Dominant Negative Form of Signal-regulatory Protein-α (SIRPα/SHPS-1) Inhibits Tumor Necrosis Factor-mediated Apoptosis by Activation of NF-κB*

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Genetic suppressor element (GSE) methodology was applied to identify new genes controlling cell response to tumor necrosis factor (TNF). A retroviral library of randomly fragmented normal human cDNA from mouse fibroblasts was screened for GSEs capable of protecting NIH3T3 cells from TNF-induced apoptosis. The most abundant among isolated GSEs represented a fragment of cDNA encoding the C-terminal cytoplasmic region of the immunoglobulin family inhibitory receptor, SHPS-1 (mouse homologue of human SIRPα). Ectopic expression of this fragment (both from human and mouse versions) increased the NF-κB-dependent transcription in three cell lines tested; this effect could be reduced by the expression of full-length SIRPα, suggesting that the isolated GSE acts through a dominant negative mechanism. GSE-mediated activation of NF-κB depended on the presence of serum, was abrogated by wortmannin, and was associated with phosphorylation of PKB/Akt, suggesting that Akt mediates it. These data indicate that SIRPα/SHPS-1 is involved in negative regulation of NF-κB signaling.

Tumor necrosis factor (TNF)¹ is involved in many disease-related processes in the organism, including inflammation, anti-viral, anti-cancer, and immune responses (1). In combination with inhibitors of transcription or translation, TNF can induce apoptosis in sensitive cells in cell culture. This apoptosis is initiated by binding TNF to its plasma membrane receptors, which belong to the death receptor family. After death receptors have been triggered, the adaptor molecules, including FADD and TRADD and the receptor proximal caspase 8, are engaged to form a death-inducing signaling complex (DISC). Upon recruitment to the DISC, caspase 8 becomes activated and mediates cleavage of procaspase 3, starting a chain of events that results in apoptotic death (2).

The key role played by TNF pathway in many pathological processes indicates the importance of its control by pharmacological approaches (3). Rational design of such approaches requires identification of cellular factors involved in regulation of TNF-mediated death, some of which have already been determined. Pro-apoptotic function of TNF can be counterbalanced by simultaneous activation of anti-apoptotic NF-κB pathway, which can effectively prevent initiation of a death cascade through transcriptional induction of a number of apoptosis inhibitors (4). NF-κB is functionally linked to Akt signaling pathway, an important sensor of environmental conditions (i.e. availability of growth factors) strongly contributing to the “to die or not to die” decision. It can greatly reduce suicidal intentions of the cell in the presence of growth factors and other ligands specific to healthy growth conditions and natural microenvironments (5).

The signaling from phosphoinositide 3-kinase (PI3K) to the protein kinase Akt is an evolutionary conserved ancient pathway that controls organism life span in invertebrates and cell survival and proliferation in mammals (6). Many cell-surface receptors induce production of second messengers, like phosphatidylinositol 3,4,5-trisphosphate (PIP₃), that convey signals to the cytoplasm from the cell surface. PIP₃ signals activate 3-phosphoinositide-dependent protein kinase-1, which in turn activates the kinase Akt, also known as protein kinase B. Akt promotes cell survival and opposes apoptosis by a variety of routes, including the activation of NF-κB through phosphorylation of inhibitory κB kinase (7).

Both NF-κB and Akt signaling are integrated into a network of cellular regulatory pathways through numerous components, only some of which have been identified. To determine new regulators of TNF apoptotic pathway, we used the genetic suppressor elements (GSE) approach, a functional genetic methodology designed for cloning genes associated with recessive phenotypes (8, 9). It was successfully used before for identification of genes involved in negative control of cell growth, including drug sensitivity and candidate tumor suppressor genes (8, 10, 11, 12). GSEs act by encoding either inhibitory antisense RNA or dominant negative truncated proteins. They are isolated from expression libraries of randomly fragmented cDNAs by functional selection. GSEs suppressing TNF-induced apoptosis would likely be derived from and act against the genes that could be important for TNF-mediated death and therefore can be used for identification and cloning.

Among genetic elements protecting cells from TNF-specific apoptosis, we isolated a GSE that encoded the cytoplasmic part...
of the inhibitory receptor SHPS1 (the mouse homologue of human SIRPα) (13, 14), known to bind and activate the SH2 domain containing protein tyrosine phosphatase, SHP-1 and SHP-2, and inositol phosphatease, SHIP (15). These phosphatases are known to be involved in cytoplasmic signaling downstream of a variety of cell surface receptors and to be capable of modulating NF-κB (16). In fact, ectopic expression of SIRPα, SHPS1, or SHPS-2 can result in the strong Akt-dependent activation of NF-κB that is likely to be responsible for the GSE-mediated TNF resistance. Overexpression of full-length SIRPα protein caused effects opposite to that of the GSE, indicating that the isolated element acts through a dominant negative mechanism. These observations suggest that SIRPα is a natural negative regulator of NF-κB signaling, presumably involved in the control of cell response to a variety of stimuli acting through this important pathway.

