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Differential Regulation of the TRAIL Death Receptors DR4 and DR5 by the Signal Recognition Particle

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TRAIL (TNF-related apoptosis-inducing ligand) death receptors DR4 and DR5 facilitate the selective elimination of malignant cells through the induction of apoptosis. From previous studies the regulation of the DR4 and DR5 cell-death pathways appeared similar; nevertheless in this study we screened a library of small interfering RNA (siRNA) for genes, which when silenced, differentially affect DR4- vs. DR5-mediated apoptosis. These experiments revealed that expression of the signal recognition particle (SRP) complex is essential for apoptosis mediated by DR4, but not DR5. Selective diminution of SRP subunits by RNA interference resulted in a dramatic decrease in cell surface DR4 receptors that correlated with inhibition of DR4-dependent cell death. Conversely, SRP silencing had little influence on cell surface DR5 levels or DR5-mediated apoptosis. Although loss of SRP function in bacteria, yeast and protozoan parasites causes lethality or severe growth defects, we observed no overt phenotypes in the human cancer cells studied—even in stable cell lines with diminished expression of SRP components. The lack of severe phenotype after SRP depletion allowed us to delineate, for the first time, a mechanism for the differential regulation of the TRAIL death receptors DR4 and DR5—implicating the SRP complex as an essential component of the DR4 cell-death pathway.

INTRODUCTION

Cytokine signaling through TNF receptors play critical roles in mammalian development and host defense by selectively eradicating infected and malignant cells from healthy cell populations (Smyth *et al.,* 2003). On binding the TNF receptor family members DR4 or DR5, TRAIL induces cell death via caspase-dependent apoptosis (LeBlanc and Ashkenazi, 2003). Nonfunctional "decoy" receptors, DcR1 and DcR2, compete for TRAIL binding, thereby promoting cell survival. However, little is known about the regulation of these receptors and why TRAIL-activated apoptosis pathways are tumor specific, whereas other pathways, such as those induced by TNF-α or Fas ligand, are not cancer cell biased. Paradoxically, many downstream components of the TNF signal transduction pathway are shared (Barnhart *et al.,* 2003). For example, the DR4, DR5, and Fas (TNFSFR6) apoptosis pathways appear nearly identical (Thorburn, 2004). Nevertheless, reminiscent of early studies examining TNF and Fas pathway agonists as potential anticancer agents, DR4 and DR5 agonists are currently shouldering the hopes for the next generation of TNF-related cancer therapeutics (LeBlanc and Ashkenazi, 2003).

The advent of RNAi technologies has inspired several attempts at genome-wide gene silencing to identify proteins whose functions influence specific cellular events or pathways (Lee *et al.,* 2003; Pothof *et al.,* 2003). In theory, small interfering RNA (siRNA) collections that specifically silence each gene in the human genome could be screened to delineate all components of any cellular pathway—given appropriate assays and elimination of current technical hurdles, such as design of efficient siRNAs to every gene (Elbashir *et al.,* 2002; Hannon, 2002; Paddison and Hannon, 2002; Deveraux *et al.,* 2003). Although far from accomplishing this goal, initial screens of smaller siRNA libraries targeted toward human genes have nonetheless proven very fruitful (Aza-Blanc *et al.,* 2003; Berns *et al.,* 2004; Paddison *et al.,* 2004).

To identify potential differences in the regulation of DR4- vs. DR5-mediated apoptosis, we screened an arrayed library of siRNAs targeted toward 543 distinct human genes. Agonistic antibodies specific for the TRAIL DR4 or DR5 receptors were used to screen the library for siRNAs that differentially influence DR4- vs. DR5-mediated reduction in human cancer cell viability. Here we show that silencing components of the signal recognition particle (SRP) strongly inhibited DR4-, but not DR5-mediated apoptosis. The lack of a severe phenotype in SRP-depleted cells allowed us to further investigate the role of the SRP complex in DR4- and DR5-mediated apoptosis and should facilitate the investigation of SRP function in global or specific aspects of mammalian cell biology.

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METHODS AND MATERIALS

Cell Culture and Materials

HeLa cells (ATCC no. CCL-2) and HCT-15 (ATCC no. CCL-225) were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 50 U ml^{-1} penicillin, and 50 g ml^{-1} streptomycin (Invitrogen). Cells were cultured at 37°C under an atmosphere of 5% CO_2.

Table 1. siRNAs that inhibit or enhance DR4- or DR5-mediated cell death

Inhibit

DR4			DR5		
Acc. number	Symbol	SR	Acc. number	Symbol	SR
NM_003844	TNFRSF10A	3.25	AB018313	VPS39	1.69
NM_003844	TNFRSF10A	3.00	NM_002211	ITGB1	1.50
NM_006947	SRP72	2.95	NM_002759	PRKR	1.49
NM_002467	MYC	2.40	NM_004071	CLK1	1.47
NM_014226	RAGE	2.32	NM_003842	TNFRSF10B	1.44

