Identification of Grb2 As a Novel Binding Partner of Tumor Necrosis Factor (TNF) Receptor I

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Summary

Tumor necrosis factor α (TNF-α) is a proinflammatory cytokine. Its pleiotropic biological properties are signaled through two distinct cell surface receptors: the TNF receptor type I (TNFR-I) and the TNF receptor type II. Neither of the two receptors possesses tyrosine kinase activity. A large majority of TNF-α–dependent activities can be mediated by TNFR-I. Recently, c-Raf-1 kinase was identified as an intracellular target of a signal transduction cascade initiated by binding of TNF-α to TNFR-I. However, the mechanism engaged in TNF-α–dependent activation of c-Raf-1 kinase is still enigmatic.

Here we report that the cytosolic adapter protein Grb2 is a novel binding partner of TNFR-I. Grb2 binds with its COOH-terminal SH3 domain to a PLAP motif within TNFR-I and with its NH$_2$-terminal SH3 domain to SOS (son of sevenless). A PLAP deletion mutant of TNFR-I fails to bind Grb2. The TNFR-I/Grb2 interaction is essential for the TNF-α–dependent activation of c-Raf-1 kinase; activation of c-Raf-1 kinase by TNF-α can be blocked by coexpression of Grb2 mutants harboring inactivating point mutations in the NH$_2$- or COOH-terminal SH3 domain, cell-permeable peptides that disrupt the Grb2/TNFR-I interaction or transdominant negative Ras. Functionality of the TNFR-I/Grb2/SOS/Ras interaction is a prerequisite but not sufficient for TNF-α–dependent activation of c-Raf-1 kinase. Inhibition of the TNFR-I/FAN (factor associated with neutral sphingomyelinase) interaction, which is essential for TNF-α–dependent activation of the neutral sphingomyelinase, either by cell-permeable peptides or by deletion of the FAN binding domain, prevents activation of c-Raf-1 kinase. In conclusion, binding of the Grb2 adapter protein via its COOH-terminal SH3 domain to the nontyrosine kinase receptor TNFR-I results in activation of a signaling cascade known so far to be initiated, in the case of the tyrosine kinase receptors, by binding of the SH2 domain of Grb2 to phosphotyrosine.

Key words: Grb2 • tumor necrosis factor • signal transduction • cell-permeable peptides • yeast two-hybrid system

TNF-α is a pleiotropic cytokine that triggers a variety of biological effects on different target cell types (1). Two different types of TNFR, which independently bind the trimeric TNF-α molecule, mediate these effects. According to their apparent molecular masses, these receptors are designated as p55 (TNFR-I) or p75 (TNFR-II) (1–3). Neither of the two receptors possesses tyrosine kinase activity. The primary amino acid (aa)[1] sequences of the cytoplasmic domains of the two receptors display no significant homology, suggesting that the two receptors interact with different cellular proteins (4). Gene knockout experiments confirmed that the two receptors control different signaling cascades (5, 6). The majority of the biological effects of TNF-α are mediated by TNFR-I.

The two types of TNFR belong to the TNFR superfamily, which includes CD40 and the Fas antigen. Binding of the ligand to these receptors results in receptor oligomerization and subsequent initiation of signal transduction cascades. Apart from the death domain present in TNFR-I and the Fas receptor, the cytoplasmic domain of TNFR-I does not share significant homology to known cell surface receptors, including the members of the TNFR superfamily. Because a broad variety of cellular responses to TNF-α are mediated by TNFR-I and due to the lack of structural similarities between TNFR-I and other cell surface receptors, the understanding of the mechanism of signal transduction through TNFR-I is of major biological interest.

TNF-α–dependent activation of phospholipase A2, protein kinase C, phosphatidylcholine-specific phospholipase C,

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[1]*Abbreviations used in this paper:* aa, amino acid; AP-1, activator protein 1; CAP, ceramide-activated protein; CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor; n, neutral; NTA, nitrilotriacetic acid; Smase, sphingomyelinase; SOS, son of sevenless.

and sphingomyelinases (Smases) is ascribed to TNFR-I (7–9). Near to the COOH terminus, the so-called death domain of ~80 amino acids was identified. In response to ligand binding, this domain recruits TNFR-associated death domain (TRADD). TRADD interacts with FLICE/MACH1, a death-promoting IL-1β–converting enzyme-like protease, which is relevant for initiation of the apoptotic pathway (10, 11). This domain is also involved in TNF-α–dependent activation of acid Smase; the TNF-α–dependent activation of the neutral (n)Smase is mediated by FAN, which binds to the membrane proximal part (8, 12, 13).

Recently, c-Raf-1 kinase was identified as an intracellular target of a signal transduction cascade initiated by binding of TNF-α to TNFR-I (14, 15). However, the mechanism engaged in TNF-α–dependent activation of c-Raf-1 kinase still remained enigmatic. In this study, a novel binding partner of TNFR-I was identified, and its relevance for the TNF-α–dependent activation of c-Raf-1 kinase was shown.

