Biochemical Properties of the 75-kDa Tumor Necrosis Factor Receptor
CHARACTERIZATION OF LIGAND BINDING, INTERNALIZATION, AND RECEPTOR PHOSPHORYLATION*

(Diane Pennica‡, Van T. Lam, Nancy K. Mize, Richard F. Weber, Martyn Lewis, Brian M. Fendly§, Michael T. Lipari†, and David V. Goeddel)

From the Departments of Molecular Biology, §§Medicinal and Analytical Chemistry and Cardiovascular Research, Genentech, Incorporated, South San Francisco, California 94080

An expression plasmid encoding the human 75-kDa tumor necrosis factor (TNF) type 2 receptor (TNF-R2) was constructed and used to generate a stable human cell line (293/TNF-R2) overexpressing TNF-R2. Ligand binding analysis revealed high affinity binding ($K_d = 0.2 \text{ nM}$) with approximately $94,000 \pm 7,500$ sites/cell for $^{125}$I-TNF-$\alpha$ and approximately 5-fold lower affinity for TNF-$\beta$ ($K_d = 1.1 \text{ nM}$) with $264,000 \pm 2,000$ sites/cell. Cross-linking of $^{125}$I-TNF-$\alpha$ and $^{125}$I-TNF-$\beta$ to 293/TNF-R2 cells yielded predominant complexes with apparent molecular weights of 211,000 for TNF-$\alpha$ and 205,000 and 244,000 for TNF-$\beta$, suggesting these complexes contain two or three TNF-R2 molecules. Immunoprecipitation of TNF-R2 from $^{32}$P-labeled 293/TNF-R2 cells demonstrated that the receptor is phosphorylated. The majority (97%) of $^{32}$P incorporation was found in serine residues with a very low level of incorporation (3%) in threonine residues. TNF-$\alpha$ treatment of 293/TNF-R2 cells did not significantly affect the degree or pattern of phosphorylation. Cell surface-bound $^{125}$I-TNF-$\alpha$ was slowly internalized by the 293/TNF-R2 cell line with a $t_{1/2} = 25 \text{ min}$. Shedding of the extracellular domain of TNF-R2 was induced by 4$\beta$-phorbol 12-myristate 13-acetate but not by TNF-$\alpha$ or TNF-$\beta$.

TNF-$\alpha$ and TNF-$\beta$ are two structurally and functionally related cytokines produced mainly by macrophages and lymphoid cells, respectively (1). They have now been shown to mediate a wide spectrum of activities including cell proliferation, cytotoxicity, antiviral responses, and activation of many cellular genes, transcription factors, and kinases (1-4). Although the molecular mechanisms underlying TNF action have not yet been elucidated, many responses have been shown to be initiated by the binding of TNF-$\alpha$ or TNF-$\beta$ to specific cell surface receptors. Receptors for both TNF-$\alpha$ and TNF-$\beta$, with dissociation constants in the nanomolar range, are characterized by 4 cysteine-rich repeat units/cell for $^{125}$I-TNF-$\alpha$ and approximately 94,000 sites/cell for $^{125}$I-TNF-$\beta$. The majority (97%) of $^{32}$P incorporation was found in serine residues with a very low level of incorporation (3%) in threonine residues. TNF-$\alpha$ treatment of 293/TNF-R2 cells did not significantly affect the degree or pattern of phosphorylation. Cell surface-bound $^{125}$I-TNF-$\alpha$ was slowly internalized by the 293/TNF-R2 cell line with a $t_{1/2} = 25 \text{ min}$. Shedding of the extracellular domain of TNF-R2 was induced by 4$\beta$-phorbol 12-myristate 13-acetate but not by TNF-$\alpha$ or TNF-$\beta$.

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† To whom correspondence should be addressed: Dept. of Molecular Biology, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, California 94080.

