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REGULATION OF TUMOR NECEOSIS FACTOR-α RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

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ABSTRACT: *Tumor necrosis factor-α(TNF), a polypeptide hormone secreted primarily by activated macrophages, was originally identified on the basis of its ability to cause hemorrhagic necrosis and tumor regression in vivo. Subsequently, TNF has been shown to be an important component of the host responses to infection and cancer and may mediate the wasting syndrome known as cachexia. These systemic actions of TNF are reflected in its diverse effects on target cells in vitro. TNF initiates its diverse cellular actions by binding to specific cell surface receptors. Although TNF receptors have been identified on most of animal cells, regulation of these receptors and the mechanisms which transduce TNF receptor binding into cellular responses are not well understood. Therefore, in the present study, the mechanisms how TNF receptors are being regulated and how TNF receptor binding is being transduced into cellular responses were investigated in rat liver plasma membranes (PM) and ME-180 human cervical carcinoma cell lines.*

^{125}I-TNF bound to high (K_d = 1.51 ± 0.35 nM) affinity receptors in rat liver PM. Solubilization of PM with 1% Triton X-100 increased both high affinity (from 0.33 ± 0.04 to 1.67 ± 0.05 pmoles/mg protein) and low affinity (from 1.92 ± 0.16 to 7.57 ± 0.50 pmoles/mg protein) TNF binding without affecting the affinities for TNF, suggesting the presence of a large latent pool of TNF receptors. Affinity labeling of receptors whether from PM or solubilized PM resulted in cross-linking of ^{125}I-TNF into M_r 130 kDa, 90 kDa and 66 kDa complexes.

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Thus, the properties of the latent TNF receptors were similar to those initially accessible to TNF. To determine if exposure of latent receptors is regulated by TNF, ^{125}I-TNF binding to control and TNF-pretreated membranes were assayed. Specific binding was increased by pretreatment with TNF ($P<0.05$), demonstrating that hepatic PM contains latent TNF receptors whose exposure is promoted by TNF. Homologous up-regulation of TNF receptors may, in part, be responsible for sustained hepatic responsiveness during chronic exposure to TNF.

As a next step, the post-receptor events induced by TNF were examined. Although the signal transduction pathways for TNF have not been delineated clearly, the actions of many other hormones are mediated by the reversible phosphorylation of specific enzymes or target proteins. The present study demonstrated that TNF induces phosphorylation of 28 kDa protein (p28). Two dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) resolved the 28 kDa phosphoprotein into two isoforms having pIs of 6.2 and 6.1. The pIs and relative molecular weight of p28 were consistent with those of a previously characterized mRNA cap binding protein. mRNA cap binding proteins are a class of translation initiation factors that recognize the 7-methylguanosine cap structure found on the 5' end of eukaryotic mRNAs. In vitro, these proteins are defined by their specific elution from affinity columns composed of 7-methylguanosine 5'-triphosphate(m^{7}GTP)-Sepharose. Affinity purification of mRNA cap binding proteins from control and TNF treated ME-180 cells proved that TNF rapidly stimulates phosphorylation of an mRNA cap binding protein. Phosphorylation occurred in several cell types that are important in vitro models of TNF action. The mRNA cap binding protein phosphorylated in response to TNF treatment was purified, sequenced, and identified as the proto-oncogene product eukaryotic initiation factor-4E(eIF-4E). These data show that phosphorylation of a key component of the cellular translational machinery is a common early event in the diverse cellular actions of TNF.

Key Words: *Tumor Necrosis Factor-α Receptors, Protein Phosphorylation, Signal Transduction, Eukaryotic Initiation Factor-4E.*

INTRODUCTION

Tumor necrosis factor-α was first identified by its ability to induce hemorrhagic

necrosis and regression of tumors in experimental animals and by the cytotoxic response that it can elicit in transformed cell *in vitro* (Old, 1985). TNF, also called cachectin, suppresses the expression of anabolic enzymes such as lipoprotein lipase in adipocytes, inducing a catabolic state and the consequent release of energy stores from adipose and other host tissues in response to infection (Beutler and Cerami, 1986; Sherry and Cerami, 1988). Thus, TNF has been proposed to mediate the wasting (cachexia) that often accompanies chronic disease states. The demonstration that the immune system produces an oncolytic agent with therapeutic potential, which may also induce significant alterations of target tissue metabolism, has produced great interest in TNF.

Chronic exposure of target cells to hormones generally results in desensitization of responsiveness to further stimulation. Down-regulation of the number of hormone receptors expressed at the cell surface is a common mechanism through which cellular responsiveness may be attenuated (Posner *et al.*, 1982; Sibley and Lefkowitz, 1985). Therefore, chronic exposure to TNF would be expected to promote receptor down-regulation and desensitization of target organ responsiveness. However, TNF has been proposed to induce metabolic changes in various host tissues during chronic diseases (Sherry and Cerami, 1988). In the liver, TNF induced the synthesis of acute phase proteins (Mackiewicz *et al.*, 1988; Romadori *et al.*, 1988; Baumann, 1988; Castell *et al.*, 1989) and lipids (Feingold and Grunfeld, 1987; Feingold *et al.*, 1989; Chajek-Shaul *et al.*, 1989), and the liver remains responsive to TNF upon repeated administration over several days (Grunfeld *et al.*, 1989). To reconcile these observations, in the present study, the mechanisms that might sustain the responsiveness of the liver to TNF during chronic exposure were investigated in the light of the presence of a large pool of latent TNF receptors and homologous up-regulation of these receptors.