EXPERIMENTAL PROCEDURES

Cells, Transfection, and Retrovirus Infection—A4 (p53-deficient mouse embryonic fibroblasts transformed by E1a + ras), NIH3T3, NIH3T3-derived amphotropic and ecotropic packaging cells GP + E6 and GP + envAm12 (17), and HeLa, 293, and Ecopack (Clontech) cell lines were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum. The transfections of NIH3T3, HeLa, 293, and Ecopack cells were conducted with LipofectAMINE PLUS reagent (Invitrogen) according to the provider’s protocols. Retroviral infection was accomplished by transferring virus-containing medium supplemented with 4 µg/ml polybrene (Sigma).

Plasmids and Libraries—The preparation of the GSE library from poly(A) + RNA of NIH3T3 cells was previously described (8). The library was made in plLNCX retroviral expression vector (18) and contains 3 x 10⁶ independent clones expressing random 150–400 bp fragments of normalized cDNA. Synthetic adapters providing initiator codons upstream of the insert in all reading frames flank each fragment. GSEs were isolated from selected cells by RT-PCR and recloned into pcDNA2 vector (Invitrogen) for sequencing. GSE2–1 and GSE5IRP, representing the entire C-terminal part of SHPS1/SIRPα, were synthesized by RT-PCR from total RNA of NIH3T3 and HeLa cells, respectively, using 5‘-GAATTCCTGCAACCATGGAACAG-3‘ sense and 5‘-GGATCCCATCACCITCGCTGCAGCT-3‘ antisense primers for GSE2–1 and 5‘-GAATTCCTGCAACCATGGAACAG-3‘ sense and 5‘-GGATCCCATCACCITCGCTGCAGCT-3‘ antisense primers for GSE5IRP. The targeted mutagenesis, which converted tyrosines to phenylalanines codons, was done by PCR. For functional testing, all the generated fragments were cloned into plLNSN retroviral expression vector, also containing G418 resistance. The complete coding region of full-length SIRPα cDNA was recloned from Expressed Sequence Tag access numbers AI357578 and BE514786 into pcDNA3 expression vector (Invitrogen). cDNA for the S32A/S36A mutant of iκBα (super-repressor mutant form of iκBα) in plBaePuro expression vector was a gift from Dr. S. S. Makarov (19).

GSE Library Screening for TNF Resistance-conferring Elements—2 x 10⁶ ECOPACK retrovirus packaging cells were transduced with 3 µg of GSE library or insert-free plLNCX plasmid (control) by standard LipofectAMINE PLUS reagent protocol. The virus-containing medium from transduced cells was transferred on 2 x 10⁶ NIH3T3 amphotropic packaging cells followed by G418 selection. 5 x 10³ G418-resistant NIH3T3 amphotropic cells per plate were treated with TNF (0.01 ng/ml) or S. S. Makarov (19). The complete coding region of full-length SIRPα cDNA was recloned from Expressed Sequence Tag access numbers AI357578 and BE514786 into pcDNA3 expression vector (Invitrogen). cDNA for the S32A/S36A mutant of iκBα (super-repressor mutant form of iκBα) in plBaePuro expression vector was a gift from Dr. S. S. Makarov (19).

RESULTS

Functional Selection of GSEs Protecting from TNF-induced Apoptosis—The scheme explaining the screening of the retroviral GSE library of normalized fragment cDNA of NIH3T3 is shown in Fig. 1A and is fully addressed under “Experimental Procedures.” 2 x 10⁶ library-producing NIH3T3-derived packaging cells sensitive to TNF were treated with TNF in the presence of inhibitor of translation CHI under conditions that resulted in killing >90% of the cells. After expansion of the surviving population, virus produced by these cells (and presumably enriched in clones conferring TNF resistance) was transferred to another type of packaging cells permissive for the infection with the isolated virus, and treatment was repeated. Up to four rounds of selection were applied before virus transfer became evidently capable of conferring TNF resistance to NIH3T3 cells as compared with control vector virus subjected to the same selection procedure (20–25% versus 2–3% of TNF-resistant cells among infected NIH3T3). At this stage, selected GSE inserts isolated by RT-PCR were recloned and sequenced. 40% of all rescued GSE clones in one of the lines of screening contained the same sequence, named GSE2. The proportion of this sequence, undetectable in the original GSE population before TNF selection, gradually increased with each next round of selection, as determined by Southern blot hybridization of PCR products of the mixture of cDNA GSE inserts isolated by PCR at different stages of screening (Fig. 1B). This GSE was subjected to a more detailed analysis.