Materials and Methods

Yeast Two-Hybrid Cloning. For construction of the lexA DNA binding domain fusion, the vector plex202 (provided by R. Brent, Harvard Medical School, Boston, MA) was used. The coding sequences for the cytoplasmic domain of TNFR-I (aa 205–426) or for the truncated form (aa 206–345) were cloned in frame into plex202. A Jurkat cDNA library fused to a synthetic activation domain (16) was screened. The yeast strain EG48/JK103 (17) was instrumental.

For analysis of the interaction of the cytoplasmic tail of (cyt)TNFR-I with full-length Grb2 and the SH3 or SH2 domain–mutated forms of Grb2, the matchmarker two-hybrid system was instrumental (Clontech). The coding sequence for the cytoplasmic domain of TNFR-I (aa 205–426) or the PLAP deletion mutant was fused to the DNA binding domain of Gal4 by subcloning pGBT9. The coding sequences for Grb2 (18), ΔNSH3Grb2, ΔSH2Grb2, or ΔCSH3Grb2 was fused to the activation domain of Gal4 by subcloning into pGAD424. Here, the yeast strain HF7c was used.

Immunoprecipitation. Lysis of the cells was performed on ice in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.05% Triton X-100, 1 mM PMSF, 0.2 U/ml aprotinin, 100 μg/ml leupeptin, and 1 mM orthovanadate. The lysate was clarified by centrifugation, and the supernatant was preabsorbed by 30-min incubation with protein A/G–agarose (Santa Cruz Biotechnology). Purified supernatants were incubated with antisera specific for c-TNFR-I (Santa Cruz Biotechnology), for full length TNFR-I, Grb2, or son of sevenless (SOS)1 (poly- and monoclonal sera from Santa Cruz Biotechnology and signal transduction laboratories), respectively. The complexes were precipitated by addition of protein A/G–agarose. The precipitates were washed twice with 0.5 M LiCl and 0.1 M Tris, pH 7.4, and once with 0.01 M Tris, pH 7.4.

Western Blot Analysis. For Western blot analysis, proteins were separated by SDS-PAGE (19) and transferred onto cellulose nitrate membranes (0.45 μm; Schleicher & Schuell). Membranes were washed for 30 min with PBST and preblocked for 15 min in PBST containing 10% nonfat dry milk powder (blocking solution). Incubation with the first antibody diluted in blocking solution was performed for 2 h at room temperature. After extensive washing with PBST, the bound antibody was detected by peroxidase-conjugated secondary antibodies (Amersham Corp.). After

45 min of washing, the blots were developed using the ECL Western blotting detection system (Amersham Corp.).

Construction of Expression Plasmids. The sequence coding for full length human TNFR-I was amplified from pHup60 (provided by P. Goodwin, Immunex Corp.) by PCR using a forward primer, which introduced the coding sequence for an NH$_2$-terminal histidine tag and subcloned into pCDNA.3. The sequence coding for the cytoplasmic domain of TNFR-I (aa 205–426) was subcloned into the bacterial expression plasmid pQe8 (QIAGEN Inc.). For construction of the PLAP deletion mutant, fragment I, nucleotide 256–1040, and fragment II, nucleotide 1050–1618, were amplified by PCR. In the case of fragment I, the backward primer introduced a HindIII site; in the case of fragment II, the forward primer harbored a HindIII site. The HindIII-restricted fragments were ligated and subcloned into the pCDNA.3 expression plasmid, generating pTNFR-IΔPLAP. The coding sequences for Grb2 and the dominant negative mutants of the SH3 and SH2 domains (provided by J. Duyster, University of Ulm) were subcloned into pCDNA.3.

Transfection Experiments. 0.8 × 10^6 70Z/3 and 293 cells were transfected by lipofection using DOTAP (Boehringer Mannheim) or the calcium phosphate procedure. For transfections, 5 μg of the expression constructs was used, with the exception of 3xAP-1(activator protein 1)-chloramphenicol acetyltransferase (CAT), where an amount of 1 μg was sufficient. Transfection efficiency was determined by transfection of the cells with green fluorescent protein. The transfection efficiency was 70–80%. Transfection efficiency was normalized for by transfection with a plasmid coding for the luciferase gene (pLuc). The CAT-ELISA was performed according to the instructions of the manufacturer (Boehringer Mannheim).

Protein Purification. Protein purification of the hexa-His–tag fusion proteins was performed as described previously (20).

Kinase Assays. 15 min after stimulation with TNF-α (100 U/ml; in the case of 70Z cells, 200 U/ml) or EGF (5 ng/ml), cells were lysed in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.2 mM EDTA, 1 mM EGTA, 10 mM sodium-β-glycerol-phosphate, 50 mM sodium fluoride, 0.5% Triton X-100, 1 mM sodium-orthovanadate, 0.25 M sucrose, 0.5 mM PMSF (Sigma Chemical Co.), 0.15 U/ml aprotinin, and 2 μg/ml leupeptin. Insoluble material was removed by centrifugation at 20,000 g at 4°C for 15 min. The protein concentration of the supernatant was determined by a Bradford assay. 500 μg total protein was incubated for 15 min at 4°C with protein A/G–agarose. The agarose beads were removed by centrifugation, and the supernatant was incubated for 2 h with c-Raf-1–specific antiserum. To precipitate the antigen–antibody complex, protein A/G–agarose was added again and incubated for 1 h at 4°C. The complexes were sedimented by centrifugation, and the pellet was washed twice in a buffer containing 100 mM Tris/HCl, pH 7.4, and 500 mM LiCl followed by a wash in 10 mM Tris/HCl, pH 7.4. The pellet was resuspended in kinase buffer as described previously (21).