The events that occur subsequent to TNF receptor binding were also examined in this study. The mechanisms which transduce TNF receptor binding into cellular responses are not well understood, but the actions of many other hormones and cytokines are mediated by the reversible phosphorylation of specific enzymes or target proteins (Rosen and Krebs, 1981). In the present study, we therefore investigated the effect of TNF on the phosphorylation of proteins in the well-characterized human ME-180 cervical carcinoma cell lines, to which TNF is cytotoxic (Sugarman *et al.*, 1985; Aggarwal and Eessalu, 1987; Aggarwal *et al.*, 1986). TNF increased the phosphorylation of a 28 kDa protein (p28) in ME-180 cells. We have partially purified p28 by affinity chromatography on 7-methylguanosine 5'-triphosphate (m^7GTP)-Sepharose, which identifies p28 as an mRNA cap binding protein. The demonstration that TNF acts upon a component of the eukaryotic initiation factor complex, an mRNA cap-binding protein, is important in identifying a possible pathway through which the TNF receptor interaction may be transduced into cellular responses.

^{125}I-TNF Binding to Several Different Preparations of Liver Plasma Membranes(PM).

Liver PM were incubated with increasing concentrations (0.04~30 nM) of ^{125}I-TNF to determine the characteristics of TNF receptors (Figure 1A). Equilibrium

binding data were analyzed by the method of Scatchard (Scatchard, 1949) and are plotted in Figure 1B. Computer analysis of binding data using LIGAND (Munson and Rodbard, 1980) distinguished high affinity, low capacity ($K_d = 1.51 \pm 0.35$ nM, $B_{max} = 0.33 \pm 0.04$ pmoles/mg protein) and low affinity, high capacity ($K_d = 13.58 \pm 1.45$ nM, $B_{max} = 1.92 \pm 0.16$ pmoles/mg protein) TNF-binding sites in liver PM (Table I).

^{125}I-TNF was covalently coupled to PM receptors using the organic cross-linking reagent disuccinimidyl suberate, and affinity-labeled ^{125}I-TNF was detected in M_r 130 kDa, 90 kDa, and 66 kDa complexes (Figure 2, lane 1). High concentrations of unlabeled TNF inhibited cross-linking of ^{125}I-TNF into each of these complexes (lane 2). Therefore, uptake of ^{125}I-TNF into these complexes was specific. In some preparations of PM, an additional specific M_r 117 kDa complex was also observed (lane 1).

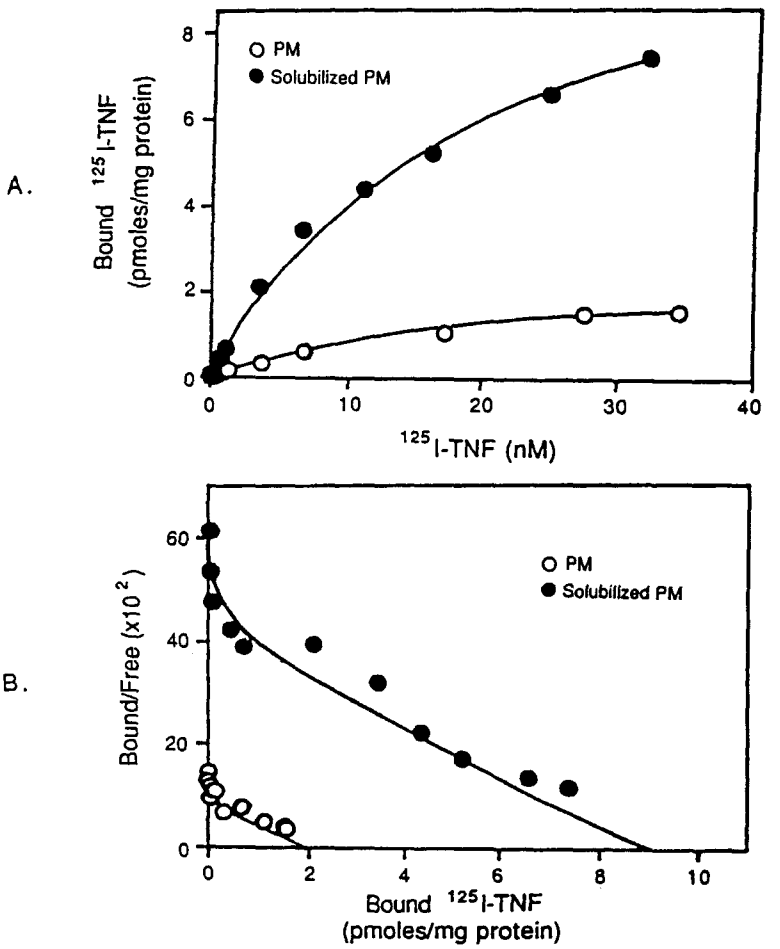

Figure 1. ^{125}I-TNF binding to PM and solubilized PM. A) PM(○) or solubilized PM(●) were incubated with ^{125}I-TNF (0.04~30 nM) at 4℃ for 24 h, and specific binding was determined. B) Scatchard analysis of equilbrium binding data. Data are representative of five (PM) and four (solubilized PM) experiments.

