-trypsin for 4 h at 37 °C. The eluted phosphoproteins were lyophilized and resuspended in 10 μl of pH 1.9 buffer, and ~1000 cpm of each sample were applied at the origin of cellulose thin-layer plates. First dimension electrophoresis was performed at 1.5 kV for 30 min in pH 1.9 buffer, and then layer chromatography for the second dimension was performed in phosphochromatography buffer (37.5% [v/v] n-butanol, 25% [v/v] pyridine, 7.5% [v/v] acetic acid) for 7 h. After drying the plates, 32P-phosphopeptides were detected by autoradiography using high performance autoradiography film (Amersham Pharmacia Biotech).

**Immunoprecipitation of 32P- and 35S-Labeled Proteins**—In vivo labeling with [32P]orthophosphate was performed using a modification of previously described methods (22). Macrophage monolayers were incubated in phosphate-free MEM for 1 h and then labeled with 1 μCi of [32P]orthophosphate dissolved in phosphate-free MEM containing 10% (v/v) dextran-coated charcoal for 10 min in 1 ml of Nonidet P-40 lysis buffer. Cell lysates were preincubated with 20 μl of protein A-Sepharose beads for 20 min, and immunoprecipitated overnight at 4 °C with 5 μg of anti-CD120a (p55) antibody or anti-CD120b (p75) antibody and 20 μl of protein A-Sepharose beads. The immune complexes were washed ten times with 1 ml of Nonidet P-40 lysis buffer. After the last wash, 2% Laemmli sample buffer containing 100 mM DTT was added, and the radiolabeled proteins were resolved by 10% SDS-PAGE under reducing conditions. The separated bands were transferred into nitrocellulose membranes for autoradiography using high performance autoradiography film (Amersham Pharmacia Biotech).

**In vitro labeling with [35S]methionine** (23) was achieved by rinsing macrophage monolayers with prewarmed Hanks’ balanced salt solution containing 0.1 mM DTT, followed by labeling for 1 h with 1 μCi of [35S]methionine dissolved in methionine-free MEM for 4–6 h. The cells were stimulated as described above under the “Results” section for removal of the [35S]methionine-containing media. The samples were processed for analysis as described above for in vivo labeling with [32P]orthophosphate.

**Construction of GST Fusion Protein Expression Vectors**—Fusion proteins comprising glutathione S-transferase and segments of CD120a (p55) and CD120b (p75) cytoplasmic domains were constructed by polymerase chain reaction and ligation into pGEX 5X-1. Polymerase chain reaction primers were synthesized to contain restriction sites for EcoRI and SalI at the 5’- and 3’-ends, respectively. The upstream and downstream primers for the construction of CD120a 184–425 (the membrane-spanning region and cytoplasmic domain) were constructed by polymerase chain reaction and ligation into pGEX 5X-1. The upstream and downstream primers for the construction of CD120a 427–707, 427–745, and 550–745 were of sequences 5'-GTTGTTAGAATTCGGATACCCGGGAGGCGGGTC-3’, 5'-GTTAGATACCCGGATACCCGGGAGGCGGGTC-3’, and 5’-GTTGGTTGTCGACCTATGCGCGCGAGGGTC-3’, respectively. The sequence of the upstream primer for the construction of CD120a 184–425 was 5’-CCACACCGAATTCGACCCGAGGTCGCAAAAGAGCCTGAG-3’, and the downstream primer was the same as that used for CD120a 427–745. The reaction mixtures were amplified with Pfu polymerase for 25 cycles containing full-length mouse CD120a (p55) as template with a profile of denaturation for 1 min at 94 °C, primer annealing at 68 °C for 45 s, and extension at 75 °C for 45 s. The polymerase chain reaction products were digested with EcoRI and SalI and ligated into EcoRI/SalI I digested pGEX-5X-1. Plasmid DNA was transformed into DH-5α cells, and the sequences of each construct were confirmed by automated DNA sequence analysis. A GST-CD120a 427–745 fusion protein was also constructed using the vector pGEX-2TK to introduce a cAMP-dependent protein kinase A motif to enable labeling of CD120a 427–745 with [32P]32P for use in far Western blots.

The expression and purification of GST fusion proteins were carried out following the modifications of a previously described method (12, 24). Transformed DH-5α cells were grown to A550 = 0.5–0.7 at 37 °C before inducing with isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.1 mM. After 3 h of induction, the cells were
Phosphorylation of TNF Receptor-associated Proteins

harvested and lysed with 10 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 1 mM EDTA, 100 μg/ml lysosome, 1.5% (v/v) sarkosyl, and 1 mM phenylmethlysulfonyl fluoride. The cell lysates were centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatants were incubated with 1.1 (v/v) slurry of glutathione-agarose beads for 1 h at 4 °C on a rotator. The beads were washed five times with cold PBS and stored at 4 °C. Unstimulated or TNFα-stimulated cells were lysed with Nonidet P-40 lysis buffer as described above. Equal amounts of whole cell lysate protein were incubated with 20 μl of Sepharose beads coated with GST-CD120a (110–425), GST-CD120a (134–425), or GST alone for 2.5 h at 4 °C. In vitro kinase reactions were then conducted as described earlier.