Enhance

Acc. number	Symbol	SR	Acc. number	Symbol	SR
NM_002958	RYK	0.30	NM_139158	ALS2CR7	0.22
XM_087575	RIKEN like	0.30	NM_007284	PTK9L	0.25
AC087742	clone RP11-104O19	0.33	NM_002110	HCK	0.26
NM_002576	PAK1	0.34	NM_003600	STK6	0.28
NM_005246	FER	0.35	NM_001229	CASP9	0.29

pMyc-SRP72 was constructed by inserting a DNA fragment encoding SRP72 into the *Bam*HI and *Xho*I sites of pcDNA-myc. Cloning the DR4 or DR5 receptor gene into the *Bam*HI and *Not*I sites of pcDNA-GFP created the pDR4-GFP and pDR5-GFP constructs.

siRNA Library Screening

The siRNA collection was prepared and plated as previously described (Aza-Blanc *et al.*, 2003). Briefly, the siRNA collection contains 543 siRNAs designed to specifically silence each of 380 known and predicted kinases, 101 genes of unknown function, and 62 known genes of interest including genes known to play a role in apoptosis pathways. This library was arrayed in 384-well microtiter plates in duplicate and transfected into HCT15 cells. Lipofectamine 2000 (650 μl, Invitrogen) was added to 65 ml Opti-MeM (Invitrogen) and then 10 μl of diluted Lipofectamine was dispensed per well in a 384-well siRNA library plate using a Titertech 96/384 microplate liquid dispenser. HCT15 cells were prepared in media containing 10% serum, and 6000 cells were seeded per well and incubated for 48 h. DR4-A and DR5-A were cross-linked with a goat anti-mouse Fc (Jackson ImmunoResearch Laboratories, West Grove, PA) with the ratio one functional antibody (DR4-A or DR5-A) to three cross-linking antibodies (goat anti-mouse Fc) at room temperature for 30 min. Treatment without or with 0.5 μg/ml cross-linked DR4-A or DR5-A was carried out for an additional 24-h period, followed by a measurement of cell viability using CellTiter-Glo (Promega, Madison, WI). For each 384-well plate the observed luminescence was normalized by dividing each well by the average of 24 wells on the same plate containing siRNAs against Luciferase and multiplying by 100 to obtain normalized viability ("% viability"). The normalized data were then used to create a "Sensitivity Ratio" by dividing the normalized values obtained in the presence of DR4-A or DR5-A by the normalized values obtained in untreated cells (see below).

Analysis of siRNA Library Screening Results

The data for each plate were normalized to the luciferase wells on each plate and then the sensitivity ratio (SR) was calculated by dividing the normalized values obtained in the presence of DR4-A or DR5-A by the normalized values obtained in untreated cells. The SRs for two independent screens were averaged and those siRNAs that caused a 20% decrease in viability of untreated cells were discarded. The remaining siRNAs were sorted by their SR and the top and bottom five siRNAs of the sorted list are shown in Table 1. Those siRNAs with a low SR enhance the ability of DR4-A or DR5-A to kill cells and those with a high SR repress the ability of DR4-A or DR5-A to induce killing of the cells.

siRNA Transfection and Creation of Clones with Stable Expression of Short-hairpin RNA's Targeting SRP Subunits

Cells were seeded into six-well plates at 300,000 cells per well. After 24 h, cells were transfected with siRNAs at a final concentration of 200 ng/ml siRNA with 2 μl Oligofectamine 2000 (Invitrogen). After 24 h of transfection, cells were trypsinized and plated at 8000 cells per well in 96-well plate or at 2500 cells per well in 384-well plate. All the siRNAs were purchased from Dharmacon Research (Lafayette, CO). The sequences of siRNAs used were as follows: siGL3: sense 5′-(CUUACGCUGAGUACUUCGAdTdT)-3′; siSRP54a: sense 5′-(GAAGAGGUAUUGAAUGCUAdTdT)-3′; siSRP54b: sense 5′-(GAAGACCUGUUUAAUAUGUdTdT)-3′; siSRP54c: sense 5′-(GAAAUGAACAGGAGUCAAUdTdT)-3′; siSRP54d: sense 5′-(GCAAGAGGAUCGGGUGUAUdTdT)-3′; siSRP72a: sense 5′-(UCUGCUGGUGCUACAUACAdTdT)-3′; siSRP72b: sense 5′-(GGAGCUUUAUGGACAAGUGdTdT)-3′; siSRP72c: sense 5′-(GGAACAAGGACAGGGAGAUdTdT)-3′; siDR4: sense 5′-(CACCAAUGCUUCCAACAAUdTdT)-3′; and siDR5: sense 5′-(AUGAGAUAAAGGUGGCUAAdTdT)-3′.

Plasmids encoding shRNA sequences for SRP54 and SRP72 were transfected into HeLa cells using Lipofectamine 2000 transfection reagent (Invitrogen). Single cell clones were selected and propagated in the presence of 1 μg/ml puromycin and then screened for SRP54 and SRP72 expression.