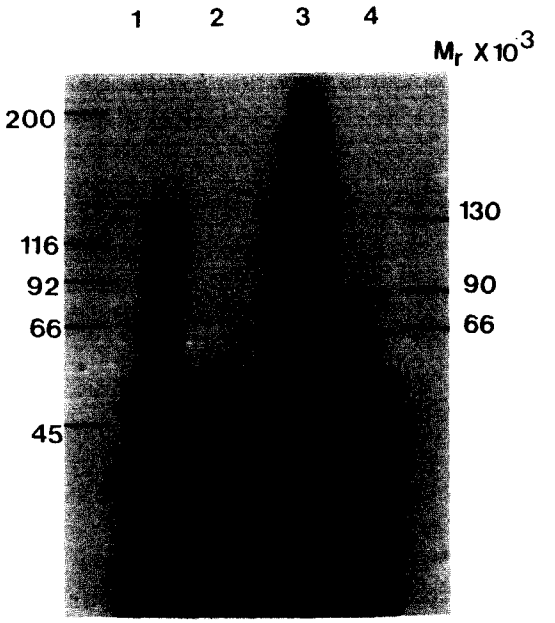

Figure 2. Affinity labeling of TNF receptors. Membranes, prepared as described under 'Experimental Procedures' were incubated with 2.5 nM ^{125}I-TNF in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 1 μM unlabeled TNF at 4℃ for 24 h. After affinity labeling, membranes were fractionated on a 4~20% acrylamide gradient gel. Lanes 1 and 2, PM; lanes 3 and 4, solubilized PM. Molecular weight standards were myosin (M_r 200 kDa), β-galactosidase (M_r 116 kDa), phosphorylase b (M_r 92 kDa), bovine serum albumin (M_r 66 kDa) and ovalbumin (M_r 45 kDa).

Table 1. Characteristics of TNF receptors in rat liver.

PM	High affinity		Low affinity	
	K_d (nM)	B_{max} (pmoles/mg protein)	K_d (nM)	B_{max} (pmoles/mg protein)
Control	1.51± 0.35	0.33± 0.04	13.58± 1.45	1.92± 0.16
Solubilized	1.94± 0.17	1.67± 0.05	11.38± 1.25	7.57± 0.50

Membranes prepared as described under 'Experimental Procedure' were incubated with increasing concentrations of ^{125}I-TNF (0.04-30 nM) at 4℃ for 24 h, and specific binding was determined. Dissociation constants (K_d) and the number of binding sites (B_{max}) were determined by the method of Scatchard (Scatchard, 1949) and computer analysis using LIGAND (Munson and Rodbard, 1980). K_d and B_{max} values are expressed as mean± S.E. from three to five separate experiments.

^{125}I-TNF Binding to Triton X-100-Solubilized Liver PM.

To examine whether there are latent receptors for TNF in PM, membranes were solubilized into 1% Triton X-100 and incubated with 0.04~30 nM ^{125}I-TNF (Figure 1A). Scatchard transformation (Scatchard, 1949) and computer analysis (Muson and Rodbard, 1980) of the equilibrium binding data resolved high and low affinity binding sites for TNF (Figure 1B and Table I). The K_d values with which these sites bound TNF were comparable to those of intact PM. However, TNF binding

capacity was significantly increased by detergent solubilization of membrane proteins. The number of high affinity binding sites increased 5.1-fold (from 0.33± 0.04 to 1.67± 0.05 pmoles/mg protein); the number of low affinity binding sites increased 3.9-fold (from 1.92± 0.16 to 7.57± 0.50 pmoles/mg protein). Thus, a large population of latent TNF receptors reside within rat hepatocyte PM.

The structural properties of the latent receptors were characterized by affinity cross-linking (Figure 2). As in PM, ^{125}I-TNF was covalently incorporated into M_r 130 kDa, 90 kDa and 66 kDa complexes in detergent extracts of PM (lane 3). However, the density of the ^{125}I-TNF-receptor complexes was increased greatly by solubilization reative to that in intact PM (compare lanes 1 and 3). Thus, results from affinity cross-linking experiments as well as from receptor binding assays show that hepatic PM possesses latent high and low affinity TNF receptors that appear structurally indistinguishable from those initially available.

Up-Regulation of TNF Receptors by Pretreatment with TNF.