For Western Blotting—A probe composed of an NH2-terminal cAMP-dependent kinase consensus phosphorylation motif fused to CD120a (110–425) was prepared by phosphorylating the parent GST-PKA-CD120a (110–425), fusion protein coated beads with bovine heart kinase in the presence of [γ-32P]ATP before digesting with thrombin to release the soluble labeled probe as described in the Amersham Pharmacia Biotech GST fusion protein technical manual. Briefly, prewashed beads (1:1 slurry) were incubated in 20 μl Tris/HCl buffer, pH 7.5, containing 100 mM NaCl, 12 mM MgCl2, 50 μl of [γ-32P]ATP, 50 units of bovine heart kinase at 4 °C for 30 min. The reactions were terminated with 5 ml of 10 mM sodium phosphate, pH 8.0, containing 10 mM sodium pyrophosphate, 10 mM EDTA, and 1 mg/ml of bovine serum albumin. The beads were washed six times with PBS and then were subjected to elution with 1 ml of lysis buffer (1 unit/ml) overnight at room temperature. The supernatants were collected and used as the probe in far Western blots.

Macrophase monolayers were lysed with 500 μl of Nonidet P-40 lysis buffer, and equal amounts of lysate protein were mixed with 5× Laemmli sample buffer containing 100 mM DTT, boiled for 5 min, and separated on a 7.5% SDS-PAGE gel under reducing conditions. The separated proteins were transferred to nitrocellulose membranes and were subjected to five cycles of denaturation/renaturation (stepwise to reduce the concentration of guanidine-HCl from 6 to 0.937 M in HB buffer (25 mM HEPES, pH 7.7, containing 25 mM NaCl, 5 mM MgCl2, and 1 mM DTT) as described previously (25). The blots were saturated with HB buffer containing 5% (w/v) milk, 0.05% (v/v) Tween-20 at 4 °C overnight before adding probe (1 × 105 cpm/ml) in H buffer (25 mM HEPES, pH 7.7, containing 75 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 5% (v/v) bovine serum albumin, 0.05% (v/v) Tween-20) for 2 h at room temperature. The blots were washed five times with H buffer (26), and then autoradiograms were prepared.

RESULTS

pp130 and pp95 Are Substrates for a CD120a (p55)-associated Protein Kinase Activity—Previous work has shown that several proteins co-immunoprecipitate with CD120a (p55) and are substrates for receptor-associated protein Ser/Thr kinase(s) (11). To define kinase substrates that interact with CD120a (p55) in mouse macrophages, we immunoprecipitated the receptor from monolayers of unstimulated mouse macrophages using an antagonistic hamster monoclonal anti-mouse CD120a (p55) antibody to prevent artifactual in vitro stimulation of CD120a (p55)-dependent signaling, as has been shown to occur with other anti-TNF receptor antibodies (27, 28). The CD120a (p55) immunoprecipitates were then subjected to an in vitro kinase assay with [γ-32P]ATP to catalyze the phosphorylation of receptor-associated substrates by receptor-associated kinase(s). As can be seen in Fig. 1A, a prominent phosphoprotein with an estimated molecular mass of ~130 kDa (designated pp130) and a less conspicuous band of ~95 kDa (designated pp95) were co-immunoprecipitated with CD120a (p55) in unstimulated macrophages. A third phosphoprotein of ~55 kDa was also detected. This phosphoprotein was found to co-localize with CD120a in anti-CD120a (p75) immunoprecipitates, the anti-CD120a (p75) antibody thus may represent either phosphorylated CD120a (p55) or an additional receptor-associated protein with a molecular mass similar to that of CD120a (p55). Immunoprecipitation of macrophase lysates with anti-CD120a (p75) antibody or nonimmune hamster IgG did not result in the phosphorylation of pp130, pp95, or any other receptor-associated substrates (Fig. 1A). Two-dimensional phosphoamino acid analyses of excised segments of poly-vinylidene difluoride membranes obtained from CD120a (p55) immunoprecipitates revealed that both pp130 and pp95 were phosphorylated on Ser and Thr residues at ratios of 6.3:1 (n = 6) and 14.2:1 (n = 6) for pp130 and pp95, respectively. Neither phosphoprotein contained detectable levels of phosphotyrosine (Fig. 1B).