‡ The abbreviations used are: TNF-$\alpha$, tumor necrosis factor-$\alpha$; TNF-$\beta$, tumor necrosis factor-$\beta$; TNF-R1, TNF receptor 1; TNF-R2, TNF receptor 2; PMA, 4$\beta$-phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; EGS, ethylene glycolbis (succinimidylsuccinate); BSA, bovine serum albumin; PE, phycoerytherin.

MATERIALS AND METHODS

Reagents—Recombinant human TNF-$\alpha$ (4.75 x $10^7$ units/mg) and TNF-$\beta$ (2.1 x $10^7$ units/mg) were provided by the Genentech, Inc.
The cell mixture was transferred to an Eppendorf tube containing 350 μl of 0.2 mM PMSF and 20 pg/ml aprotinin was added to each plate and the reaction continued for 1 h at 4 °C. The cells were washed once with cold PBS, and the amount of 125I-TNF-α or 125I-TNF-β bound to the cells was determined by counting the cell pellets in an Isodata gamma counter. The data were plotted with the Scatchard equation using nonlinear least-squared regression.

Cross-linking of TNF-α and TNF-β to TNF-R2-1. To 1 × 10⁶ TFN-R2 cells were washed and resuspended in 0.2 ml of PBS. 125I-TNF-α (5.2 nM, 15.9 pCi/μg) or 125I-TNF-β (0.4 nM, 32.8 pCi/μg) was added and the cells incubated at room temperature for 1 h in the presence or absence of 0.1 μM unlabeled TNF-α or TNF-β. The crosslinking reagent, EGS (8.1 mM), was added to each reaction and incubation continued for an additional 30 min at room temperature. Unbound 125I-labeled ligand was removed by washing the cells twice with 0.2 ml of 1 mM glycine (0.1 M, pH 10.0, final concentration). The pellets were washed three times by resuspending in 0.2 ml of cold PBS and spinning for 1 min at 12,400 × g at 4 °C. To the final cell pellet, 20 μl of a solution containing 1% Triton X-100, 1 μM PMSF, was added and the cells lysed by incubating at 4 °C for 1 h. After lysis, the cell debris was removed by centrifuging at 15,800 × g for 4 min at 4 °C. Immunoprecipitation of the supernatant using 2 μl of rabbit anti-human sTNF-R2-1.2-mAbs and 2 μl of anti-TNF-α or anti-TNF-β polyclonal antisera was performed using the same protocol. The reaction was centrifuged for 2 min at 15,800 × g. The Protein A-Sepharose pellet was washed four times with 500 μl of 1× lysis buffer. The final pellet was resuspended in 25 μl of 2× SDS-PAGE sample buffer, heated at 100 °C for 10 min, and then centrifuged for 2 min at 15,800 × g. The supernatant was subjected to electrophoresis on a 4–20% Tris/glycine polyacrylamide gel. The gel was fixed, incubated in Amplify (Amersham Corp.), dried, and exposed to X-ray film.