We next conducted experiments to determine if exposure of latent receptors could be regulated by TNF. Thus, PM were incubated in the absence or presence of TNF (50 nM) for 3 h at 23℃ (Phase I incubation). Unbound TNF during the Phase I incubation was washed from the membranes. Control experiments (not shown) demonstrated that this condition permits dissociation of virtually all TNF bound to membranes during the Phase I incubation. Control and TNF-treated membranes were finally incubated (Phase II incubation) with 5 nM ^{125}I-TNF for 24 h at 4℃. The low temperature was used to 'freeze' the membranes, thereby preventing possible return to latency of receptors 'exposed' during the Phase I incu-

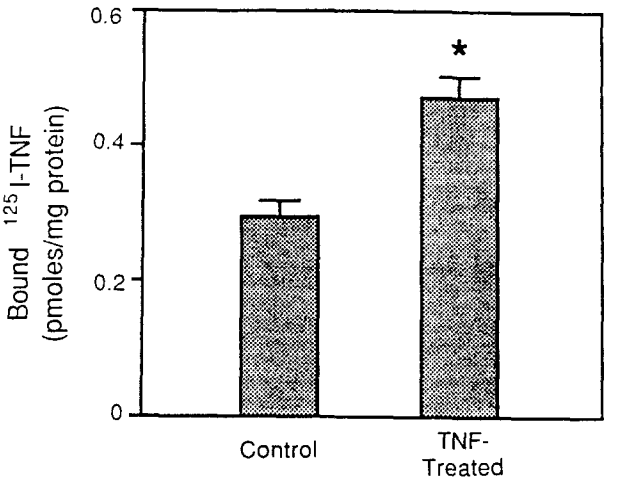

Figure 3. Binding of ^{125}I-TNF to PM pretreated with unlabeled TNF. PM were incubated at 23℃ in the absence or presence of 50 nM unlabeled TNF. Unbound TNF was removed by centrifugation, and the membranes were resuspended in binding buffer. Bound TNF was then allowed to dissociate from its receptor during a 30 min incubation at 23℃. The membranes were centrifuged, resuspended, and incubated with 5 nM ^{125}I-TNF at 4℃ for 24 h; and specific binding was determined. Data shown are mean± S.E. of four experiments. The asterisk indicates a significant difference from the control membranes ($P<0.05$, two-tailed t-test).

bation. As shown in Figure 3, significantly more ^{125}I-TNF bound to TNF-pretreated PM than to control PM ($P<0.05$, two-tailed t-test). These results show that TNF had recruited receptors into the exposed pool from the latent pool.

Characterization of TNF-Induced Phosphorylation in ME-180 Cells.

Phosphoproteins from control and TNF-treated ME-180 cells were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. TNF enhanced the phosphorylation of a 28 kDa protein within minutes (Figure 4). Maximal stimulation was induced after 15 min. Subsequently, phosphorylation of p28 diminished but remained above the basal level 2 h after TNF addition. Extent of the TNF-induced phosphorylation of p28 depended on the TNF concentration to which cells were exposed (Figure 5). Each lane in the autoradiograph of Figure 5 and in five replicate experiments was scanned with a densitometer to quantitate protein phosphorylation. Maximal stimulation in ME-180 cells (4.4 ± 0.8, $n=6$) was elicited by 15 min incubation with 1 nM TNF (Figure 5).

Identification of p28 as an mRNA Cap-Binding Protein.

Fractionation of phosphoproteins rom control (Figure 6A) or TNF-treated (Figure 6B) ME-180 cells by two-dimensional gel electrophoresis confirmed that p28 was the predominant substrate for TNF-induced phosphorylation. Two variants of p28 (pI 6.2 and 6.1) were resolved by isoelectric focusing in the first dimension;