Results

Grb2 Interacts with the Cytoplasmic Domain of TNFR-I. To elucidate the mechanism of TNF-α–dependent activation of c-Raf-1 kinase, a yeast two-hybrid screen using the lexA system was performed to identify adapter molecules interacting with the membrane proximal region of TNFR-I. Using a human Jurkat T cell cDNA library (16), a clone encoding the COOH-terminal part of the adapter protein Grb2 (aa 113–217) was identified. The coding sequence

Table I. *Identification of the COOH-terminal SH3 Domain of Grb2 As a Binding Partner of the Cytoplasmic Domain of TNFR-I Using the Yeast Two-Hybrid System*

DNA binding domain hybrid	Activation domain hybrid	Growth on Leu⁻ medium	Colony color
cTNF-RI aa 206–345	–	–	white
cTNF-RI aa 206–345	ΔGrb2 aa113–217	+	blue
cTNF-RI aa 206–426	ΔGrb2 aa113–217	+	blue
SYK	ΔGrb2 aa113–217	–	white
–	ΔGrb2 aa113–217	–	white

DNA binding domain hybrid	Activation domain hybrid	Growth on Leu⁻/His⁻/Trp⁻ medium	Colony color
cTNF-RI aa 206–426	Grb2	+	blue
cTNF-RI aa 206–426	ΔNSH3Grb2	+	blue
cTNF-RI aa 206–426	ΔCSH3Grb2	–	white
cTNF-RI aa 206–426	ΔSH2Grb2	+	blue

Yeast strains harboring constructs encoding fusion proteins of the DNA binding domain of lexA and various fragments encoding the cytoplasmic domain of TNFR-I (aa 206–345 and aa 206–426) were transfected with constructs coding for fusion proteins of the Grb2 COOH-terminal SH3 domain and the lexA-activating domain.
Yeast strains transfected with pGBT9cTNFR-I (aa 205–426) were transformed with the vector containing the activation domain of Gal4 fused to the coding sequences for (top) truncated Grb2 (aa 113–217), (bottom) full length Grb2, or (bottom) the inactivating point mutations in the SH3/SH2 domains or SH2 domain. The experiments summarized at top were performed using the lexA system, and the experiments described at bottom were performed using the Gal4 system.

consists of a truncated middle SH2 domain and a complete COOH-terminal SH3 domain (Table I, top). The complete adapter protein Grb2 consists of a middle SH2 domain known to bind to phosphotyrosine residues and an NH$_2$- and COOH-terminal SH3 domain. The TNFR-I/Grb2 interaction was confirmed using the Gal4 system by cotransfection of the yeast strain HF7c with pGAD424Grb2 and pGBT9cytTNFR-I (Table I, bottom). Only double-transfected cells grew under the selection conditions.

To further confirm the interaction of Grb2 with the cytoplasmic domain of TNFR-I in another experimental system, coprecipitation experiments using highly purified, bacteria-derived Grb2 and cytTNFR-I were carried out. These experiments demonstrated that (i) Grb2 is indeed a binding partner of TNFR-I and (ii) no additional factors are necessary for the binding of Grb2 to TNFR-I (Fig. 1 A).

The in vivo interaction of endogenous Grb2 with endogenous TNFR-I was studied by immunoprecipitation of cellu-
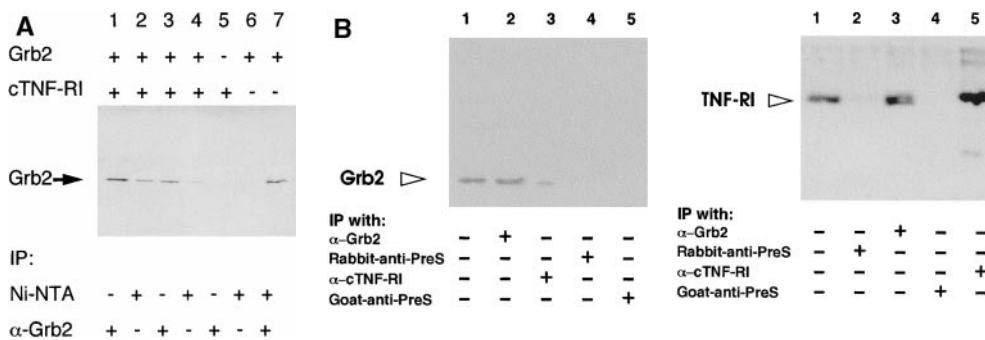