Immunoprecipitation of 32P-Labeled TNF-R2 and sTNF-R2-1.2. TNF-R2 cells were seeded in 1.5 × 10⁶ cells/cm² on a 1-cm² glass coverslip for 24 h. The cells were washed once with PBS and then incubated in 2 ml of phosphate-free media for 30 min at 37 °C. The media was replaced with 1 ml of phosphate-free media containing 0.5 μCi of 32P orthophosphate and the cells incubated for 4 h at 37 °C. The labeled cells were washed with 0.4 ml of PBS/EDTA and the plates rinsed with 0.2 ml of PBSA. The cell mixture was transferred to an Eppendorf tube and the cells washed once with 1 ml of cold PBSA by centrifuging at 800 × g for 5 min at 4 °C. The cell pellet was lysed by adding 250 μl of lysis buffer (0.2 M NaCl, 1 mM EDTA, 50 mM Tris, pH 8.0, 1% Nonidet P-40) containing the phosphatase inhibitors phenylmethylsulfonyl fluoride (1 mM), sodium orthovanadate (2 mM), ZnCl₂ (1 mM), and okadaic acid (1 mM), and the protease inhibitors, leupeptin (50 μg/ml), EGS (1 mM), pepstatin (0.7 mg/ml), and aprotinin (0.2 μg/ml) was added to each plate and the cell mixture was transferred to an Eppendorf tube containing 350 μl of 2× lysis buffer (2% Triton X-100, 1% deoxycholate, 10 mM EDTA, 250 mM NaCl, and 25 mM Tris, pH 7.5). After vortexing, the cells were lysed for 10 min at 4 °C. Nuclear and cell debris were removed by centrifugation at 10,000 × g for 10 min at 4 °C. SDS was added to the supernatant to a final concentration of 0.1%. One ml of 500 μl of anti-TNF-R2 monoclonal antibody 9H9 (IgG1 isotype) and 500 μl of 1× lysis buffer. The final pellet was resuspended in 25 μl of 2× SDS-PAGE sample buffer, heated at 100 °C for 10 min, and then centrifuged for 2 min at 15,800 × g. The supernatant was subjected to electrophoresis on a 4–20% Tris/glycine polyacrylamide gel. The gel was fixed, incubated in Amplify (Amersham Corp.), dried, and exposed to film.

Binding Analysis—293/TNF-R2 cells were harvested with PBS containing 1 mM EDTA (PBS/EDTA) and resuspended in PBS, 0.1% BSA, 0.02% sodium azide (PBSA buffer). Saturation isotherm experiments were performed in duplicate by incubating 2 × 10⁵ cells in 0.2 ml of PBSA with increasing concentrations of 125I-TNF-α or 125I-TNF-β. The cross-linking reagent, EGS (8.1 mM), was added to each reaction and incubation continued for an additional 30 min at 37 °C. Unbound 125I-labeled ligand in the supernatant was removed by aspiration. The pellets were washed twice with PBSA and the amount of ligand bound to the cells was determined by counting the cell pellets in an Isodata gamma counter. The data were plotted with the Scatchard equation using nonlinear least-squared regression.

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whereas the level of TNF-R2 expression on 293/TNF-R2 cells sequentially incubated with anti-TNF-R2 monoclonal antibody 9H9 and PE-conjugated goat anti-mouse IgG and analyzed by flow cytometry as described under "Materials and Methods." The unshaded area represents the level of TNF-R2 expression on 293/TNF-R2 cells whereas the black area represents the endogenous level of TNF receptor expression on untransfected 293 cells.

R1 monoclonal antibody 4E4 (27 \( \mu \)g/ml) for 1 h at 37 °C before the addition of \(^{125}\)I-TNF-\( \alpha \). After incubation, the cells were washed twice with cold PBS, 0.1% BSA, resuspended in the same buffer, and 250-\( \mu \)l aliquots of 5 \( \times \) 10^5 cells were removed in duplicate for determining the total amount of \(^{125}\)I-TNF-\( \alpha \) bound. The cells were then shifted to 37 °C and at various times, duplicate samples of 5 \( \times \) 10^5 cells (250-\( \mu \)l aliquots) were removed and washed twice with 0.5 ml of cold 0.5 M NaCl, 0.2 M acetic acid to remove surface-bound plus released \(^{125}\)I-TNF-\( \alpha \). The supernatants from the acid-washed pellets were precipitated with 10% trichloroacetic acid for 1 h on ice. Acid-washed pellets (internalized cmp), trichloroacetic acid pellets (cell surface-associated/released cmp), and trichloroacetic acid supernatants (degraded cmp) were counted in an Isodata gamma counter.