To investigate whether pp130 and pp95 were related phosphoproteins, we conducted high resolution two-dimensional tryptic phosphopeptide mapping on excised segments of nitrocellulose membrane. As shown in Fig. 2A, pp130 yielded 7 major tryptic peptides and ~8–10 less distinct peptides that were only apparent upon extended autoradiographic exposures. The tryptic phosphopeptide map of pp95 (Fig. 2B) was markedly different and exhibited only two highly phosphorylated peptides, the mobilities of which were not superimposed with any of the phosphopeptides obtained by tryptic hydrolysis of pp130. Extended exposure of the pp95 phosphopeptides revealed ~15 less intense 32P-labeled tryptic phosphopeptides, which also exhibited a markedly different pattern compared with pp130. pp130 and pp95 thus appear to be distinct protein Ser/Thr kinase substrates that are constitutively associated with CD120a (p55) but not CD120b (p75).

Exposure to TNFα Induces a Bimodal Change in the Level of In Vitro Phosphorylation of pp130 and pp95—We next determined whether the level or pattern of phosphorylation of pp130 and pp95 was altered in response to ligation of TNF receptors with TNFα. Macrophage monolayers were incubated with TNFα (10 ng/ml) for time intervals up to 24 h prior to lysis, immunoprecipitation with anti-CD120a (p55) antibody, and in vitro kinase reactions with [γ-32P]ATP. As can be seen in Fig. 3A, exposure to TNFα induced a bimodal change in the level of phosphorylation of both pp130 and pp95. Following stimulation for 5–10 min, a modest, although consistent, decrease in the level of phosphorylation of both pp130 and pp95 was detected in comparison with unstimulated cells. However, after 30 min of incubation with TNFα, the level of phosphorylation of pp130 and pp95 had returned to that of unstimulated cells and then began to increase before peaking ~4–12 h after exposure to TNFα. As shown in Fig. 3B, the level of phosphorylation of pp130 and pp95 was increased in a concentration-dependent fashion compared with unstimulated macrophages following
Phosphorylation of TNF Receptor-associated Proteins

18 h of exposure to increasing concentrations of TNFα (0.01–100 ng/ml). Incubation with TNFα did not result in the phosphorylation of CD120b (p75)-associated proteins following exposure for 18 h (Fig. 3 and data not shown). Phosphoamino acid analysis of pp130 and pp95 at each time point revealed no gross changes in the ratio of phosphoserine to phosphothreonine, nor was phosphorylation of tyrosine residues seen at any time point following stimulation with TNFα (Fig. 4). In addition, we did not observe any significant changes in the pattern of peptides phosphorylated in pp130 and pp95 following stimulation with TNFα compared with unstimulated cells (data not shown). Thus, the bimodal changes in the level of phosphorylation of pp130 and pp95 do not appear to be associated with gross changes in the pattern of phosphorylation of pp130 and pp95 and are suggestive of changes in the overall level of phosphorylation of acceptor sites.

In Vivo Labeling with [32P]Orthophosphate and [35S]Methionine—To investigate the effect of TNFα receptor ligation on the level of phosphorylation in vivo, macrophage monolayers were labeled with [32P]orthophosphate (1 mCi for 6 h) and incubated with TNFα or medium alone for 10 min or 6 h. The cells were then lysed, immunoprecipitated with anti-CD120a (p55) antibody, and analyzed by SDS-PAGE and autoradiography. Immunoprecipitation with anti-CD120b (p75) antibody was used as a control, as previously reported studies have shown this TNF receptor to be constitutively phosphorylated in vivo (13). As can be seen in Fig. 5A, [32P]-labeled pp130 and pp95 were detected, albeit at low levels in unstimulated macrophages. Following incubation with TNFα for 10 min, the level of phosphorylation of pp130 and, to a lesser extent, pp95 was increased in comparison with unstimulated cells but returned to the level of unstimulated macrophages after 6 h exposure to TNFα. As expected, CD120b (p75) immunoprecipitates exhibited a heavily phosphorylated band of ~80 kDa consistent with this phosphoprotein being CD120b (p75) (13) (data not shown). We also investigated the effect of TNFα on the association of pp130 protein with CD120a (p55) by biosynthetic labeling with [35S]methionine and co-immunoprecipitation with anti-CD120a (p55) antibody. As can be seen in Fig. 5B, similar amounts of [35S]methionine-labeled pp130 were associated with CD120a (p55) in both unstimulated and TNFα-stimulated cells, pp95 was not detected in co-immunoprecipitates of [35S]methionine-labeled cells suggesting either (i) a low turnover rate, (ii) a low abundance, or (iii) insufficient methionine residues to enable adequate labeling. Collectively, these findings indicate that pp130 is constitutively associated with the CD120a (p55) receptor complex and that exposure to TNFα initiates a rapid and transient increase in the level of phosphorylation of pp130, and to a lesser extent pp95, in intact cells.