Analysis of Cell Death

After 48 h, siRNA-transfected cells were treated with various amounts of TRAIL (Calbiochem, San Diego, CA), CH11 (MBL International Corp, San Diego, CA), TNF-α (Sigma-Aldrich, St. Louis, MO), DR4-A antibody, DR5-A antibody, UV radiation, cycloheximide (Sigma-Aldrich), or staurosporine (Sigma-Aldrich). DR4 or DR5 mAb was cross-linked with goat anti-mouse Fc (Jackson ImmunoReseach Laboratories, West Grove, PA) as described above for siRNA library screening. Twenty-four hours after treatment, cell viability was assessed by MTT assay (Pierce, Rockford, IL) or CellTiter-Glo luminescent cell viability assay (Promega) according to manufacturer's instructions. For caspase activity assays, siRNA transfected cells in six-well plates were induced for apoptosis by addition of indicated concentrations of TRAIL, DR4-A, or DR5-A. Caspase 3/7 activity assays were determined as previously described (Deveraux *et al.*, 1999) and using Caspase-Glo 3/7 assay according to the manufacturer's instructions in 96-well plates (Promega).

Western Blotting

Cells in six-well plate were washed with ice-cold PBS and lysed in a 1% Triton X-100 hypotonic lysis buffer (HEPES [pH 7.2] 20 mM, $MgCl_2$ 1.5 mM, KCl 10 mM, EDTA 1 mM) or RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 50 mM Tris-HCl at pH 8, 0.1% SDS, 10% glycerol, 5 mM EDTA, 20 mM NaF, and 1 mM Na_3VO_4). Cell lysates were cleared by centrifugation at 16,000 $\times$ *g* for 5 min at 4°C. Total protein of lysates were then quantified using BCA Protein Assay (Pierce), and equal amounts of protein (40–60 μg) were loaded into Novex Tris-Gly SDS-PAGE gels (Invitrogen). The primary antibodies used were purchased from: anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA); anti-SRP54, anti-GM130, anti-Caspase 3, anti-caspase 8, and anti-Bid from BD Biosciences Pharmingen (San Diego, CA); anti-DR4 from Imgenex (San Diego, CA); anti-DR5 from Cayman (Ann Arbor, MI); anti-GFP from Transduction Laboratories (Lexington, KY); anti-Calnexin from Stressgen (Victoria, BC, Canada); anti-Golgi 97 from Molecular Probes (Eugene, OR); antipan-cadherin from Zymed Laboratories (South San Francisco, CA), anti-Myc and anti-Caspase 9 from Cell Signaling (Beverly, MA) and MBL International Corp. (Watertown, MA). The secondary antibodies conjugated to HRP were obtained from Bio-Rad (Hercules, CA) and used as suggested. Signals were detected using the ECL system (Amersham Biosystems, Buckinghamshire, United Kingdom) and Kodak Biomax films (Eastman Kodak, Rochester, NY).

RT Quantitative and Semiquantitative PCR

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. Equal amounts of total RNA (2 μg) were used as template for first-strand synthesis with oligo dT primers (SuperScript first-strand synthesis system for RT-PCR, Invitrogen). The resulting first-strand cDNA was diluted and used as template in the RTQ-PCR and semiquantitative PCR analysis. Custom primers and probes were designed for targeted transcripts with use in the ABI 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Results were normalized with GAPDH control amplification. Reactions for each sample were performed in triplicate using equal amounts of template cDNA. For semiquantitative RT-PCR amplifications were carried out for indicated cycles. The sequences of the PCR primer pairs were as follows: SRP54 sense 5′-(CCTGGAGTTAAGGCATGGA)-3′; antisense5′-(CCTCTTGCTACTCTTTGGAT)-3′; SRP72 sense 5′-(ATGGCGAGCGGCGGCAGC)-3′; antisense5′-(CAGCCACCTTTTCCACCTT)-3′; GAPDH sense 5′-(ATGTCGAAGCGCGACATC)-3′; antisense5′-(TCACTTGTCTCGGCTGAA)-3′; and SRP68 sense 5′-(GGATAGCCTGAGTTTGGAG)-3′; antisense5′-(GCCGTTGATAGCTTGATGTA)-3′.

FACS Analysis

Surface expression of the TNF family receptors DR4, DR5, TNFSFR6, and TNFSFR1 was determined by florescence-activated cell sorting (FACS) using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA). HeLa cells, transfected with indicated siRNAs for 24, 48, and 72 h or cells stably expressing shRNAs, were twice washed with PBS and incubated with 10 μg/ml primary antibody in PBS supplemented with 5% FBS, 1 mM EDTA, 0.05% NaN_3 for 1 h at 4°C. Cells were then washed with PBS only and incubated with secondary goat anti-mouse antibody conjugated with allophycocyanin (AFC; Molecular Probes) for 30 min at 4°C. Cells were stained with propidium iodide (50 μg/ml) and analyzed by flow cytometry using a FACS Calibur flow cytometer. Anti-TNFSFR6 antibody and anti-TNFSFR1 antibody were purchased from Apo-1–3, Alexis Biochemicals (San Diego, CA) and R&D Systems (Minneapolis, MN), respectively.