Analysis of TNF-R2 Shedding—TNF-R2-expressing 293 cells were plated 24 h before induction in 60-mm dishes at a density of 1.5 \( \times \) 10^6 cells/plate in 2 ml of media. At time zero, cells were induced with either media alone, TNF-\( \alpha \) (1 or 10 nM final concentration), TNF-\( \beta \) (10 nM), or PMA (32 nM final concentration). At various times after induction, 15 \( \mu \)l of media were collected and electrophoresed on a 4–12% Tris/glycine SDS gel (Novex). Detection of the shed sTNF-R2 was by Western blot analysis using monoclonal antibody 2E8 (1 \( \mu \)g/ml) essentially as described previously (40), using the ECL detection reagent, according to the manufacturer's instructions.

RESULTS

Generation of a TNF-R2 Overexpressing Cell Line—A cDNA encoding full-length human TNF-R2 was isolated from a U-R937 cDNA library, inserted into a mammalian expression vector, and transfected into the human embryonic kidney 293 cell line. 293 cells were selected because of their high transfection efficiency and relatively low numbers of endogenous TNF receptors (approximately 800–1000 total sites/cell). A population of cells overexpressing TNF-R2 was isolated by three rounds of fluorescence-activated cell sorting using a monoclonal antibody specific for the extracellular domain of TNF-R2. The level of TNF-R2 expression in this stable 293/TNF-R2 cell population is shown by the shift to the right of the fluorescence histogram (Fig. 1, unshaded cell population). Untransfected 293 cells (shaded population) display a low level of binding of the TNF-R2 monoclonal antibody. The intensity of fluorescent staining in the 293/TNF-R2 cells is reduced to background levels by omitting the primary antibody and incubating the cells with PE-conjugated goat anti-mouse IgG second antibody only, demonstrating that the observed binding is specific.

Radioimmunoprecipitation of TNF-R2—In order to confirm overexpression of full-length TNF-R2, 293/TNF-R2 cells were metabolically labeled with [\(^{35}\)S]methionine and [\(^35\)S]cysteine. Labeled cell extracts and media were immunoprecipitated with anti-TNF-R2 polyclonal antiserum and the precipitates analyzed by SDS-PAGE. A major polypeptide of 72 kDa was specifically precipitated from extracts of 293/TNF-R2 cells but not untransfected 293 cells (Fig. 2). This size is consistent with the previous estimates of about 75 kDa for TNF-R2 (41–43). A polypeptide of approximately 39 kDa is specifically immunoprecipitated from the media of 293/TNF-R2 cells but not 293 control cells (Fig. 2) suggesting that this protein is the shed extracellular domain of TNF-R2. Consistent with these results, other investigators have reported a size of about 40 kDa for the soluble form of TNF-R2 (sTNF-R2) (21, 44).

Analysis of TNF-\( \alpha \) and TNF-\( \beta \) Binding to 293/TNF-R2 Cells—Saturation isotherms for the binding of \(^{125}\)I-TNF-\( \alpha \) and \(^{125}\)I-TNF-\( \beta \) to 293/TNF-R2 cells were performed. In both cases, binding was specific and saturable (Fig. 3). Scatchard analysis of the data (Fig. 3A, inset) indicates a single class of binding sites with a \( K_d \) of 0.18 ± 0.01 nM for TNF-\( \alpha \) which is similar to that reported previously in transiently transfected COS-1 cells (22). The number of binding sites for TNF-\( \alpha \) was estimated at approximately 94,000 ± 7,500/cell. The Scatchard analysis of the binding data for TNF-\( \beta \) (Fig. 3B, inset) also indicates a single class of binding sites but with a lower affinity (\( K_d \) 1.05 ± .05 nM) than that observed for TNF-\( \alpha \). Approximately 264,000 ± 2,000 TNF-\( \beta \)–binding sites/cell were observed.