Okadaic Acid Mimics the Increased Phosphorylation of pp130 by TNFα—Although seemingly discordant, the findings of a decrease in the phosphorylation of pp130 and pp95 following 10 min of stimulation with TNFα as detected with the immunoprecipitation-in vivo kinase assay and an increase in the phosphorylation of pp130 as detected by in vivo labeling with [32P]orthophosphate are consistent with the interpretation that the activity of a CD120a (p55) receptor-associated kinase(s) is counteracted by a non-receptor-bound Ser/Thr phosphatase that exhibits constitutive activity in the absence of TNFα and that becomes inactivated or rendered unavailable following ligation of CD120a (p55). This model predicts that pp130 would be phosphorylated by constitutively active receptor-associated kinase(s) subsequent to immunoprecipitation from unstimulated cells. However, following phosphorylation in intact cells, phosphate acceptor sites would become occupied and less available for phosphorylation in the in vivo kinase assay. Thus, the results from the immunoprecipitation-in vitro kinase experiments would be expected to be the reciprocal of the results from in vivo labeling.

To determine whether a PP2A/PP1-related Ser/Thr phosphatase was involved in the regulation of phosphorylation of pp130 and pp95, we have determined (i) whether the [32P]-labeled pp130 and pp95 are sensitive to hydrolysis with purified PP2A,
and (ii) whether okadaic acid mimics the increase in phosphorylation of pp130 and pp95 detected in response to stimulation with TNFα, whereas the level of pp130 associated with the receptor complex is unchanged. A, macrophages were labeled with [32P]orthophosphate and stimulated with TNFα for the indicated times. Lysates were immunoprecipitated with either anti-CD120b (p75) (all lanes labeled A) as a control or anti-CD120a (p55) (all lanes labeled B). The positions of pp130 and pp95 are indicated by the arrows. B, macrophages were labeled with [35S]methionine and stimulated in the presence and absence of TNFα as indicated. pp130 was co-immunoprecipitated with CD120a (p55). Lysates were also immunoprecipitated with nonimmune IgG as a control as indicated.

FIG. 5. Biosynthetic labeling with [32P]orthophosphate (A) and [35S]methionine (B) reveals a rapid and transient phosphorylation of pp130 and pp95 in response to stimulation with TNFα, whereas the level of pp130 associated with the receptor complex is unchanged. A, macrophages were labeled with [32P]orthophosphate and stimulated with TNFα for the indicated times. Lysates were immunoprecipitated with either anti-CD120b (p75) (all lanes labeled A) as a control or anti-CD120a (p55) (all lanes labeled B). The positions of pp130 and pp95 are indicated by the arrows. B, macrophages were labeled with [35S]methionine and stimulated in the presence and absence of TNFα as indicated. pp130 was co-immunoprecipitated with CD120a (p55). Lysates were also immunoprecipitated with nonimmune IgG as a control as indicated.

FIG. 6. A, sensitivity of phosphorylated pp130 and pp95 to dephosphorylation with purified PP2A and alkaline phosphatase. Immunoprecipitates of CD120a (p55) from unstimulated macrophages were subjected to in vitro kinase reactions in the presence or absence of PP2A (0.2 units) or alkaline phosphatase (0.2 units). As a control, okadaic acid (1 μM), a PP1/PP2A inhibitor, was added as a control to verify that the reduced level of phosphorylation of pp130 and pp95 was due to PP2A. B, okadaic acid stimulates the phosphorylation of pp130 and pp95 in intact [32P]orthophosphate-labeled mouse macrophages. Macrophage monolayers were labeled with [32P]orthophosphate (1 μCi) for 4 h and stimulated with okadaic acid (1 μM) for the indicated time points before lysing in Nonidet P-40 buffer. CD120a (p55) was immunoprecipitated and separated by SDS-PAGE through a 7.5% gel. TNFα (10 ng/ml) was included as a control.

FIG. 7. Tryptic phosphopeptide maps of pp130 following labeling in an in vitro kinase assay using [γ-32P]ATP (A) and biosynthetic labeling with [32P]orthophosphate (B) in intact mouse macrophages. The numbered arrows indicate seven superimposable tryptic phosphopeptides.

mixture, the level of phosphate incorporation into pp130 and pp95 was fully restored to that seen in the absence of PP2A. The inclusion of alkaline phosphatase in the incubation mixture also resulted in dephosphorylation of pp130 and pp95. Thus, the phosphorylated residues of both pp130 and pp95 are sensitive to removal by PP2A, and okadaic acid inhibits the PP2A-dependent dephosphorylation of these CD120a (p55)-associated phosphoproteins in vitro.