Subcellular Fractionation, Immunoprecipitation, and Microscopic Visualization of DR4 and DR5 Receptors

For immunoprecipitation of plasma membrane and total DR4 and DR5, 24 h after transfection with the indicated siRNAs, 1×10^7 HeLa cells were washed twice with PBS supplemented with 1% FBS. To immunoprecipitate membrane DR4 or DR5, cells were first resuspended in 1 ml PBS supplemented with 1% FBS and 10 μg/ml mouse anti-human DR4-A or DR5-A mAb for 2 h. Cells were washed with PBS three times to remove excess antibody and lysed in hypotonic lysis buffer. The heavy membrane fraction was isolated by centrifugation at 16,000 $\times$ *g* for 10 min at 4°C and washed with hypotonic lysis buffer twice before suspension in 1 ml of hypotonic lysis buffer containing 1% Triton X-100. Soluble material was saved for incubation with protein G-Sepharose beads. For total protein extracts, 1×10^7 HeLa cells were lysed with 1 ml of hypotonic lysis buffer containing 1% Triton X-100. Cell lysate or membrane lysate was incubated with 30 μl protein G-Sepharose beads from Zymed overnight at 4°C. Beads were washed with 1% Triton X-100 hypotonic lysis buffer and boiled in SDS sample buffer. Proteins were analyzed by Western blotting.

For sucrose gradient fractionation and immunoprecipitation of DR4 and DR5, control or shSRP stable cell lines were cultured in a 10-cm dish were washed with PBS and then collected by centrifugation. The cells were resuspended in 0.85 ml HE buffer (10 mM HEPES-KOH, pH 7.5, and 1 mM EDTA) containing 20 μg/ml α2-macroglobulin, homogenized by 10 times aspiration through a 27-gauge needle, and then mixed with 0.17 ml HE buffer containing 60% sucrose and centrifuged at 600 $\times$ *g* for 10 min to obtain a postnuclear supernatant. The supernatant was layered over a discontinuous gradient of 40 and 60% sucrose in HE buffer (6.6 and 2.2 ml, respectively). All solutions contained a mammalian proteinase inhibitor cocktail (Sigma) according to the manufacturer's instructions. After centrifugation at 100,000 $\times$ *g* for 3 h, 1-ml aliquots were collected from the top of the tube and combined with 1 ml RIPA buffer (300 mM NaCl, 1% NP-40, 1% Triton X-100, 0.5% DOC, 0.1% SDS, and 50 mM Tris-HCl) subject to immunoprecipitation.

To immunoprecipitate DR4 or DR5, fractions were incubated with 10 μg/ml mouse anti-human DR4-A or DR5-A mAb for 2 h. Each fraction was subsequently incubated with 30 μl protein G-Sepharose beads from Zymed overnight at 4°C. Beads were washed with 1% Triton X-100 hypotonic lysis buffer and boiled in SDS sample buffer. Proteins were analyzed by Western blotting using anti-DR4 (Imgenex) or anti-DR5 (Cayman) according to the manufacturer's protocol.

For microscopic examination the indicated stable shRNA clones were first cultured in Falcon CultureSlides (Becton Dickinson Labware, Franklin Lakes, NJ) overnight. Cells were then transfected with 0.5 μg/ml pDR4-GFP or pDR5-GFP with 50 μM in final concentration of Z-VAD. After 24 h of transfection cells were rinsed with PBS and fixed using Cytofix/cytoperm Kit (BD Biosciences PharMingen). Fixed cells were incubated with 2.5 μg/ml anti-GM130 as Golgi marker in PBS buffer supplemented with 5% FBS, 1 mM EDTA, 0.05% NaN_3 for 1 h, washed three times with PBS, and incubated with secondary cy3 AffiniPure donkey anti-mouse from Jackson ImmunoResearch Laboratories for 30 min. After washing three times with PBS, cells were covered with coverslips in Vectashield (Vector Laboratories, Burlingame, CA) and examined under confocal microscope.

Note that immunoprecipitation and GFP-expression constructs were used for these studies because all DR5 antibodies marketed primarily recognize and apparent artifact band on direct Western blots. This apparent artifact migrates at ~60–65 kDa, whereas the predicted mass DR5 and DR5 isoforms range from ~50 to 55 kDa. Although the larger product may represent a modified version of these receptors, it was not depleted by siRNAs specific for DR5, nor can it be immunoprecipitated using our functional DR5-A antibodies, whereas proteins migrating at the expected molecular masses of 50–55 kDa were. Thus, we used functional antibodies to immunoprecipitate DR4 and DR5, followed by commercial antibodies for Western blot analysis application and ectopically expressed GFP fusion constructs for microscopic examination of DR4 and DR5 receptors. Notably these DR4-GFP and DR5-GFP constructs induce apoptosis when ectopically overexpressed and sensitize the respective transfected cells to DR4-A or DR5-A antibodies.