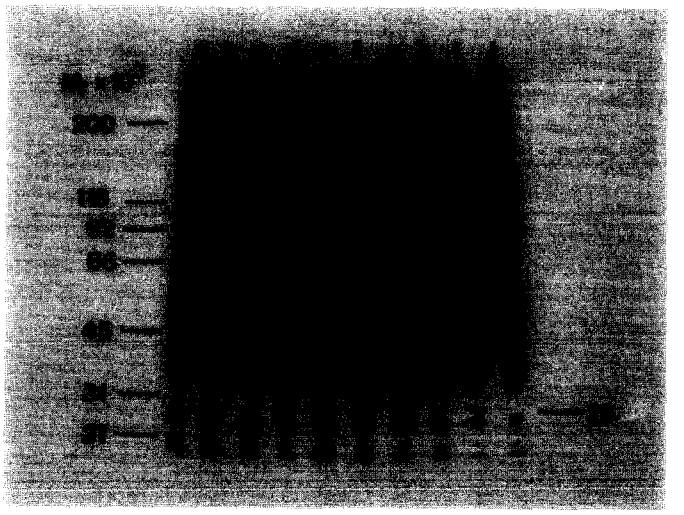

Figure 4. TNF-induced phophorylation in ME-180 cervical carcinoma cells. After equillibration with ^{32}P, cells were incubated with TNF (1 nM) at 37℃ for 0 time control (lanes a and J), 1 min (lane b), 5 min (lane c), 10 min (land d), 15 min (lane e), 30 min (lane f), 45 min (lane g), 60 min (lane h) and 120 min (lane i). Reactions were terminated by aspirating the medium and freezing the monolayers on dry ice/isopropanol. Frozen monolayers were thawed and solubilized into 3% SDS/10% (vol/vol) glycerol/10 nM P_i/2% 2-mercaptoethanol/0.01% bromophenol. Phosphoproteins were fractionated on 1.5 mm linear gradient gels of 5~15% acrylamide (acrylamide/bisacrylamide ratio of 37.5 : 1) by using the discontinuous buffer system described by Laemmli (Laemmli, 1970). Phosphoproteins were detected after SDS/PAGE and autoradiography.

these may be mono- and diphosphorylated isoforms of p28.

The M_r and pI of p28, the substrate for TNF action seen here, are similar to those of eukaryotic initiation actor-4E (eIF-4E), and mRNA cap binding protein the phosphorylation state of which has been found altered during heat shock in

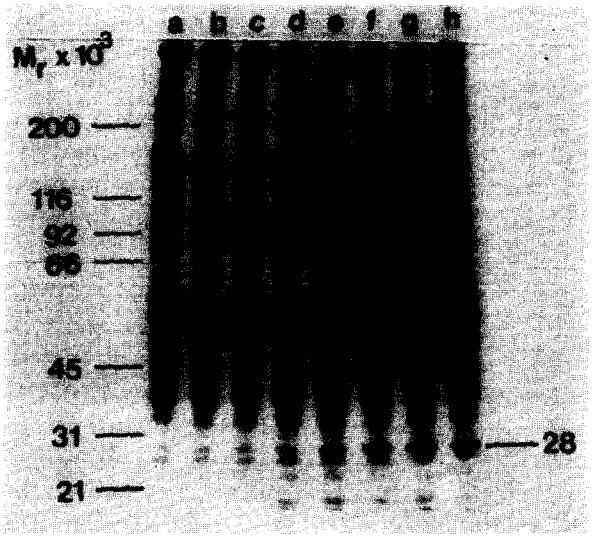

Figure 5. Dose-response of TNF-induced phosphorylation. Cells were equilibrated with ^{32}P and then incubated at 37℃ for 15 min with no TNF (control) (lane a), 0.1 pM TNF (lane b), 1 pM TNF (lane c), 0.01 nM TNF (lane d), 0.1 nM TNF (land e), 1 nM TNF (lane f), 10 nM TNF (lane g) and 100 nM TNF (lane h). (Phosphoproteins were fractionated by SDS/PAGE as described in the legend to Figure 4.

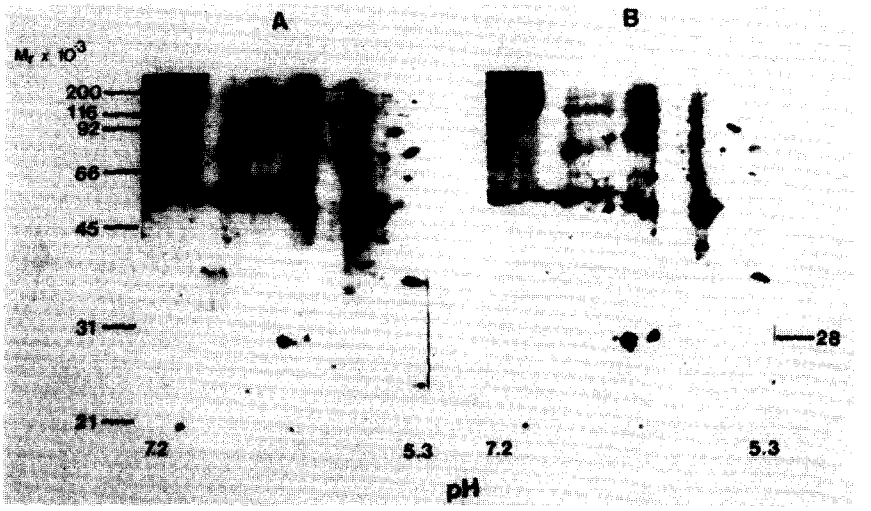

Figure 6. Two-dimensional gel electrophoresis of phosphoproteins. Control (A) or TNF-treated (B) ME-180 cells were solubilized and fractionated by two-dimensional gel electrophoresis according to O'Farrell (O'Farrell, 1975) with modifications (Yamada *et al.*, 1987). The approximate pH range of the first dimension was 7.2(left)~5.3(right).

HeLa cells (Duncan *et al.*, 1987). *In vivo*, such cap binding protein bind specifically to the 7-methylguanosine cap found on the 5' end of eukaryotic mRNAs. *In vitro*, cap binding proteins are defined on the basis of (i) cross-linking to the 5' cap of mRNAs or (ii) binding and specific elution from affinity columns, such as m^7GTP-Sepharose, which contain immobilized cap analogs (Sonenberg, 1988; Webb *et al.*, 1984).