Cross-linking of TNF-R2 with TNF-\( \alpha \) and TNF-\( \beta \)—Cross-linking experiments were performed to visualize the complex formation between TNF-R2 and TNF-\( \alpha \) or TNF-\( \beta \). 293/TNF-R2 cells were incubated at room temperature with \(^{125}\)I-TNF-\( \alpha \) or \(^{125}\)I-TNF-\( \beta \) in the presence or absence of an excess of unlabeled TNF-\( \alpha \) or TNF-\( \beta \) to determine the specificity of the binding. The complexes were cross-linked by the addition of EGS and the labeled proteins analyzed by SDS-PAGE. As illustrated in Fig. 4, cross-linking of \(^{125}\)I-TNF-\( \alpha \) to 293/TNF-R2 cells showed one major high molecular mass cross-linked product.
TNF-R2 cells. 293/TNF-R2 cells were incubated with \(^{125}\text{I-TNF-\(\alpha\)}\) or \(^{125}\text{I-TNF-\(\beta\)}\) in the presence or absence of unlabeled TNF-\(\alpha\) or TNF-\(\beta\), and the specific binding at each concentration was determined. The inset presents the data transformed by Scatchard analysis. All binding experiments were performed two to three times with similar results. The results from a single representative experiment are shown.

![Graph](image)

**FIG. 3.** Binding of \(^{125}\text{I-TNF-\(\alpha\)}\) and \(^{125}\text{I-TNF-\(\beta\)}\) to 293/TNF-R2 cells. Saturation isotherms of the specific binding of \(^{125}\text{I-TNF-\(\alpha\)}\) and \(^{125}\text{I-TNF-\(\beta\)}\) were performed by incubating duplicate samples of 2 x 10^5 293/TNF-R2 cells with increasing concentrations of \(^{125}\text{I-TNF-\(\alpha\)}\) or \(^{125}\text{I-TNF-\(\beta\)}\) alone or in the presence of 100-fold molar excess of unlabeled TNF-\(\alpha\) or TNF-\(\beta\), and the specific binding at each concentration was determined. The inset presents the data transformed by Scatchard analysis. All binding experiments were performed two to three times with similar results. The results from a single representative experiment are shown.

![Graph](image)

**FIG. 4.** Cross-linking of \(^{125}\text{I-TNF-\(\alpha\)}\) and \(^{125}\text{I-TNF-\(\beta\)}\) to 293/TNF-R2 cells. 293/TNF-R2 cells were incubated with \(^{125}\text{I-TNF-\(\alpha\)}\) or \(^{125}\text{I-TNF-\(\beta\)}\) in the presence or absence of unlabeled TNF-\(\alpha\) or TNF-\(\beta\) and treated with the cross-linking reagent EGS. Cell lysates were resolved on 4-12% Tris/glycine polyacrylamide gel followed by autoradiography. Lane 1, \(^{125}\text{I-TNF-\(\alpha\)}\) only; lane 2, \(^{125}\text{I-TNF-\(\alpha\)}\) + 0.1 \(\mu\text{M}\) unlabeled TNF-\(\alpha\); lane 3, \(^{125}\text{I-TNF-\(\beta\)}\) only; lane 4, \(^{125}\text{I-TNF-\(\beta\)}\) + 0.1 \(\mu\text{M}\) unlabeled TNF-\(\beta\). The positions of \(^{14}\text{C\text{m}}\) molecular weight standards are indicated.
cells and appearing in the media was measured by Western analysis. Constitutive shedding of TNF-R2 occurred at low but detectable levels throughout the duration of the experiment (Fig. 7). TNF-α at 1 nM concentration did not affect the amount of sTNF-R2 released. Treatment of cells with 10 nM TNF-α or 10 nM TNF-β also had no effect on the rate of shedding. However, within 5 min of PMA treatment, there was a rapid release of sTNF-R2 from the cells which continued to accumulate over the course of the experiment (Fig. 7).

**DISCUSSION**

We have constructed a human 293 cell line that expresses the human TNF-R2 (75-kDa TNF receptor) at levels more than 100-fold above the endogenous level of TNF receptors on 293 cells. This high level of overexpression facilitated an examination of the biochemical characteristics of TNF-R2.