RESULTS

Screening an siRNA Library for Genes that Differentially Influence DR4- vs. DR5-mediated Apoptosis

To identify potential differences in the DR4 and DR5 apoptosis pathways, we developed functional antibodies that specifically activate DR4 (DR4-A) or DR5 (DR5-A), and not other TNF related receptors (Nasoff and Knee, unpublished data). An arrayed library of siRNAs (Aza-Blanc *et al.*, 2003) was transfected into HCT15 colon carcinoma cells and DR4-A or DR5-A antibodies were used to screen for siRNAs that differentially influence DR4- vs. DR5-dependent reduction in cell viability. Of the siRNAs screened (see *Materials and Methods*), ~1% significantly enhanced or inhibited the reduction in cell viability after treatment with DR4-A or DR5-A (Figure 1, A and B). Included in this group of siRNAs that influenced TRAIL sensitivity were several targeted toward genes known to be essential for DR4- and DR5-mediated apoptosis, such as caspase-8 or known to inhibit both pathways, such as CFLAR (c-FLIP), which inhibited or enhanced both pathways, respectively (unpublished data). Although few siRNAs were identified that differentially influenced DR4-A– vs. DR5-A–induced reduction in cell viability (Table 1), we were encouraged that siRNAs targeted toward the DR4 (TNFRSF10A) or DR5 (TNFRSF10B) receptors specifically inhibited DR4-A or DR5-A, respectively (Table 1).

Silencing SRP72 Inhibits DR4-, but not DR5-mediated Apoptosis

Among the siRNAs that differentially affected DR4 vs. DR5, we focused our attention on a siRNA homologous to the SRP 72-kDa subunit SRP72 (siSRP72), which was one of the top-ranked RNA duplexes that specifically suppressed DR4-A–mediated apoptosis in HCT15 cells (Table 1). To confirm these results, we measured the influence of siSRP72 on HCT15 viability over a spectrum of DR4-A or DR5-A concentrations. Consistent with our initial screen data, siSRP72 provided significant protection against cell death over a wide range of DR4-A concentrations (Figure 1C). However, siSRP72 had little effect on DR5-A–induced cell death at any of the concentrations tested (Figure 1D)—supporting the initial observation that SRP72 regulates the DR4, but not the DR5, cell-death pathway. Similar results were obtained in the pancreatic carcinoma cell line T3M4, indicating that these results are not confined to a single cell line (unpublished data).

In an independent screen of this siRNA library, siSRP72 also suppressed TRAIL-induced cell death in HeLa cells (Aza-Blanc *et al.*, 2003). To determine if those results were attributed to the preferential inhibition of the DR4 pathway, we transfected HeLa cells with siSRP72 and then treated

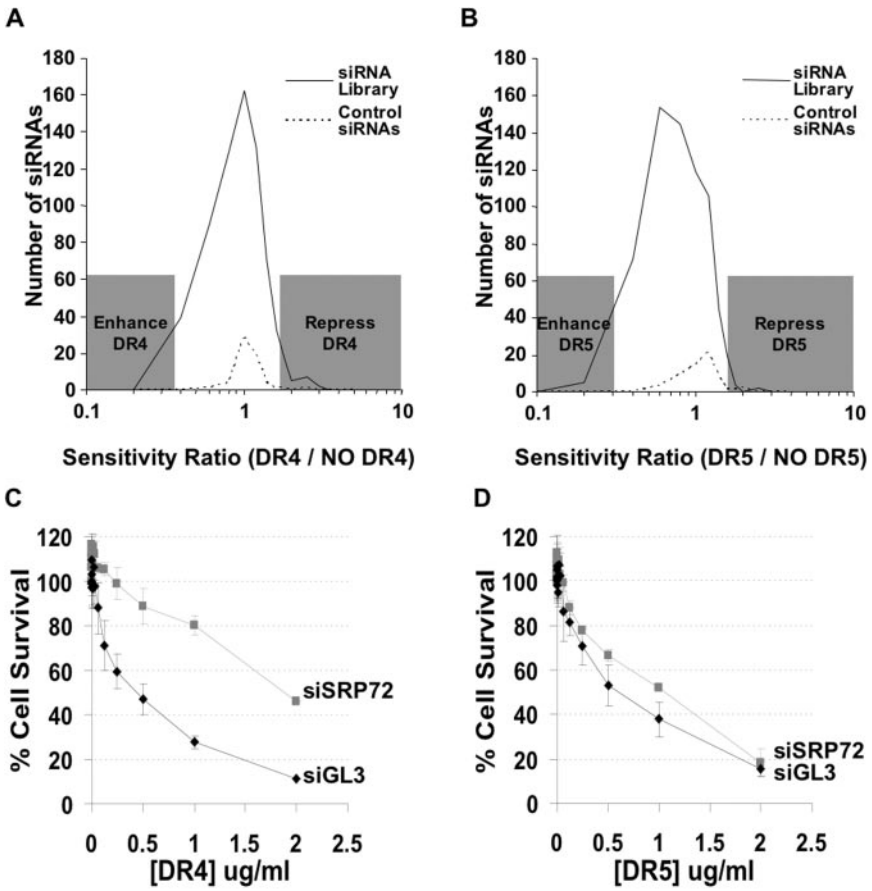

Figure 1. Screening for siRNAs that differentially affect DR4- vs. DR5-mediated reduction in cell viability. (A and B) A histogram was plotted showing the distribution of the sensitivity ratios (see *Materials and Methods*) across the siRNA collection (solid line) and compared with negative control siRNAs targeting luciferase (hatched line). (C and D) Silencing SRP72 suppresses viability reduction of DR4-A– but not DR5-A–treated cells. 48 h after transfection with siSRP72 or siGL3, HCT15 cells were treated with the indicated concentrations of DR4-A (C) or DR5-A (D) for 24 h. Cells were incubated for 48 h to allow target decay and treated with DR4-A, DR5-A, or left untreated. Twenty-four hours later cell viability was measured using CellTitre-Glo (Promega). Experiments were performed in triplicate and represent average and SD from the average.