To address the question of whether an okadaic acid-inhibitable Ser/Thr phosphatase was involved in controlling the activity of the CD120a (p55)-associated kinase in mouse macrophages, we investigated the effects of okadaic acid on the level of phosphorylation of pp130 and pp95 in vivo. Macrophage monolayers were labeled with [32P]orthophosphate and incubated with okadaic acid (1 μM) for up to 3 h or with TNFα (10 ng/ml) as a positive control. The cells were then lysed, and the level of incorporation of 32P into pp130 and pp95 was determined by co-immunoprecipitation with CD120a (p55). As can be seen in Fig. 6B, exposure to okadaic acid resulted in a marked increase in the level of phosphorylation of pp130 and, to a lesser extent, pp95. We also subjected the gel-purified 32P-labeled pp130 from okadaic acid stimulated cells to tryptic phosphopeptide mapping and compared the results to phosphopeptides obtained from tryptic digests of gel purified pp130 following labeling in an in vitro kinase assay. As can be seen in Fig. 7, the qualitative pattern or phosphopeptides between the two digests was very similar with conservation of at least seven phosphopeptides. These data thus suggest that the same
protein was labeled by both techniques. Interestingly, on a semiquantitative basis, some phosphopeptides appeared to be more heavily phosphorylated in preparations of pp130 from $^{32}$P-labeled macrophages compared with when the protein was labeled in the in vitro kinase assay (e.g. phosphopeptide 3). We were unable to obtain sufficient labeling with $^{32}$Porthophosphate to be able to detect labeled phosphopeptides from unstimulated or TNFα-stimulated macrophages.

The Okadaic Acid-sensitive pp130 and pp95 Phosphatase Is Not Associated with the CD120a (p55) Receptor Complex—The detection of pp130 and pp95 Ser/Thr kinase activity in immunoprecipitates and the reciprocal nature of the findings from the in vivo labeling with $^{32}$Porthophosphate labeling are suggestive that the okadaic acid-sensitive protein Ser/Thr phosphatase is not associated with the CD120a (p55) receptor complex. To directly address this issue, we determined the effect of okadaic acid on the pp130 kinase activity of anti-CD120a (p55) immunoprecipitates because if the phosphatase were bound to the receptor complex, the level of phosphorylation of pp130 and pp95 would be expected to increase in the presence of okadaic acid. pp130 and pp95 were co-immunoprecipitated from monolayers of unstimulated and TNFα-stimulated (10 ng/ml, 10 min) macrophages and subjected to an in vitro kinase assay in the presence or absence of okadaic acid. The cell lysates were then incubated with GST-CD120a 207–425-coated Sepharose beads (GST-CD120a207–425), GST-coated Sepharose beads (GST alone) served as a negative control. In contrast, both pp95 and pp130 were detected by co-immunoprecipitation with CD120a (p55) antibody (i/p with anti-CD120a (p55)), far Western blot using $^{32}$P-labeled CD120a207–425 as a probe. Macrophage lysates were separated by SDS-PAGE through a 7.5% gel, electroblotted onto nitrocellulose, and subjected to denaturation/renaturation in guanidine hydrochloride. The blots were then probed with $^{32}$P(CD120a207–425) washed, and exposed to x-ray film. GST (GST alone) was used as a negative control, and GST-CD120a207–425 was used as a positive control.

Mechanism of Interaction between pp130, pp95, and CD120a (p55)—The in vitro and in vivo findings presented above have relied on the ability of anti-CD120a (p55) antibody to co-immunoprecipitate a receptor complex that contains pp130 and pp95, and although the method has proven useful in delineating the characteristics of these phosphoproteins, it did not allow us to determine whether their interaction with CD120a (p55) was direct or indirect. To address this question, we have adopted two approaches. First, we constructed fusion proteins in which GST was fused to residues 207–425 of mouse CD120a (p55). As can be seen in Fig. 8A, pp130 kinase activity was not increased in the presence of okadaic acid, suggesting the absence of an okadaic acid-sensitive phosphatase from the receptor complex. To further investigate whether cytosolic phosphatases were capable of dephosphorylating pp130, we incubated CD120a (p55) immunoprecipitates labeled with $^{32}$P by in vitro kinase assay, with macrophage cytosolic extracts. As can be seen in Fig. 8B, incubation in lysis buffer for 60 min did not result in any significant dephosphorylation of pp130 and pp95, also supporting the observation that a pp130/pp95 phosphatase is not associated with the receptor complex. However, when the immunoprecipitates were incubated with cytosolic extract, pp130 and pp95 were dephosphorylated. (Fig. 8B).
tion of pp130 and pp95 in both unstimulated and TNFα-stimulated macrophages. However, we did not detect any phosphorylated pp130 or pp95 following co-precipitation of cell lysates with GST-CD120a207–425-coated beads in either unstimulated or TNFα-stimulated cells. Similar results were obtained using a fusion protein containing both the transmembrane domain and the cytoplasmic domain (GST-CD120a184–425) (data not shown). Unlike the lysates from unstimulated macrophages, lysates from TNFα-stimulated cells were found to support phosphorylation of residues present within the cytoplasmic domain of CD120a (p55) as previously reported (12), indicating that the fusion proteins were capable of interacting with other cytoplasmic proteins and/or kinase(s). These findings imply that the interaction between CD120a (p55) and pp130 and pp95 may be indirect.