To determine whether p28 is an mRNA cap binding protein, we investigated the ability of p28 to specifically bind to m^7GTP-Sepharose. Radiolabeled proteins from control and TNF-treated cells were incubated with m^7GTP-Sepharose. The affinity matrix was then exhaustively washed until supernatants were essentially free of phosphoproteins. Nonspecifically absorbed proteins were first eluted with GTP, and then specifically bound proteins were eluted with m^7GTP (Figure 7, ELUTION). The major phosphoprotein that was specifically eluted had an M_r of 28 kDa. Comparison of the m^7GTP eluate (plus and minus TNF) indicates that TNF-treatment resulted in a 2.1-fold stimulation of p28 phosphorylation. This stimulation in affinity eluates as compared with the 4.4-fold stimulation in crude cell lysates may reflect the action of phosphatases during the affinity purification.

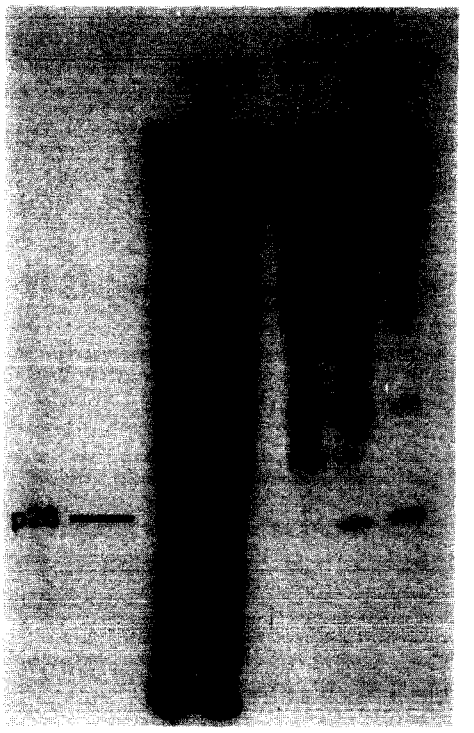

Figure 7. Binding and elution of p28 from m^7GTP-Sepharose. Diluted lysates of control of TNF-treated cells were fractionated by SDS/PAGE to illustrate the effect of TNF on p28 phosphorylation (LYSATE-or+TNF). Undiluted lysates were incubated with m^7GTP-Sepharose, which was sequentially washed with buffer until supernatants were essentially free of phosphoproteins. Elution from the affinity matrix was accomplished by incubation with GTP and then with m^7GTP, as described (ELUTION). Phosphoproteins were fractionated by SDS/PAGE (Figure 4, legend) on a linear gel of 10% acrylamide.

TNF-Induced Phosphorylation of an mRNA Cap Binding Protein in Various Cells

To explore the possibility that mRNA cap binding protein phosphorylation is a common early cellular response to TNF, we purified mRNA cap binding protein from control and TNF-treated bovine aortic endothelial cells (BAEC), human foreskin fibroblast (FS), HeLa and ME-180 human cervical carcinoma cells, and U937 histiocytic lymphoma cells, each of which serves as a model of TNF action *in vitro* (Figure 8) (Rosenblum and Donato, 1989). These cells were equilibrated with ^{32}P-labeled inorganic phosphate and then incubated in the absence (−) or presence (+) of TNF for 15 min. Cell extracts were prepared and incubated with m^7GTP-Sepharose which was then washed exhaustively. Specifically bound mRNA cap binding protein was eluted with m^7GTP and separated by SDS-PAGE. Analysis of autoradiographs revealed that TNF promotes the phosphorylation of a 28 kDa mRNA cap binding protein in endothelial cells (3-fold), fibroblasts (4-fold), HeLa cells (3-fold), ME-180 cells (3-fold) and U937 cells (3-fold) (Figure 8). These observations showed that TNF rapidly promotes phosphorylation of an mRNA cap binding protein in several cell types in which it induced distinct biologic effects.

Amino Acid Sequence Analysis of an mRNA Cap Binding Protein.

To identify the mRNA cap binding protein phosphorylated in response to TNF treatment, we analyzed amino acid sequences derived from the purified protein. Toward this end, cell extracts prepared from ME-180 cells were incubated with m^7GTP-Sepharose. After the m^7GTP-Sepharose was washed free of nonspecifically absorbed proteins, specifically bound proteins were eluted with m^7GTP.