them with TRAIL, DR4-A, or DR5-A. Figure 2A shows that siSRP72 suppressed TRAIL and DR4-A, but not DR5-A–induced cell rounding and detachment—cellular morphologies indicative of apoptosis. Next, we designed two additional nonoverlapping siRNA duplexes homologous to SRP72 (siSRP72b and SRP72c) and tested their ability to inhibit TRAIL-, DR4-A–, or DR5-A–induced caspase activation—the biochemical hallmark of apoptosis. The original siRNA, siSRP72a, and the newly designed siSRP72b and siSRP72c diminished caspase activation induced by TRAIL and DR4-A treatment (Figure 2B), which correlated with their abilities to inhibit TRAIL or DR4-A reduction of cell viability (unpublished data). Consistent with our previous results, these SRP72 siRNAs did not inhibit DR5-A–induced caspase activation (Figure 2B). Thus, the observed suppression of TRAIL-induced apoptosis, mediated by silencing SRP72, appears to occur through preferential inhibition of the DR4 pathway.

Because of the lack of available antisera for Western blot analysis of SRP72 protein levels, we ectopically expressed recombinant myc-tagged SRP72 in 293 cells and monitored myc-SRP72 protein levels using antibodies specific for myc. Cotransfection of myc-SRP72 with siSRP72a, siSRP72b, or siSRP72c dramatically reduced myc-SRP72 expression compared with cells cotransfected with myc-SRP72 and control siRNAs (Figure 2C). The capacities of these siRNAs to inhibit myc-SRP72 expression correlated with their abilities to suppress DR4-A–induced caspase activation (Figure 2B) and to diminish endogenous SRP72 mRNA as measured by Taqman PCR and semiquantitative PCR methods (unpublished data). Together, these data indicate that suppression of apoptosis induced by TRAIL and DR4-A antibody is attributed to SRP72 silencing and not "off-target" effects.

The SRP Complex Is Necessary for DR4-mediated Apoptosis

SRP72 is a subunit of a ribonucleoprotein complex that facilitates targeting of nascent secretory and membrane proteins to the endoplasmic reticulum (ER), thus initiating the protein sorting process (Keenan *et al.*, 2001; Pool *et al.*, 2002; Koch *et al.*, 2003; Nagai *et al.*, 2003). Because silencing of SRP72, one of six protein components of the SRP, resulted in the selective attenuation of DR4-mediated apoptosis in HCT15 and HeLa cells, we asked whether this effect was caused by disruption of SRP function or attributed to an additional, as yet unknown role of SRP72. To address this question we used siRNAs homologous to the SRP core subunit SRP54, which is the most conserved component in the SRP complex and essential for SRP function (Bernstein *et al.*, 1989; Romisch *et al.*, 1990). Several of the designed siRNAs diminished endogenous SRP54 protein levels by more than 80% in transiently transfected cells (Figure 3A). We used these siRNAs to show that SRP54 silencing effectively reduced TRAIL and DR4-A–, but not DR5-A–induced caspase activation (Figure 3B) and reduction in cell viability (unpublished data), similar to siRNAs specific for SRP72. On the basis of these data, we conclude that the SRP complex, rather than SRP72 itself, is essential to the DR4 apoptosis pathway.