To investigate this issue further, we conducted far Western blotting experiments using 32P-labeled CD120a207–425 as a probe. Equal amounts of protein from lysates of unstimulated and TNFα-stimulated cells (10 ng/ml) were separated by SDS-PAGE through a 7.5% gel, blotted onto nitrocellulose membranes, and then subjected to denaturation followed by renaturation through a graded series of solutions containing guanidine hydrochloride. We also subjected a sample of GST-CD120a207–425 to the same procedure to serve as a positive control because the cytoplasmic domain of CD120a (p55) has been previously shown to self-associate (4, 29). The blots were then probed with CD120a207–425 labeled to high specific activity with 32P using bovine heart kinase in the presence of [γ-32P]ATP. As can be seen in Fig. 9B, blotting with [32P]CD120a207–425 failed to detect either pp130 or pp95. In contrast, self-association of the CD120a207–425 with the labeled probe was readily detected, indicating that the labeled probe was capable of interacting with itself.

DISCUSSION

As might be expected for a cytokine that initiates such a broad and diverse spectrum of biological activities, the intracellular signaling mechanisms that control downstream responses to TNFα have proven to be equally complex (as reviewed in Ref. 30). In the work presented herein, we have characterized two apparently unrelated CD120a (p55)-associated proteins, pp130 and pp95, that are substrates for a CD120a (p55)-associated Ser/Thr kinase. In addition, we have investigated the mechanisms controlling the phosphorylation of pp130 and pp95 in macrophages. Although the sequence and identity of pp130 and pp95 were not determined, the apparent molecular masses of these phosphoproteins support the contention that they are unrelated to TRADD, TRAP1, TNF-receptor associated factor-2, or RIP, because each of these previously cloned proteins exhibit molecular masses less than 100 kDa (4, 5, 15, 31), whereas MADD has a molecular mass of 176 kDa (32). pp130 has a similar molecular mass to 55.11 (TRAP2) (33, 34). However, immunoblotting of anti-CD120a (p55) co-immunoprecipitates with an anti-TRAP2 antiserum failed to provide an indication that pp95 and 55.11 (TRAP2) were related.3 Previously reported studies by VanArsdale and Ware (11) have confirmed the existence of phosphoproteins of similar molecular mass to pp130 and pp95 in CD120a (p55)-co-immunoprecipitates from the human histiocytic lymphoma cell line, U937. Thus, pp130 and pp95 appear to specifically interact with CD120a (p55) and serve as substrates for a receptor-associated kinase activity.

---

2 Van Linden, A., Cottin, V., and Riches, D. W. H. J. Biol. Chem. in press.

3 S.-t. Uh, D. B. Donner, and D. W. H. Riches, unpublished observations.

pp130 and pp95 were found to be phosphorylated on Ser and Thr residues in in vitro kinase assays in unstimulated macrophages. We specifically used an antagonistic monoclonal anti-CD120a (p55) antibody to prevent artificial cross-linking of CD120a (p55), pp130, pp95, and the associated Ser/Thr kinase activity during immunoprecipitation. These findings suggest that both proteins constitutively interact with CD120a (p55) in the absence of ligand, a conclusion supported in the case of pp130, by the finding of a constitutive association of 32P-labeled pp130 with CD120a (p55). Although these findings contrast with observations made for some CD120a (p55)-associated proteins, including TRADD and RIP for which interaction with CD120a (p55) has been shown to occur in a ligand-dependent fashion (4, 6), other proteins, especially those that bind to the membrane proximal region of the cytoplasmic domain, such as phosphatidylinositol 4,5-bisphosphate kinase, appear to interact in a constitutive fashion, similar to pp130 and pp95 (35).

The relative amount of 32P-labeled pp130 protein associated with CD120a (p55) was not influenced by the presence or absence of TNFα. However, the level of phosphorylation of pp130 and pp95 as seen in [γ-32P]orthophosphate-labeled macrophages was rapidly and transiently increased in response to TNFα, peaking at 10 min. In contrast, when CD120a (p55) was immunoprecipitated from unlabelled cells and subjected to an in vitro kinase assay in the presence of [γ-32P]ATP, the level of phosphorylation of pp130 and pp95 underwent a transient and concurrent decrease in response to TNFα that was maximal at 10 min and that was followed by a recovery and ultimately an increase in overall phosphorylation several hours after the addition of TNFα. In an attempt to reconcile these findings, we propose that the reduced phosphorylation detected in the in vitro kinase assays reflects increased net phosphorylation of pp130 and pp95 in response to TNFα in intact cells prior to lysis, thereby reducing the number of phosphate acceptor sites available for phosphorylation in vitro. Thus, the pattern of phosphorylation in the in vitro kinase assays was the converse of that seen using in vivo labeling with [32P]orthophosphate.