Sequence analysis of GSE2 showed that it represents a sense-oriented fragment of cDNA encoding a cytoplasmic part of a known protein, inhibitory receptor SHPS1 (accession number NM007547). (The human homologue is known as signal regulatory protein α or SIRPα). The SHPS1/SIRPα corresponding open reading frame in GSE2 starts from the second initiator codon of the adapter and encodes 99 amino acid resi-
dues from the cytoplasmic part of SHPS-1, with the exception of the very C-terminal part that includes the last C-terminal tyrosine site for phosphorylation (Fig. 1 C). Structurally, SHPS-1/SIRP/H9251 belongs to the immunoglobulin family of receptors. When activated by Src-mediated phosphorylation of a specific tyrosine residue, SHPS-1/SIRP/H9251 can affect the signaling pathways between epidermal growth factor receptor and PI3K (22) through binding the SH2-domain containing phosphotyrosine phosphatases SHP-1 and SHP-2. No connection has been reported so far between SHPS-1/SIRP/H9251 and TNF-dependent apoptosis.

Cytoplasmic Domain of SHPS-1/SIRP/H9251 Protects Mouse Fibroblasts from TNF-induced Apoptosis—GSE2 in the selected clone was in sense orientation and could potentially express the majority of the cytoplasmic part of SHPS-1/SIRP/H9251 if translated from the second ATG codon of the adapter. However, two other start codons in alternative frames could, in principle, initiate the translation of SHPS-1/SIRP/H9251-unrelated peptides, which could be responsible for the observed effect. To rule out this possibility, we modified the GSE2 sequence to eliminate two alternative initiator codons and cloned it into another retroviral vector (pLXSN) under the control of the long terminal repeat promoter. In parallel, we synthesized by RT-PCR and cloned into the same vector the fragment of SHPS-1/SIRP/H9251 cDNA that encodes the entire C-terminal region of the protein, supplying it with the initiator ATG codon (GSE2–1) (Fig. 1 C).

All these constructs, including insert-free vector, were converted into retroviruses by transfection of packaging cells and transduced into two mouse cell lines (NIH3T3 and A4) that were then tested for TNF resistance. Only GSE2 and GSE2–1 efficiently protected the infected cells from TNF-specific apoptosis (Fig. 2). Cells infected with GSE2–1 in antisense orientation showed the same sensitivity to TNF as the cells infected with empty pLXSN vector and the original cells. No difference in the potency of TNF protection was found between GSE2 and GSE2–1, indicating that the last tyrosine of SHPS-1/SIRP/H9251, which was reported to be important for SHPS-1/SIRP/H9251 function (23), is dispensable in the GSE activity. Thus, biological activity of the isolated GSE is indeed the function of the C-terminal fragment of SHPS-1/SIRP/H9251 protein expression.

Cytoplasmic Domain of SHPS-1/SIRP/H9251 Can Activate NF-kB—TNF is known to stimulate activation of NF-kB possessing pro-inflammatory and anti-apoptotic functions (24, 25). NF-kB activation by TNF and platelet-derived growth factor requires

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**Fig. 1. Isolation of SHPS-1 fragment conferring resistance to TNF.**

A, scheme of selection of GSEs protecting against TNF-induced apoptosis. B, enrichment of GSE2-expressing cells during selection against TNF-specific apoptosis. The results of Southern hybridization of RT-PCR products generated using primers specific for LNCX vector sequences flanking the library inserts. 1 μg of total RNA from cells infected with GSE library before TNF selection (0) and after 1, 2, or 3 rounds of selection (lanes 1–3) was used for RT-PCR. C, the amino acid sequence of SHPS-1 (in bold) aligned with the GSEs isolated or generated. The cytoplasmic part of the protein is underlined. Asterisks mark the homologous amino acids between mouse and human proteins. Bold letter in GSEISIRP marks the tyrosine substitution with phenylalanine in GSEIFSIRP.
Akt serine-threonine kinase (26, 27). SIRPα was reported to have negative regulatory effects on cellular responses to growth factors (13) and to inhibit EGF-induced PI3K activation (22). Because SIRPα affects the PI3K/Akt pathway, we speculated that it can modulate TNF signaling through Akt-dependent NF-κB activation. To test this hypothesis we analyzed NF-κB activity in the cells overexpressing GSE2–1 and full-length SIRPα, using specific reporter constructs expressing NF-κB-dependent luciferase. The results of these experiments are presented in Fig. 3A. They show that GSE2–1 stimulates basal activity of NF-κB up to 8 times. At the same time, overexpression of the full-length SIRPα protein had the effect opposite to that of the GSE, causing a decrease in the activity of NF-κB (Fig. 3A). Neither construct had any effect on the expression of luciferase reporter with minimal promoter (data not shown).