Figure 1. Grb2 and TNFR-I are direct binding partners. (A) Purified hexa-His–tagged cytoplasmic domain of TNFR-I (cTNFR-I; aa 205–426) (lanes 1–3 and 5, 1 μg; lane 4, 0.1 μg) was added to purified Grb2 (lanes 1–4, 6, and 7, 0.8 μg) in a volume of 300 μl. The mixtures were precipitated by addition of Ni-NTA–agarose (lanes 2, 4, 6, and 7) (specific for hexa-His–tagged c-TNFR-I), by a polyclonal sera specific for Grb2. Analysis of the precipitates for the presence of Grb2 was performed by Western blot using a monoclonal Grb2-specific antiserum. In lane 7, one-fifth of the precipitate was loaded to avoid overloading. Lanes 5 and 6 served as negative controls. (B) Coimmunoprecipitation of Grb2 and TNFR-I from the lysate of 10⁶ CCL13 cells. The lysates were precipitated by polyclonal rabbit-derived sera against Grb2 or goat-derived serum against TNFR-I and analyzed for the presence of Grb2 (left) or TNFR-I (right) by Western blot, using a mouse mAb specific for Grb2 or a polyclonal rabbit-derived serum specific for TNFR-I. Precipitations with an unrelated rabbit serum (rabbit anti-PreS2; left, lane 4; right, lane 2) or an unrelated goat serum (goat anti-PreS2; left, lane 5; right, lane 4) served as negative control. Total cellular lysates (left and right, lane 1) were used as positive controls.

lar lysates derived from CCL13 cells. Grb2 was coprecipitated by a TNFR-I–specific antibody and, vice versa, TNFR-I was coprecipitated by a Grb2-specific antiserum (Fig. 1 B). These results demonstrate that Grb2 binds to TNFR-I in vivo. Moreover, binding of Grb2 to TNFR-I seems to be constitutive, as the presence of TNF-α did not influence the amount of coprecipitated TNFR-I by a Grb2-specific antiserum.

The COOH-terminal SH3 Domain of Grb2 Binds to a PLAP Motif within TNFR-I. In the next set of experiments, the structural basis of the observed Grb2/TNFR-I interaction was analyzed. SH3 domains are known to bind to PXXP motifs (22–24). The membrane-proximal domain of TNFR-I harbors a PLAP motif within aa 237–240 as a potential binding site for the COOH-terminal SH3 domain of Grb2. Indeed, increasing concentrations of peptides covering this sequence (functional peptide TTK-PLAP) were able to compete the interaction of Grb2 with the cytoplasmic domain of TNFR-I, as demonstrated by coprecipitation experiments in vitro (Fig. 2 A).

The significance of this PLAP motif for the Grb2/TNFR-I interaction was confirmed using the yeast two-hybrid system. A deletion mutant of cytTNFR-I lacking the PLAP motif (pGBT9cytTNFR-IΔPLAP) failed to interact with full length wild-type Grb2 (pGAD424Grb2) (Table I, bottom). Furthermore, in vivo Grb2 could not be coprecipitated by Ni-NTA (nitrilotriacetic acid)–agarose from the lysate of transfected 293 cells overproducing hexa-His–tagged TNFR-I lacking the PLAP motif (Fig. 2 B). Ni-NTA–agarose specifically precipitated the hexa-His–tagged mutated receptor but not the wild-type receptor.

Essential structural requirements of the Grb2 adapter protein for interaction with TNFR-I were analyzed using the yeast two-hybrid system. Functional inactivating mutations of the NH$_2$-terminal SH3 domain (pGADGrb2ΔNSH3) or of the middle SH2 domain (pGADGrb2ΔSH2) did not af-

fect the interaction of Grb2 with cytTNFR-I. The inactivating mutation of the COOH-terminal SH3 domain (pGADGrb2ΔCSH3), however, abolished the interaction with cytTNFR-I under these conditions (Table I, bottom). These results confirm the dependence of the Grb2/TNFR-I interaction on the COOH-terminal SH3 domain of Grb2.

Binding of Grb2 to the PLAP Motif within TNFR-I Is Essential for the Activation of c-Raf-1 Kinase by TNF-α. The significance of TNFR-I/Grb2 interaction for TNF-α–dependent activation of c-Raf-1 kinase was investigated by different experimental approaches. Cell-permeable peptides were applied to disrupt the TNFR-I/Grb2 complex. These peptides consist of two domains, a signal sequence derived from the third helix of the antennapedia homeodomain, which mediates membrane translocation (25), and the functional domain covering the PLAP sequence of TNFR-I. Presence of these peptides abolished TNF-α–dependent activation of c-Raf-1 kinase (Fig. 3 A). This effect is specific, as the EGF-dependent activation of c-Raf-1 kinase was not affected by the presence of these peptides. Moreover, a mutated peptide (KLAP) did not affect the TNF-α–dependent activation of c-Raf-1 kinase.

In addition, transfection of 70Z/3 cells, which lack functional TNFR-I, with an expression plasmid encoding wild-type TNFR-I restores the capability of TNF-α–dependent activation of c-Raf-1 kinase. However, 70Z/3 cells transfected with an expression plasmid encoding the PLAP deletion mutant of TNFR-I failed to respond to TNF-α stimulation with an activation of c-Raf-1 kinase (Fig. 3 B).