The level of phosphorylation of protein substrates reflects a balance of the activities of the appropriate protein Ser/Thr kinases and phosphatases. Given the findings that the activity of the CD120a (p55)-associated Ser/Thr kinase was detected in unstimulated cells, we tested the hypothesis that the increased phosphorylation of pp130 and pp95 in intact cells was mediated by a cytosolic protein Ser/Thr phosphatase. Several lines of evidence supported this hypothesis. First, incubation of macrophages with okadaic acid, a specific PP1/PP2A inhibitor, was found to increase the phosphorylation of pp130, and to a lesser extent, pp95, in intact 32P-labeled macrophages. Consistent with this finding, okadaic acid has been shown to mimic several TNF-signaling responses, including activation of phosphorylation of p38MAPK and p70S6K (36, 37). Okadaic acid also shares in common with TNFα an ability to activate NF-κB (38) and induce down-modulation of TNFα receptors (39). Second, purified 32P-labeled pp130 and pp95 as substrates, a pp130/ pp95-phosphatase activity was detected in macrheage cytosolic extracts. Third, the pp130/pp95 phosphatase did not appear to interact with the CD120a (p55)-receptor complex because (i) the phosphorylation of pp130 or pp95 was not increased when okadaic acid was added to in vitro kinase assays using immunoprecipitates of CD120a (p55) as a source of pp130, pp95, and the kinase, and (ii) dephosphorylation of pp130 and pp95 could be demonstrated upon incubation with total cell cytosolic extract but was not detected in the absence of cytosolic extract. A similar model of rapid inactivation of a Ser/Thr phosphatase following stimulation with TNFα has been proposed by Guy et al. (36), who have additionally shown the phosphatase to be
redox- and okadaic acid-sensitive; the identity of this phosphatase has been proposed to be PP2A or PP1 (36, 40).

As more has been learned about the components of the TNF-receptor complex, it has become clear that the interaction of signaling proteins with the receptor occurs in a hierarchical fashion in which signaling proteins recruit each other through specific and shared docking motifs. To address the question of whether or not pp130 and pp95 were able to directly interact with the cytoplasmic domain of CD120a (p55), we conducted (i) in vitro kinase assays using the GST fusion proteins containing the cytoplasmic domain of CD120a (p55) as an affinity matrix and (ii) far Western blots using $^{32}$P-labeled CD120a207–425 as a probe. Although predictable interactions were detected in the control studies, we were unable to detect pp130 or pp95 using either approach. We speculate that these findings suggest that the interaction between pp130 and pp95 with CD120a (p55) is indirect and may involve either novel or previously described adaptor proteins. In summary, we have characterized the mechanism of phosphorylation of two phosphoproteins that specifically interact with the cytoplasmic domain of CD120a (p55). Increased Ser/Thr phosphorylation of two phosphoproteins that specifically interact with the cytoplasmic domain of CD120a (p55) as an affinity matrix and (ii) far Western blots using $^{32}$P-labeled CD120a207–425 as a probe. Although predictable interactions were detected in the control studies, we were unable to detect pp130 or pp95 using either approach. We speculate that these findings suggest that the interaction between pp130 and pp95 with CD120a (p55) is indirect and may involve either novel or previously described adaptor proteins.

In summary, we have characterized the mechanism of phosphorylation of two phosphoproteins that specifically interact with the TNF-receptor CD120a (p55). Increased Ser/Thr phosphorylation of both proteins occurs in response to stimulation of macrophages with TNFα through a mechanism that is proposed to involve inactivation of a cytosolic Ser/Thr phosphatase, thereby enabling the receptor-associated kinase to express dominant activity. Clearly, it remains a possibility that either pp130 or pp95 may be the kinase.

Acknowledgments—We thank Linda Remigio and Cheryl Leu for excellent technical assistance and Drs. Vincent Cotto, Surinder Sood, and Ed Chan for helpful discussions during the course of this work. We are also indebted to Dr. David Donner, Department of Physiology and Biophysics, Indiana University School of Medicine (Indianapolis, IN) for providing anti-TRAP2 antiserum.