The TNF-R2 expressed on the surface of 293 cells was found to bind both TNF-α (K_d = 0.2 nM) and TNF-β (K_d = 1.1 nM) with high affinity. Others (16, 22), using transiently expressing TNF-R2 in COS cells, have reported similar binding constants. The number of binding sites on the 293/TNF-R2 cells for TNF-α was found to be lower than for TNF-β (94,000 versus 264,000). All 264,000 TNF-β sites can be competed effectively by TNF-α, demonstrating that these sites are not specific for TNF-β only. Two possible explanations for the discrepancy in number of sites are that TNF-α and TNF-β may bind with different stoichiometries or that not all of the 125I-TNF-β is active. We favor the latter interpretation, since Scatchard analysis, size exclusion chromatography, and cross-linking suggest that two or three molecules of the recombinant extracellular domain of TNF-R2 (sTNF-R2) can bind to a single trimer of TNF-α or TNF-β.

A range of sizes for cross-linked TNF receptor-ligand complexes using labeled TNF-α and TNF-β on human and murine cell lines has been reported (45, 46). We observe a single

\[ \text{FIG. 5. Panel A, } [\text{32P}] \text{orthophosphate labeling of TNF-R2. 293/}
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\[ \text{TNF-R2 cells were labeled with } [\text{32P}], \text{for } 4 \text{ h as described under }
\]

\[ \text{"Materials and Methods." Cells were either stimulated with media alone or 2 nM TNF-α for 15–30 min. Cell lysates were prepared from the labeled cells and immunoprecipitated with TNF-R2 polyclonal antiserum. The immunoprecipitated samples were resolved on a 4–}
\]

\[ \text{20% Tris/glycine polyacrylamide gel and the gel exposed to film. Lanes } 1–3, \text{ immunoprecipitates from cells treated with media alone; lanes } 4–6, \text{ immunoprecipitates from cells treated with 2 nM TNF-α. The positions of prestained molecular weight standards are indicated on the right. Panel B, phosphoamino acid analysis of TNF-R2. 293/}
\]

\[ \text{TNF-R2 cells were metabolically labeled with } [\text{32P}], \text{and processed as described above. Immunoprecipitated } [\text{32P}]-\text{labeled TNF-R2 was excised from the gel, eluted, and then analyzed for phosphoamino acid content by two-dimensional electrophoresis as described previously (39). The thin layer chromatography plates were exposed to film for 13 days. The migration positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated.}
\]

\[ \text{FIG. 6. 125I-TNF-α internalization and degradation by 293/}
\]

\[ \text{TNF-R2 cells. 293/TNF-R2 cells were incubated with 0.25 nM 125I-}
\]

\[ \text{TNF-α for 1 h at 4 °C. Cells were washed twice then shifted to 37 °C}
\]

\[ \text{for 0, 5, 15, 30, 60, 120, and 240 min. At the indicated times, duplicate}
\]

\[ \text{samples of } 5 \times 10^5 \text{ cells were processed as described under "Materials}
\]

\[ \text{and Methods." Circles, acid-washed pellet cpm (internalized 125I-TNF-}
\]

\[ \text{α); squares, non-trichloroacetic acid-precipitable cpm (degraded 125I-}
\]

\[ \text{TNF-α); and triangles, trichloroacetic acid-precipitated supernatant}
\]

\[ \text{cpm derived from acid-washed cell supernatants (cell surface-associated}
\]

\[ \text{125I-TNF-α). Open symbols represent data obtained in the presence}
\]

\[ \text{of anti-TNF-R1 monoclonal antibody (4E4). Closed symbols are}
\]

\[ \text{without antibody.}
\]

\[ \text{FIG. 7. Induction by PMA of TNF-R2 shedding. 293/TNF-}
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\[ \text{R2 cells were incubated with media only, TNF-α (1 nM) or PMA (32}
\]

\[ \text{nM) and 15-μl aliquots of the media removed at 0, 5, 15, 30 min and}
\]