Finally, transfection of 293 cells with expression plasmids encoding inactivating point mutations in the NH$_2$- or COOH-terminal SH3 domain of Grb2 in both cases caused a loss of TNF-α–dependent induction of c-Raf-1 activity. The mutation of the middle SH2 domain, however, did not affect the TNF-α–dependent activation of c-Raf-1 kinase (Fig. 3 C).
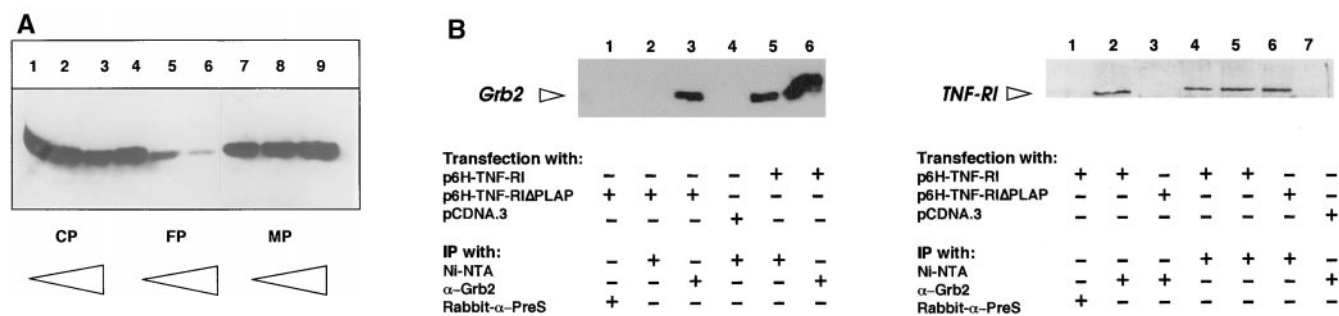