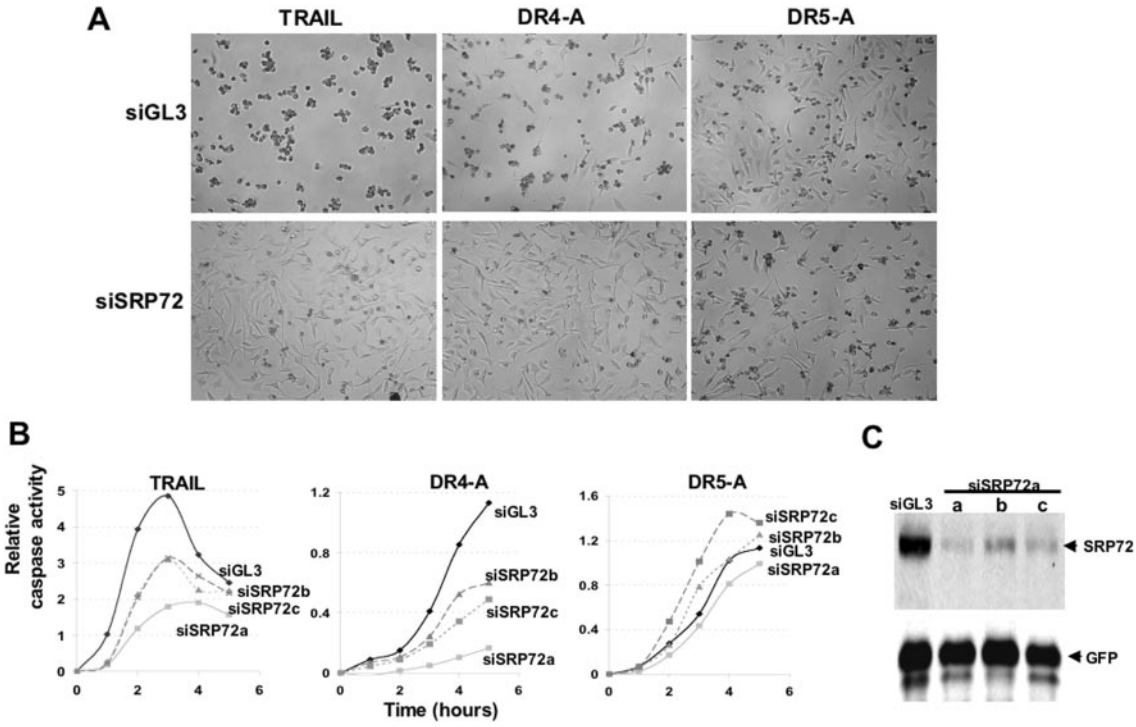

Figure 2. TRAIL and DR4-A, but not DR5-A–induced apoptosis is suppressed by siRNAs that silence siSRP72 expression. (A) Forty-eight hours after transfection with siGL3 (control) or siSRP72, HeLa cells were treated with 100 ng/ml TRAIL, 1 μg/ml DR4-A, or 1 μg/ml DR5-A, respectively, for 18 h and viewed by phase microscopy for rounded and detached cells or (B) caspase activity was measured in cell lysates prepared at 0, 2, 4, and 6 h. (C) Western blot analysis of ectopically coexpressed Myc-SRP72 with control siRNA, siSRP72a, b and c, respectively, in 293 HEK cells. GFP was coexpressed as a transfection control. Data are representative of multiple experiments.

Requirement for SRP Function before Caspase-8 Processing

After ligand or agonistic antibody binding to DR4 and DR5, the apical caspase-8 zymogen is recruited to the receptor complex and activated, resulting in its autocatalytic processing (Boatright *et al.*, 2003; LeBlanc and Ashkenazi, 2003). Active caspase-8 initiates the proteolytic activation of executioner caspases and in parallel cleaves the proapoptotic BCL-2 family member Bid. The newly generated truncated Bid (tBid) promotes the release of mitochondrial factors that initiate caspase-9 activation and amplify the proteolytic maturation of the downstream executioner caspases, such as caspase-3 (Denault and Salvesen, 2002; Shi, 2002). We monitored the cleavage of these apoptosis molecules by Western blot analysis to determine where SRP silencing affects the TRAIL pathway. Both siSRP72 and siSRP54 blocked TRAIL (Figure 3C) and DR4-A (Figure 3D) induced apoptosis at the apical point in this proteolytic cascade, before procaspase-8 processing, whereas procaspase-8, Bid, procaspase-9, and procaspase-3 cleavage were unaffected by control siRNAs (Figures 3, C–E). In accordance with our previous results, siSRP72 or siSRP54 did not impact the DR5-A–induced proteolytic cascade (Figure 3E).

The SRP Complex Is Necessary for DR4 Cell Surface Localization

Because the SRP complex can facilitate protein trafficking to the plasma membrane and silencing the expression of SRP subunits blocks the DR4 pathway before caspase-8 activation, we monitored DR4 and DR5 cell surface receptor levels after treatment with siSRP72 (Figure 4). DR4 and DR5 were readily detected on the surface of HeLa cells by flow cytometry using DR4-A or DR5-A antibodies. siSRP72 transfection resulted in dramatic reduction of cell surface–localized DR4 in a time-dependent manner (Figure 4, A and C). Analogous results were obtained by silencing SRP54 expression (unpublished data). Conversely, siSRP72 did not influence cell surface DR5 (Figure 4, B and D). Notably, silencing SRP function potently suppressed TRAIL-induced apoptosis, albeit not completely, without influencing DR5 cell surface receptors.

Next we questioned whether SRP function regulates other TNF receptor family members. Comparable to DR5, SRP function does not appear to be necessary for cell surface localization of other TNF receptors because silencing SRP72 did not influence TNF-α or Fas receptor (TNFRSF1 and TNFRSF6, respectively) levels (Figure 4, E and F) or correspondingly, cell-death mediated by engagement of these TNF receptor family members (unpublished data). Based on our combined results, expression of physiological concentrations of the SRP complex is necessary for cell surface localization and function of DR4, but not DR5, TNF-α, or Fas receptors.

siRNAs targeting DR4 (siDR4) reduced cell surface expression of DR4 but not DR5, and siDR5 suppressed DR5 but not DR4 cell surface levels (Figure 4, G and H), underscoring the specificity of the reagents used in these studies. Moreover, these data indicate that the observed differential regulation of TRAIL receptors by the SRP complex is not attributed to differences in receptor stability, because siDR5 readily down-regulated cell surface DR5 within 48 h, whereas siSRP72 or siSRP54 failed reduce DR5 receptor