\[ \text{1, 2, 4, 6, and 19 h after induction. The samples were resolved on a}
\]

\[ \text{4–20% Tris/glycine polyacrylamide gel followed by Western blot}
\]

\[ \text{analysis. One ng of purified sTNF-R2 was also resolved on the gel as}
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\[ \text{an internal control and appears on the right hand side of each row.}
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distinct species after cross-linking TNF-R2 with TNF-α and two major bands after cross-linking with TNF-β. The complexes we observe appear to be significantly larger than what has been previously reported (205–244 versus ~75–100 kDa). The most likely explanation for this discrepancy is that our cross-linking studies were done at room temperature rather than at 4 °C. When we perform cross-linking at 4 °C, the major species observed are smaller sized complexes, with sizes consistent with those reported previously (45, 46). At room temperature the fluidity of the cell membrane could allow lateral movement and ligand-mediated oligomerization of TNF-R2, resulting in higher molecular mass ligand-receptor (205–244 kDa) complexes. One can speculate that the major high molecular weight complexes observed are composed solely of a single trimer of TNF-α or TNF-β bound to two or three TNF-R2 molecules. It is possible that only this one type of complex exists and that the minor products observed at smaller sizes are a result of incomplete cross-linking of receptor molecules with ligand. The complexes might also contain receptors cross-linked to associated regulatory proteins since the cross-linking reagent used in these studies is membrane permeable. Hayakawa et al. (47) have suggested that TNF receptors purified from human placental membranes may be complexes with a guanine nucleotide-binding protein (47) and other workers have suggested that the Fas antigen may be associated with TNF receptors (48).

The mechanism of signal transduction through TNF-R2 remains obscure. One interesting feature of the cytoplasmic region of TNF-R2 is that it contains an unusually high number of serine, threonine, proline, and glutamic acid residues. Proteins that are rich in these residues are often rapidly degraded intracellularly (49). Whether these residues are important in TNF-R2 degradation, or signaling, however, remains to be determined. Another feature of the intracellular domain of TNF-R2 is the lack of any tyrosine residues or of any potential phosphorylation sites for CAM-dependent kinase. There is, however, one potential site for protein kinase C phosphorylation at Ser-415 (16, 50). TNF-R2 isolated from 32P-labeled cells was found to be phosphorylated largely on serine residues with very low levels of phosphorylation on threonine residues. This pattern of phosphorylation appeared to be unchanged upon TNF-α treatment. Others have reported the constitutive phosphorylation of natural TNF-R2 in SW480T cells (51). However, the significance of TNF-R2 phosphorylation in 293 or SW 980T cells is unclear since no TNF-R2-specific responses have yet been demonstrated in these cells. A serine-rich region in the cytoplasmic domain of the human interleukin-2 receptor β chain was found to be essential for ligand-mediated signal transduction through this receptor (52). CDw40, another member of the TNF receptor superfamily, has been found to be constitutively phosphorylated in the cytoplasmic domain (53). Although the importance of serine and threonine phosphorylation in mediating signal transduction through TNF-R2 is not known, the fact that the serine and threonine residues are highly conserved between mouse and human (19) suggests a possible role in signaling.

Previous reports have indicated that following binding to cell surface receptors, TNF-α is rapidly internalized at temperatures above 15 °C and subsequently degraded (9, 10, 54). Electron microscopy studies using gold particle-labeled TNF-α have shown that TNF-α is internalized in L929 cells through clathrin-coated pits and endosomes and ends up in lysosomes where it is degraded (55). It was not determined in these studies, however, whether internalization occurred through TNF-R1 or TNF-R2. However, Yoshie et al. (10) used Hela cells which have predominantly TNF-R1 and undetectable TNF-R2 (42). Two other reports investigated internalization of human TNF-α in mouse cells (9, 54). As human TNF-α cannot recognize mouse TNF-R2 (19), these results were also specific for TNF-R1. In all three cases, 80–90% of the TNF-α bound to TNF-R1 was rapidly internalized and degraded. Our results indicate that TNF-α bound to 293/TNF-R2 cells is more slowly internalized and much less efficiently degraded. After 2 h at 37 °C, only about 40% of the 32P-TNF-α initially bound at the cell surface was internalized, and less than 10% was degraded after 4 h. Unlike TNF-R1, TNF-R2 has no tyrosines in its cytoplasmic domain and therefore lacks a consensus sequence for rapid cellular internalization via coated pits (56, 57). This suggests that internalization and degradation of TNF-α proceeds by different mechanisms depending on whether it is bound to TNF-R1 or TNF-R2.