Figure 2. A PLAP motif in the cytoplasmic domain of TNFR-I mediates the interaction with Grb2. (A) Purified hexa-His–tagged cytTNFR-I (1 μg) was added to cellular lysate (500 μg) derived from TNF-α–stimulated 293 cells. Coprecipitation of Grb2 by addition of Ni-NTA–agarose, which precipitates the 6H-cytTNFR-I, was inhibited by increasing concentrations of a peptide (functional peptide [FP], lanes 4–6) harboring the recognition sequence TTKPLAP, whereas a mutated peptide (MP) (TTKKLAP, lanes 7–9) did not significantly affect the interaction of Grb2 and TNFR-I. An unrelated peptide served as additional control (CP) (DRQIKIWFQNRRMKWKK; lanes 1–3). The peptides were used in concentrations of 0.1 (lanes 2, 5, and 8) and 4 μg/ml (lanes 3, 6, and 9). The precipitates were analyzed by Western blot using a Grb2-specific antiserum. (B) Left: after transient transfection of 293 cells with p6H-TNFR-I (encoding for a fusion protein of an NH$_2$-terminal hexa-His tag and full length TNFR-I) (lanes 5 and 6), with p6H-TNFR-IΔ-PLAP (encoding for the hexa-His–tagged PLAP deletion mutant (lanes 1–3), or with the vector pCDNA.3 (lane 4), cellular lysates were precipitated by addition of Ni-NTA–agarose (lanes 2, 4, and 5) by a Grb2-specific polyclonal serum (lanes 3 and 6) or by an unrelated rabbit-derived serum (anti-PreS2; lane 1). The subsequent Western blot analysis was performed using a Grb2-specific, mouse-derived mAb. Right: lysates derived from p6H-TNFR-I– (lanes 1, 2, 4, and 5), p6H-TNFR-IΔPLAP– (lanes 3 and 6), or pCDNA.3- (lane 7) transfected cells were precipitated with Ni-NTA–agarose (lanes 4–6), a Grb2-specific polyclonal rabbit derived serum (lanes 2, 3, and 7), or an unrelated rabbit serum (anti-PreS2; lane 1). The precipitates were analyzed using a hexa-His tag–specific antibody.
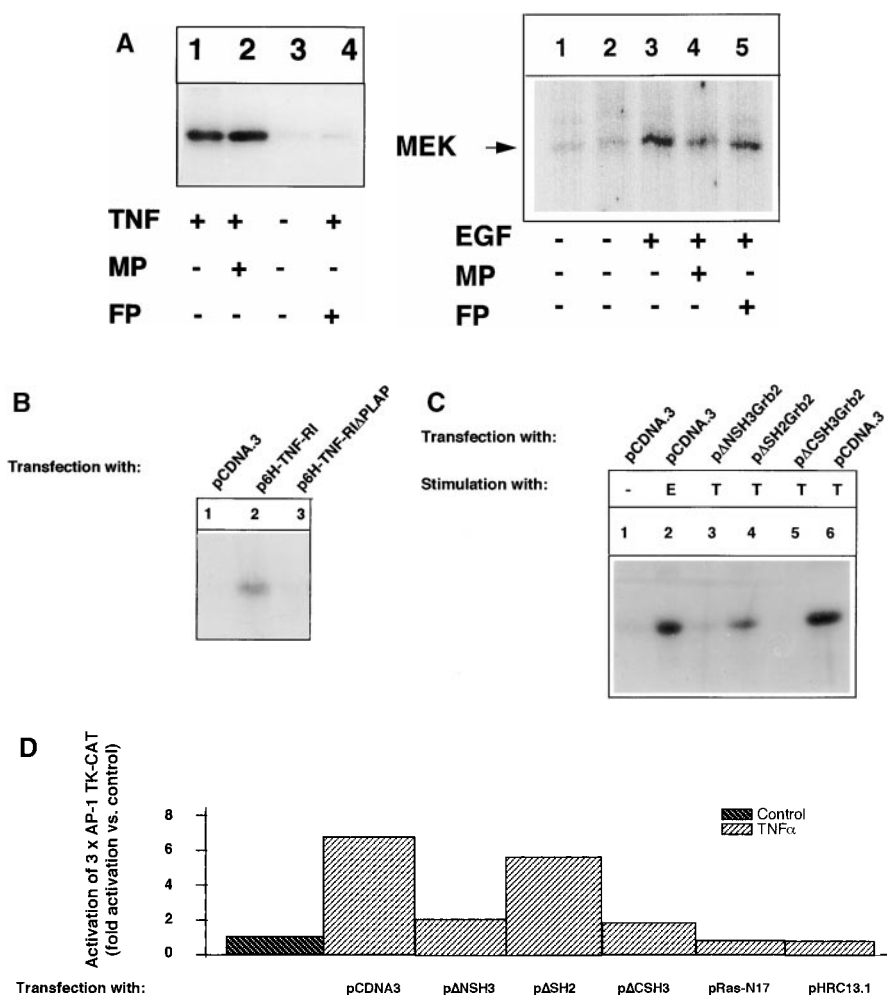
Figure 3. The TNFR-I/Grb2 interaction is essential for TNF-dependent activation of c-Raf-1 kinase and subsequent activation of AP-1. (A) 12-h serum-deprived 293 cells were grown for 6 h in the presence of cell-permeable peptides (15 μg/ml) covering the PLAP sequence (functional peptide [FP]: DRQIKI-WFQNRRMKWKKTTKPLAP; left, lane 4; right, lane 5) or a mutated peptide ([MP]: DRQIKIWFQNRRMKWKKTTKKLAP; left, lane 2; right, lane 4). Thereafter, cells were exposed for 15 min to TNF-α (100 U/ml) (left, lanes 1, 2, and 4) or to EGF (5 ng/ml) (right, lanes 3–5) in addition to the cell-permeable peptides. Activity of c-Raf-1 kinase was determined by immunocomplex assay using 6H-MEK as substrate and compared with unstimulated cells (left, lane 3; right, lanes 1 and 2). (B) 70Z3 cells were transfected with pCDNA.3 (lane 1), p6H-TNFR-I (lane 2), and p6H-TNFR-IΔPLAP (lane 3). After stimulation with TNF-α (200 U/ml), the activity of c-Raf-1 kinase was determined by immunocomplex assay. Activity of c-Raf-1 kinase in pCDNA.3-transfected cells was set arbitrarily as 1. (C) Immunocomplex assay of c-Raf-1 kinase activity in 293 cells transiently transfected with pCDNA.3 (lanes 1, 2, and 6), pΔNSH3-Grb2 (lane 3), pΔSH2Grb2 (lane 4), and pΔCSH3Grb2 (lane 5) after TNF-α (lanes 3–6) or EGF (lane 2) stimulation. Unstimulated cells (lane 1) served as negative control. (D) Reporter gene assay in 293 cells transiently cotransfected with pCDNA.3, pΔNSH3-Grb2, pΔSH2Grb2, pΔCSH3Grb2, and pHCR13.1 encoding a transdominant negative mutant of c-Raf-1 kinase 20 or pRasN17 and the reporter plasmid p3xAP-1–CAT after TNF-α stimulation (100 U/ml) for 24 h. The determination of the induced CAT amount was performed using a commercially available ELISA system. Activities given as fold induction are mean values of two independent experiments.

SOS Is Part of the Grb2/TNFR-I Complex. In the case of tyrosine kinase receptor–dependent activation of c-Raf-1 kinase, the SH3 domains of Grb2 are known to interact with the SOS proteins, which interfere with Ras (22, 26, 27). Based on the observation that integrity of the NH$_2$-terminal SH3 domain of Grb2 is essential to trigger TNF-α–dependent activation of c-Raf-1 kinase, it was investigated whether Grb2, while bound to TNFR-I with its COOH-terminal SH3 domain, still interacts with SOS1/2 proteins with its NH$_2$-terminal SH3 domain. Hexa-His–tagged cytoplasmic domain of TNFR-I was added to cellular lysates derived from 293 cells. The mixture was precipitated using either anti-Grb2– or anti-SOS1/2–specific antibodies, or Ni-NTA–agarose, which specifically precipitates 6H-cytTNFR-I. Western blot analysis of the precipitates demonstrated that cytTNFR-I indeed coprecipitates with both Grb2 and SOS (Fig. 4 A).