The mutated form of IκB known as “super-repressor,” which can effectively inhibit NF-κB transcriptional activity at the step of translocation of NF-κB to the nucleus (19), has often been used to prove the NF-κB-specificity of the studied effects (19, 27). We expressed the super-repressor form of IκB in combination with GSE2–1 and found that it can neutralize the NF-κB-activating effect of the GSE as well as the activation of NF-κB by TNF (Fig. 3B).

The activity of full-length SIRPα depends on the phosphorylation of tyrosine residues in the cytoplasmic part of the protein by Src tyrosine kinase (13); only the phosphorylated form of SIRPα can bind SHP-1 and -2 protein phosphatases (13, 22). We checked whether this phosphorylation is crucial to the NF-κB-inducing activity of the cytoplasmic fragment of SHPS-1/SIRPα. In these experiments we used the construct expressing the human homologue, named GSE/SIRPα, of mouse GSE2–1 that has a similar biological effect on NF-κB. Substitution of tyrosine for phenylalanine within the consensus of immunoreceptor tyrosine-based inhibitory motif (ITIM) (V/LXXXYV/L) decreased the ability of GSE1FSIRP to activate NF-κB in all cell lines tested (Fig. 4). The mutation form of GSE with three tyrosines changed for phenylalanines completely lost the ability to activate NF-κB (data not shown). It is noteworthy that all the versions of GSE2 and GSE/SIRPα were expressed in our experiments at a level comparable with those of endogenous SHPS-1/SIRPα, whereas ectopic expression of full-length constructs resulted in high levels of SHPS-1/SIRPα that significantly exceeded the level of endogenous protein (Fig. 3C).

The Cytoplasmic Domain of SHPS-1/SIRPα Is Likely to Act through a Dominant Negative Mechanism—Expression of the cytoplasmic portion of SHPS-1/SIRPα protected cells against TNF-induced apoptosis, presumably through its ability to activate NF-κB. On the contrary, ectopic expression of full-length SHPS-1/SIRPα makes cells more sensitive to TNF-stimulated apoptosis (Fig. 5A). This effect was stronger in HeLa than in NIH3T3 cells because of higher sensitivity of NIH3T3 cells to TNF-specific apoptosis (Fig. 5A). Consistently, full-length SHPS-1/SIRPα caused an inhibitory effect on NF-κB activity (Fig. 3A). These observations suggested that the cytoplasmic portion of SHPS-1/SIRPα acts as a dominant negative mutant. In fact, cotransfection of cells with different ratios of plasmids encoding truncated and full-length versions of SHPS-1/SIRPα showed that the excess of full-length SHPS-1/SIRPα decreased the ability of GSE2–1 to activate NF-κB (Fig. 5B).

Activation of NF-κB by the Cytoplasmic Portion of SHPS-1/SIRPα is Akt-dependent—GSE2–1 did not stimulate NF-κB-dependent transcription in serum-free medium in NIH3T3 cells (Fig. 6A), suggesting the involvement of PI3K/Akt signaling in the GSE activity. We used wortmannin, an inhibitor of PI3K/ Akt to activate NF-κB. Cells were transfected and maintained in serum-free medium overnight. An additional 10% of fetal calf serum activated NF-κB-mediated transcription of the reporter, and the expression of GSE2–1 had an additive effect.
HeLa, NIH3T3, and 293 cells were cotransfected with the combination were lysed 4 h after treatment with 0.01 ng/ml TNF followed by luciferase assay. Bars represent average results of two experiments. The average data of four experiments are presented. Wortmannin completely inhibited NF-κB activation by TNF (26, 28). Activated Akt phosphorylates the IκB complex, which phosphorylates NF-κB inhibitor IkB and stimulates its degradation (29), which leads to a release of NF-κB that accumulates in the nucleus and activates transcription of the target genes.