Radiolabeled mRNA cap binding protein purified from cells equilibrated with ^{32}P-labeled inorganic phosphate comigrated during two-dimensional gel electrophoresis with the mRNA cap binding protein subjected to sequence analysis (data

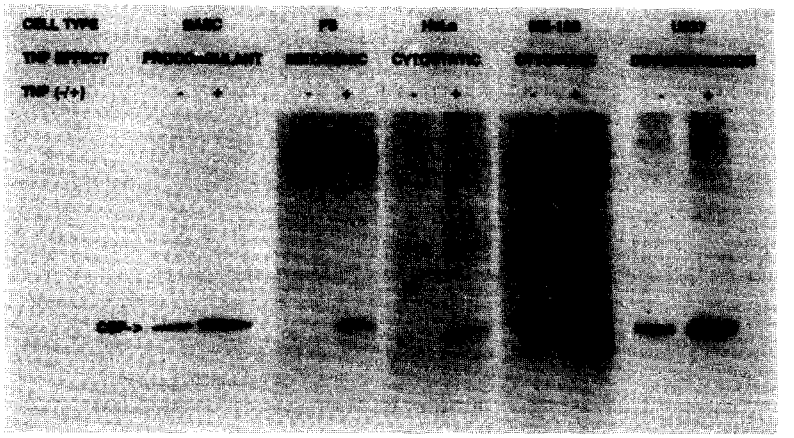

Figure 8. Autoradiographs showing the effect of TNF on the phosphorylation of mRNA cap binding protein in various cell types. 10^7 cells were seeded into 100 cm^2 plates. The following day, cells were equilibrated with ^{32}P and then incubated in the absence (−) or presence (+) of 1 nM TNF for 15 min at 37°C. mRNA cap binding proteins were purified from each incubate by affinity chromatography on m^7GTP-Sepharose and fractionated by SDS/PAGE (12%).

not shown). This verifies that the sequenced protein was the substrate for TNF promoted phosphorylation.

An attempt to directly sequence the 28 kDa mRNA cap binding protein purified by affinity chromatography and SDS-PAGE was unsuccessful, presumably due to a blocked amino terminus. Instead, purified mRNA cap binding protein was trans-

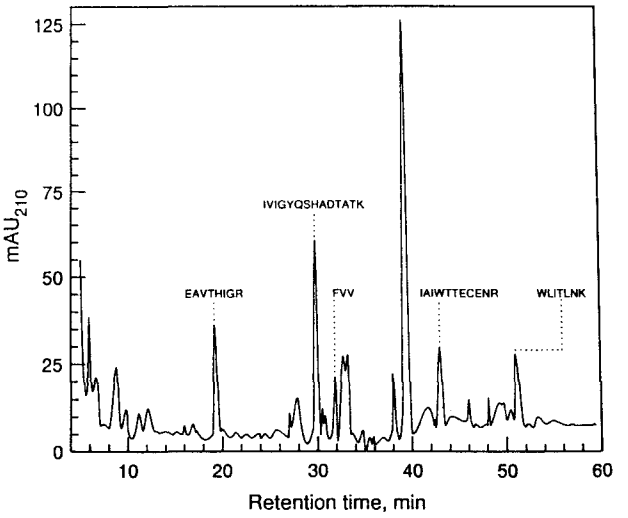

Figure 9. HPLC fractionation and amino acid sequence analysis of peptides from trytic digestion of mRNA cap binding protein. mRNA cap binding protein was purified from ME-180 cells (80×10^8 cells) by affinity chromatography on m^7GTP-Sepharose. mRNA cap binding protein fractionated by SDS-PAGE was transferred to nitrocellulose and then digested with trypsin. Fragments were separated by HPLC and sequenced.

M A T V E P E T T P T P N P P T T E E E K • T E S N Q E V A N P E H Y

I K • H P L Q N R • W A L W F F K • N D K • S K • T W Q A N L R • L I

S K • F D T V E D F W A L Y N H I Q L S S N L M P G C D Y S L F K • D

G I E P M W E D E K • N N R • G G R • W L I T L N K • Q Q R • R • S D

L D R • F W L E T L L C L I G E S F D D Y S D D V C G A V V N V R • A

K • G D K • I A I W T T E C E N R • E A V T H I G R • V Y K • E R • L

G F P P K • I V I G Y Q S H A D T A T K • S G S T T K • N R • F V V

Figure 10. Primary structure of human eIF-4E (Rychlik *et al.*, 1987) showing the amino acid sequences derived from purified mRNA cap binding protein (boxed). Trypsin cleavage sites within the eIF-4E sequence are indicated(●). The one-letter code is used to specify the amino acids.

ferred onto nitrocellulose after SDS-PAGE, and the immobilized mRNA cap binding protein was digested with trypsin to allow characterization of internal amino acid sequences (Aebersold *et al.*, 1987). Tryptic fragments were separated by HPLC (Figure 9), and six peptides were chosen for sequence analysis on the basis of purity and relative abundance. The peptide eluting at 39.0 min (Figure 9) could not be sequenced and probably contains the blocked amino-terminal tryptic fragment. The primary sequences of the remaining analyzed tryptic peptides (Figure 9, boxed in Figure 10) were identical to predicted products of tryptic digestion of the human cap binding protein eIF-4E (Rychlik *et al.*, 1987). In addition, the peak eluting at 29.8 min yielded a second amino acid sequence (Thr-Trp-Gln-Ala-Asn-Leu-Arg) which also corresponded to an eIF-4E tryptic peptide. Thus, purification and sequence analysis demonstrate that it is the 28 kDa mRNA cap binding protein eIF-4E which is phosphorylated in response to TNF treatment.