Transfection of 293 cells with an expression plasmid coding for a transdominant negative Ras mutant (RasN17) caused a loss of TNF-α–dependent activation of c-Raf-1 kinase. These results demonstrated that the TNF-α–dependent activation of c-Raf-1 kinase requires the integrity of the TNFR-I-Grb2-SOS-Ras pathway.

The TNFR-I/Grb2 Interaction Is Essential for the TNF-dependent Activation of AP-1. The physiological significance of Grb2-mediated activation of c-Raf-1 kinase upon stimulation with TNF-α was investigated by reporter gene assays. Cotransfection of an AP-1–driven reporter gene plasmid (p3xAP-1–CAT) (28) with the expression plasmids encoding the three Grb2 mutants harboring the inactivating point mutations in the NH$_2$- or COOH-terminal SH3 domain or those in the middle SH2 domain, with a transdominant negative Ras mutant (pRasN17), or with a transdominant negative mutant of c-Raf-1 kinase (pHCR13.1) (29) caused, with exception of pΔSH2Grb2, a loss of TNF-α–dependent induction of the reporter gene (Fig. 3 D). This shows (i) that the integrity of the TNFR-I/Grb2 interaction is a prerequisite for the TNF-α–dependent activation of AP-1, (ii) that, in addition to the COOH-terminal SH3 domain, the NH$_2$-terminal SH3 domain of Grb2 is essential for the activation of AP-1 by TNFR-I, and (iii) that Ras is involved in TNF-α–dependent signal transduction.

Activation of c-Raf-1 Kinase Requires TNFR-I/FAN Interaction. The PLAP-dependent Grb2/TNFR-I interaction is essential but not sufficient for TNF-α–dependent acti-
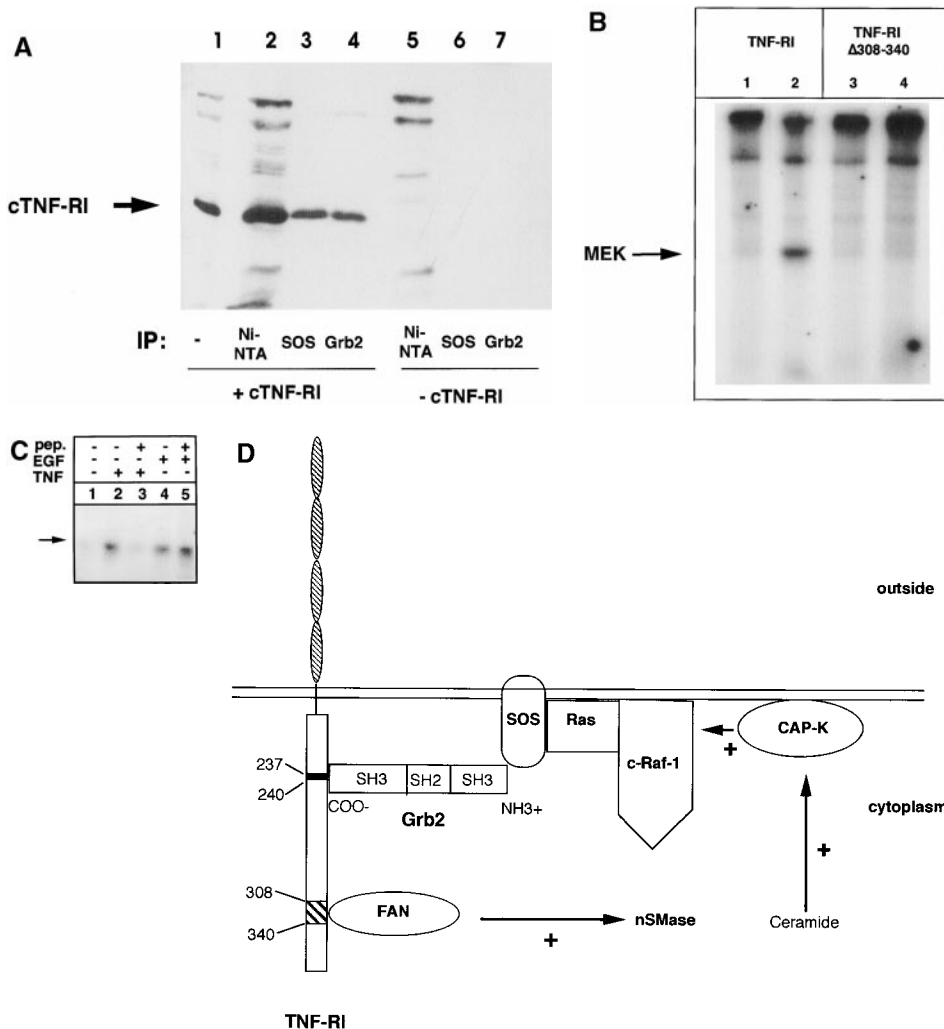
Figure 4. SOS coprecipitates with Grb2 and TNFR-I. (A) In the presence (+; lanes 1–4) or absence (−; lanes 5–7) of purified hexa-His–tagged cyt(c)TNFR-I (1 μg), cellular lysates derived from 293 cells were precipitated with Ni-NTA–agarose (lanes 2 and 5), SOS1/2-specific antiserum (lanes 3 and 6), and Grb2-specific antiserum (lanes 4 and 7). The precipitates were analyzed for the presence of cytTNFR-I by Western blot using a hexa-His tag–specific antibody. In lane 1, the mixture of cytTNFR-I combined with cellular lysate was loaded directly as the positive control. (B) 70Z3 cells stably producing wild-type TNFR-I or a deletion mutant (TNFRID308–340) lacking the nSMase-activating domain were stimulated with TNF-α (200 U/ml) for 15 min, and c-Raf-1 kinase activity was determined by immunocomplex assay. (C) 12-h serum-deprived 293 cells were grown for 6 h in the presence of cell-permeable peptides (lanes 2 and 4) covering the FAN binding sequence (functional peptide [FP], 5 μg/ml). Thereafter, cells were exposed for 15 min to TNF-α (100 U/ml; lanes 2 and 3) or EGF (5 ng/ml; lanes 1 and 5) in addition to the cell-permeable peptides. Activity of c-Raf-1 kinase was determined by immunocomplex assay using 6H-MEK as substrate. (D) Schematic representation of the proposed model of TNF-α–dependent activation of c-Raf-1 kinase. A PLAP motif recruits the Grb2 adapter protein through its COOH-terminal SH3 domain to TNFR-I. The NH₂-terminal SH3 domain of Grb2 interacts with SOS, which recruits via Ras c-Raf-1 kinase to the membrane. TNF-α–dependent activation of nSMase is mediated by binding of FAN to a distinct domain of TNFR-I (12, 13). Activation of nSMase results in the production of ceramide and, consequently, in an activation of CAP kinase. Both TNF-α–responsive pathways, the activation of Ras via Grb2 and the activation of nSMase, cooperatively stimulate c-Raf-1 kinase.