Discussion

In our search for GSEs protecting cells against TNF-induced apoptosis, we isolated a genetic element encoding the cytoplasmic part of an already known protein, SHPS-1/SIRPα. The isolated GSE protected cells from TNF and strongly induced transcriptional activity of NF-κB in a PI3K/Akt-dependent manner. Because NF-κB is known to protect cells from TNF, GSE-mediated activation of this transcription factor is presumably responsible for the inhibition of TNF-mediated apoptosis. Full-length SHPS-1/SIRPα shows effects opposite to those of the isolated GSE, suggesting that the GSE-encoded truncated form of this protein acts through a dominant negative mechanism, interfering with the function of the full-length protein.

What is the mechanism linking SHPS-1/SIRPα to NF-κB? The PI3K/Akt pathway is important for NF-κB activation by TNF (26, 28). Activated Akt phosphorylates the IκB complex, which phosphorylates NF-κB inhibitor IkB and stimulates its degradation (29), which leads to a release of NF-κB that accumulates in the nucleus and activates transcription of the target genes. The functional connection of SIRPα with the PI3K/Akt pathway was shown. Overexpression of SIRPα, but not of its tyrosineless mutant form, inhibits PI3K activation and stimulation of proliferation by growth factors (13, 22). On the contrary, ectopic expression of the cytoplasmic portion of SHPS1/SIRPα stimulates growth factor-dependent Akt phosphorylation. NF-κB activation seems to be the result of this effect because it was completely suppressed by the PI3K inhibitor wortmannin. Importantly, this effect of the GSE-encoded truncated protein was also dependent on the presence of tyrosine residues. These observations are consistent with the hypothetical model that the truncated dominant negative form of SHPS1/SIRPα competes with the full-length membrane-bound protein for some factors that are bound to these proteins in a tyrosine phosphorylation-dependent manner. There are two proteins belonging to the same family that are known to bind with SHPS1/SIRPα and mediate its biological functions, tyrosine phosphatases SHP-1 and -2 (15). SHPS1/SIRPα can bind both proteins when its own tyrosine residues in ITIM signals are phosphorylated with Src (13, 14, 22).

SHP-1 and -2 are involved in many pathways, the function of which requires tyrosine phosphorylation (30–32), including NF-κB signaling (16, 33). Despite significant homology between SHP-1 and -2, these proteins have opposite effects on cell viability. SHP-1 is pro-apoptotic and can suppress cell growth (34–37), and its absence stimulates NF-κB activation by TNF (33). In contrast, SHP-2 has an anti-apoptotic effect (38), stimulating Akt activation by growth factors (16, 39); its absence inhibits NF-κB activation by TNF (16). NF-κB activity is increased in cells from the Mev strain of mice deficient in SHP-1 (33). The opposite biological effect is associated with SHP-2 gene deficiency (16).

SHP-1 is linked to NF-κB through modulation of PI3K/Akt signaling. It causes dephosphorylation of tyrosine 688 inside the N-terminal SH2 domain of the regulatory subunit of PI3K p85 that turns it into an inhibitor of the catalytic subunit p110 (40, 41). The depletion of SHP-1 by the GSE-encoded cytoplasmic portion of SHPS1/SIRPα might lead to the activation of the p110 subunit of PI3K and phosphorylation of Akt. Thus,
dephosphorylation of the p85 regulatory subunit of PI3K might be one of the mechanisms through which dominant negative SHPS-1/SIRP could activate NF-κB and promote TNF resistance. However, in our experiments GSE2 caused only a slight increase in the level of Akt phosphorylation in 293 cells, making it unlikely that this effect is solely responsible for the dramatic activation of NF-κB-dependent transcription. There could be additional targets for GSE2 downstream of Akt that remain to be identified.

Although the function of SHP-1 is consistent with the observed biological effects, the role of SHP-2 in our model remains less defined. There are contradictory reports on the activity of this phosphatase. There is evidence indicating that inactivation of SHP-2 decreases the transactivating abilities of NF-κB in response to interleukin 1 (16). At the same time, other reports claim that inactivation of SHP-2 increases the PI3K-dependent Akt activation with EGF (42) and that cooperation of SIRPα and SHP-2 had a negative effect on EGF-specific activation of PI3K (22). According to these data, the functions of the two phosphatases are similar and titration of...
SHP-2 by GSE2 could also stimulate EGF/Pi3K-dependent activation of NF-κB. This apparent controversy has to be resolved in future experiments.

In summary, the biological effects of full-length SHPS-1/SIRPα can be explained through its ability to bind and compartmentalize SHP-1, and possibly SHP-2, with growth factor receptors causing their inactivation. We hypothesize that GSE-encoded proteins act as scavengers of SHP-1 and -2 in the cell, affecting proper targeting of these phosphatases to plasma membrane and thereby interfering with their inhibitory activity against growth factor-mediated signaling.

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