REFERENCES

1. Beutler, B., and Cerami, A. (1988) *Annu. Rev. Biochem.* 57, 505–518
2. Fiers, W. (1991) *FEBS Letts.* 285, 199–212
3. Riches, D. W. H. (1996) in *The Molecular and Cellular Biology of Wound Repair* (Clark, R. A. F., ed) 2nd Ed., pp. 95–141, Plenum Press, New York
4. Hsu, H., Xiong, J., and Goeddel, D. V. (1995) *Cell* 81, 495–504
5. Hsu, H., Shu, H.-B., Pan, M.-G., and Goeddel, D. V. (1996) *Cell* 84, 299–308
6. Hsu, H., Huang, J., Shu, H.-B., Bai, C., and Goeddel, D. V. (1996) *Immunity* 4, 387–396
7. Kelleher, M. A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B. Z., and Leder, P. (1996) *Immunity* 8, 297–303
8. Yeh, W.-C., Shahinian, A., Speiser, D., Krausus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hum, B., Iscove, N., Ohashi, P., Rothe, M., Goeddel, D. V., and Mak, T. W. (1997) *Immunity* 7, 715–725
9. Zhang, J., Cado, D., Chen, A., Kabra, N. H., and Winoto, A. (1998) *Nature* 392, 296–300
10. Mizu, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) *J. Biol. Chem.* 273, 2926–2930
11. VanArsdale, T. L., and Ware, C. F. (1994) *J. Immunol.* 153, 3043–3050
12. Darnay, B. G., Reddy, S. A. G., and Aggarwal, B. B. (1994) *J. Biol. Chem.* 269, 2909–2914
13. Beyerb, R., Vanhaesebroeck, B., Deleger, V., Van Lint, I., Vandenberghe, P., Agustinis, P., Vandenhende, J. R., and Fiers, W. (1995) *J. Biol. Chem.* 270, 229–2302
14. Wu, M.-Y., Hsu, T.-L., Lin, W.-W., Campbell, R. D., and Haseh, S.-L. (1997) *J. Biol. Chem.* 272, 17154–17159
15. Stanger, B. Z., Leder, P., Lee, T. H., Kim, E., and Seed, B. (1995) *Cell* 81, 513–523
16. McCarthy, J. V., Ni, J., and Dixit, V. M. (1998) *J. Biol. Chem.* 273, 16968–16975
17. Pary, L., Russell, S. W., LeBlanc, P. A., and Murasko, D. M. (1985) *J. Immunol.* 134, 977–981
18. Riches, D. W. H., and Underwood, G. A. (1991) *J. Biol. Chem.* 266, 24785–24792
19. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gantner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85
20. Hunter, T., and Sefton, B. M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 77, 1311–1315
21. Van Der Geer, P., Luo, K., Sefton, B. M., and Hunter, T. (1993) in *Protein Phosphorylation* (Hardie, D. G., ed) pp. 38–40, Oxford University Press, Oxford, United Kingdom
22. Winchester, B. W., and Riches, D. W. H. (1995) *J. Biol. Chem.* 270, 27391–27394
23. Winstein, B. W., Remigio, L. K., and Riches, D. W. H. (1985) *J. Biol. Chem.* 270, 27291–27294
24. Frangioni, J. V., and Neel, B. G. (1993) *Anul. Biochem.* 210, 179–187
25. Vinson, C. R., LaMarre, K. L., Johnson, P. E., Landschulz, W. H., and McK- inght, S. L. (1988) *Genes Dev.* 2, 801–806
26. Cavaillles, V., Dauvois, S., Danielain, P. S., and Parker, M. G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 10009–10013
27. Sohn, H. Y., Tagartia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H. W., Chen, E. Y., and Goeddel, D. V. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 2830–2834
28. Song, H. Y., Dunbar, J. D., and Donner, D. B. (1994) *J. Biol. Chem.* 269, 22492–22495
29. Darnay, B. G., and Aggarwal, B. B. (1997) *J. Leukocyte Biol.* 61, 559–566
30. Song, H. Y., Dunbar, J. D., Zhang, Y. X., Guo, D., and Donner, D. B. (1995) *J. Biol. Chem.* 270, 3574–3581
31. Schierville, A. R., Chen, J. H., Graham, J. R., and Lin, L.-L. (1997) *J. Biol. Chem.* 272, 12069–12075
32. Dunbar, J. D., Song, H. Y., Guo, D., Wu, L.-W., and Donner, D. B. (1997) *J. Immunol.* 158, 4252–4259
33. Boldin, M. P., Mett, I. L., and Wallach, D. (1995) *FERS Letts.* 367, 39–44
34. Castellino, A. M., Parker, G., Borenkoven, I. V., Anderson, R. A., and Chao, M. V. (1997) *J. Biol. Chem.* 272, 5861–5870
35. Guy, G. R., Cairns, J., Ng, S. B., and Tan, Y. H. (1993) *J. Biol. Chem.* 268, 2141–2148
36. Guy, G. R., Cao, C., Chua, S. P., and Tan, Y. H. (1992) *J. Biol. Chem.* 267, 1846–1852
37. Sun, S. C., Maggirwar, S. B., and Harbaj, E. (1995) *J. Biol. Chem.* 270, 18347–18351
38. Higuchi, M., and Aggarwal, B. B. (1993) *J. Biol. Chem.* 268, 5624–5631
39. Hunter, G. R., Philip, H., and Tan, Y. H. (1995) *Eur. J. Biochem.* 229, 503–511