The soluble form of TNF-R2 was slowly, but spontaneously, released into the media from 293/TNF-R2 cells, and treatment of these cells with the phorbol ester PMA rapidly and dramatically increased the rate of TNF-R2 shedding (from < 0.3 ng/ml/106 cells at time 0 to approximately 20 ng/ml/106 cells after 30 min). Neither TNF-α nor TNF-β induced the formation of soluble TNF-R2. Down-regulation of cell surface TNF-binding sites by phorbol esters and interleukin 1 has been reported previously, although neither the specific TNF receptor involved nor the mechanism of loss of TNF binding were known (58–60). Proteolytic release of many membrane proteins is a specific and regulated event and is enhanced after activation of the host cell. This has been found to be a protein kinase C-dependent process for TNF receptors (58–61), the Mel-14 antigen (62), CSF-1 receptor (63), and pro-TGF-α (64). It has recently been determined that the protease elastase is responsible for the shedding of the extracellular domain of TNF-R2 from activated neutrophils while having no effect on the shedding of TNF-R1 from endothelial cells (65). Soluble TNF-binding proteins have been associated with certain disease states. For example, increased levels of both soluble TNF-R1 and TNF-R2 have been found in the sera of cancer patients (66). Although the physiological significance of TNF receptor shedding is unknown, the production of soluble receptor forms may be involved in the modulation of TNF activities.

The individual role of TNF-R1 and TNF-R2 in initiating signal transduction is only beginning to be understood. For example, antibodies against TNF-R1 were found to act as specific agonists for this receptor and initiate several TNF responses (30, 67–70). More recently, we have found that rabbit polyclonal antibodies to human and murine TNF-R1 and TNF-R2 each acted as specific receptor agonists and induced a subset of TNF activities (32, 71). Taken together, these studies provide evidence that TNF-R1 and TNF-R2 are not redundant in mediating cellular responses and support the existence of receptor-specific signal transduction pathways.

The mechanism(s) by which TNF-R2 signals these cellular responses, however, has not yet been well defined. Recent evidence suggests that, similar to TNF-R1 (30, 32, 68), the initiation of signal transduction by TNF-R2 involves receptor aggregation. These studies (32) have shown that TNF-R2-specific polyclonal antibodies act as receptor agonists suggesting that receptor clustering provides a sufficient signal for certain TNF effects. Data from our cross-linking experiments also provide evidence for TNF-R2 aggregation induced by both TNF-α and TNF-β. Studies with a recombinant extracellular domain of TNF-R2 show that one trimer of TNF-α or TNF-β can simultaneously bind two to three sTNF-R2 molecules, thereby providing a mechanism for TNF-
induced receptor cross-linking. Ligand-induced receptor aggregation has been observed for a number of growth factor receptors including epidermal growth factor receptor, platelet-derived growth factor receptor, colony-stimulating factor 1 receptor, and the insulin receptor and is thought to be the initiating event in the signal transduction process (72–74).

Although the early stages of signal transduction by TNF-R1 and TNF-R2 may be initiated similarly by receptor aggregation, the wide diversity of cellular responses mediated through the two TNF receptors will probably be shown to be dependent on a diversification of post-receptor signaling mechanisms.

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