vation of c-Raf-1 kinase (15). Transfected 70Z/3 cells producing a deletion mutant of TNFR-I (TNFR-IΔ308–340), which lacks the ability to activate nSMase, fail to respond with activation of c-Raf-1 kinase upon stimulation of TNFR-I (8, 12). This mutant lacks the FAN binding domain (13, 30). FAN was recently discovered to mediate the TNF-α–dependent activation of nSMase (12, 13) (Fig. 4 B). In addition, disruption of the TNFR-I/FAN interaction by cell-permeable peptides (25) covering the binding domain blocked the TNF-α–dependent activation of c-Raf-1 kinase (Fig. 4 C). These data confirm the relevance of TNF-α–dependent activation of nSMase for induction of c-Raf-1 kinase activity.

Discussion

Based on our results, we propose that the TNF-α–dependent activation of c-Raf-1 kinase is mediated through at least two cooperative domains that are both indispensable.

The cytoplasmic tail of TNFR-I interacts via a PLAP motif with the COOH-terminal SH3 domain of the adapter protein Grb2. The NH₂-terminal SH3 domain of Grb2 interferes with SOS, therefore linking TNFR-I to c-Raf-1 kinase involving Ras (26). In contrast to the situation, in the case of tyrosine kinase receptors, in which Grb2 interacts with its middle SH2 domain with phosphotyrosine, this Grb2/SOS/Ras-dependent pathway is not sufficient for activation of c-Raf-1 kinase. A second domain is required. This domain was recently shown to bind FAN (12, 13). The TNFR-I/FAN interaction is essential for TNF-α–dependent activation of nSMase (8, 12, 13, 31, 32). The hydrolysis of sphingomyelin catalyzed by nSMase generates ceramide, which can finally activate ceramide-activated protein (CAP) kinase. CAP kinase recently has been shown to bind to TNF-α–dependent activated c-Raf-1 kinase (15). Both pathways, the Grb2-SOS-RAS pathway and the activated CAP kinase pathway, might cooperatively mediate TNF-α–dependent activation of c-Raf-1 kinase (Fig. 4 D).

Although TNFR-I does not belong to the tyrosine kinase receptor family, Grb2 has been identified as a binding partner. In contrast to the interaction with phosphotyrosine, which is mediated through its middle SH2 domain, Grb2 interacts here with TNFR-I via its COOH-terminal SH3 domain. Although there is an obvious difference on the molecular level of the receptor/adapter interaction, in both cases, binding of Grb2 to the receptor is essential for triggering activation of c-Raf-1 kinase. Whereas for tyrosine kinase receptors the Grb2-SOS-Ras pathway seems to be sufficient for activation of c-Raf-1 kinase, in the case of TNFR-I, a second pathway via CAP kinase is involved.

One possible explanation is that the interaction of SOS with the NH_2-terminal SH3 domain of Grb2 is not strong enough to recruit c-Raf-1 kinase to the membrane and, in addition, the membrane-associated CAP kinase is required. Moreover, the binding of Grb2 to the PLAP motif in the cytoplasmic domain of TNFR-I is constitutive. Therefore, TNF-α–dependent activation of nSMase provides a trigger for the TNF-α–dependent activation of c-Raf-1 kinase.

In conclusion, we demonstrate that the tyrosine kinase adapter protein Grb2 is a novel binding partner of TNFR-I and is essential for TNF-α–dependent activation of c-Raf-1 kinase.

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