CONCLUSION

In this study, we have identified a novel population of latent TNF receptors that reside in rat liver PM. Isolation of latent TNF receptors within PM distinguishes these sites from newly synthesized receptors in transit to the cell surface and from receptors in the process of recycling. The exposed and latent populations of hepatic TNF receptors were characterized as high affinity, low capacity and low affinity, high capacity TNF receptors. TNF receptors solubilized from liver PM faithfully reflect the receptor heterogeneity of intact PM, although binding capacity is substantially increased upon exposure of the latent receptor population.

Affinity labeling experiments present further insight into the nature of the latent and exposed populations of TNF receptors. ^{125}I-TNF was detected in M_r 130 kDa and 66 kDa complexes after incubation with liver PM and solubilized PM. Consistent with the presence of latent receptors several-fold more abundant than those initially accessible to TNF, solubilization of PM resulted in increased cross-linking of ^{125}I-TNF into all three complexes relative to uptake by intact PM. In control experiments, we have found that a variety of protease inhibitors increase the specific binding of ^{125}I-TNF to PM and its cross-linking into each of the covalent complexes, eliminating the possibility that the M_r 66 kDa complex is produced by proteolysis of the higher M_r species. Thus, affinity labeling and equilibrium binding assays demonstrate the presence of a heterogeneous pool of latent TNF receptors in hepatic PM.

The cDNAs for two types of TNF receptors have recently been cloned (Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990). These receptors differ in transcript size, amino acid sequence, potential glycosylation sites, and cellular distribution. The relationship between these cloned receptors and the receptors we have characterized in rat liver PM remains to be determined.

Another novel observation described in this study is the demonstration that TNF promotes exposure of latent receptors from a pre-existing pool present in the hepatic cell membrane. Once solubilized from the membrane, latent and exposed receptor populations were indistinguishable. The present results lead us to speculate that TNF binding to accessible receptors may induce allosteric conformational

changes leading to exposure of latent sites. TNF-induced up-regulation of TNF receptors may play a role in sustained hepatic responsiveness to TNF during chronic infection and cancer.

In the present study, we have also investigated the role of protein phosphorylation in TNF signal transduction using *in vitro* models in which TNF induces cytotoxicity and cytostasis (MF-180 and HeLa cells, respectively), differentiation (U937 cells), mitogenesis (fibroblasts) and inflammatory responses (FS, U937 and endothelial cells). In each of these cell lines, TNF promotes the rapid phosphorylation of the 28-kDa mRNA cap binding protein eIF-4E.

eIF-4E is thought to be the first initiation factor to interact with the mRNA (Rhoads, 1988) and may have a discriminatory role in selecting specific messages for translation initiation (Ray *et al.*, 1983; Lawson *et al.*, 1988). *In vitro* studies suggest that phosphorylation 'activates' eIF-4E and correlates with recruitment of translationally controlled mRNAs to active ribosomes (Kaspar *et al.*, 1990; Joshi-Barve *et al.*, 1990). Central to the TNF-induced proinflammatory response of the macrophage the induction of several important inflammatory mediators which include interleukin-1, TNF and several colony-stimulating factors has been reported in endothelial cells and fibroblasts (Kunkel *et al.*, 1989). Recent evidence suggests that these mediators are encoded by mRNAs which are regulated at the translational level (Han *et al.*, 1990). It is therefore of particular interest that TNF promotes the phosphorylation of eIF-4E, a factor implicated in the regulation of translation in these cells.

eIF-4E phosphorylation also appears to play an important role in the regulation of cell growth. In yeast, eIF-4E is involved in controlling the transition of cells from G_1-into S-phase of the cell cycle (Brenner *et al.*, 1988). Mutation of the yeast eIF-4E gene leads to growth arrest in G_1 but can be complemented by a mutation which leads to constitutive activation of the catalytic subunit of cyclic AMP dependent protein kinase (Brenner *et al.*, 1988). In fibroblast, the phosphorylation state of eIF-4E increases during mitogen stimulation of quiescent cells has been reported in endothelial cells and fibroblasts (Kaspar *et al.*, 1990) and decreases during mitosis (Bonneau and Sonenberg, 1987). Recently, transfection and overexpression of eIF-4E were shown to lead to malignant transformation of NIH 3T3 cells (Lazaris-Karatzas *et al.*, 1990), establishing that eIF-4E is the product of a proto-oncogene. Transformation did not occur when cells were transfected with an eIF-4E mutant ($Ser^{53} \rightarrow Ala^{53}$) lacking the major eIF-4E phosphorylation site (Lazaris-Karatzas *et al.*, 1990). Thus, the phosphorylation of eIF-4E promoted by TNF may prove relevent to the growth effects induced by TNF in ME-180 cells (cytotoxicity), HeLa cells (cytostasis), fibroblasts (mitogenesis) and U937 cells (differentiation). In conclusion, our study suggests that TNF promoted phosphorylation of eIF-4E may mediate or modulate some of TNF's effects on cell growth and metabolism.

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