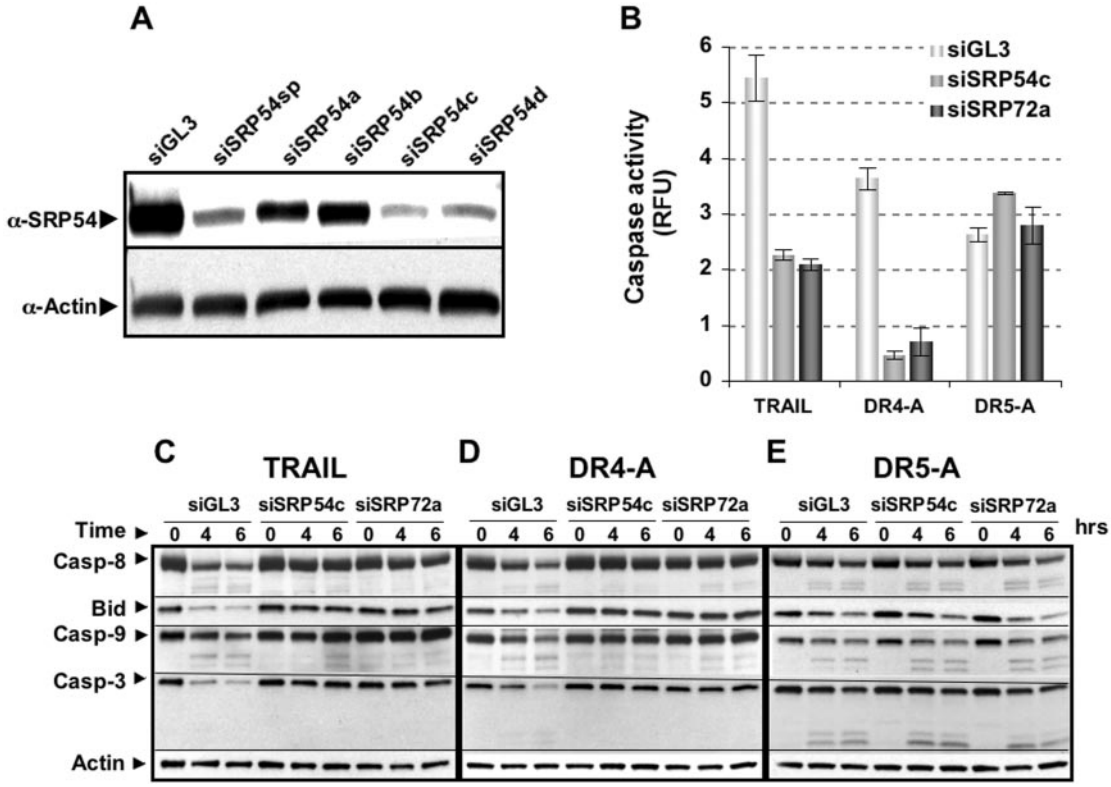

Figure 3. Silencing SRP72 or SRP54 suppresses TRAIL and DR4-A–, but not DR5-A–induced apoptosis before caspase-8 activation. (A) Development of siRNAs targeting SRP54. Forty-eight hours after transfection with siGL3, a pool of 4 siRNA oligo duplexes specific for SRP54 (siSRP54sp) or each individual siRNA in the pool (siSRP54a-d), corresponding cell lysates were analyzed on Western blots using antibodies specific for SRP54. Actin antibodies were used to visualize protein loading. (B) Forty-eight hours after transfection with siGL3, siSRP72a, or siSRP54c, TRAIL (100 ng/ml), DR4-A (1 μg/ml), or DR5-A (1 μg/ml) was added and caspase activities were measured after 4 h using DEVD-AFC or analyzed on Western blots (C–E) using antibodies specific for caspase-8, bid, caspase-9, caspase-3, or, as a loading control, actin. Experiments were performed in triplicate.

levels up to 72 h after transfection with siSRP72 or siSRP54 (Figure 4, C and D, and unpublished data).

Using alternative methods to observe DR4 and DR5 protein levels and their cell surface localization, we immunoprecipitated these receptors from total protein extracts or from the cell surface after transfection with siSRP72 or control siRNA (Figure 5). In agreement with our FACS data, cell surface DR4 was down-regulated in siSRP72-transfected cells, whereas DR5 cell surface receptor levels were unchanged. Total DR4 or DR5 protein was found in the membrane fraction and concentrations remained unchanged regardless of siSRP72 transfection. Collectively, these data indicate that SRP function is essential for DR4 cell surface receptor localization but not its expression or association with the membrane fraction.

Stable Depletion of SRP72 or SRP54 Severely Inhibits DR4 Cell Surface Localization, but Not Its Expression

To address the long-term effects of SRP depletion on DR4- and DR5-mediated apoptosis, we created HeLa cell lines that stably express short hairpin RNAs (shRNA) (Paddison *et al.*, 2002) specific for SRP54 (shSRP54) or SRP72 (shSRP72). Correspondingly, HeLa cell subclones with severely reduced mRNA expression of SRP54 or SRP72 were selected from shSRP54 or shSRP72 expressing clones (Figure 6A). In these SRP depleted subclones, mRNA levels of DR4 and DR5 were not significantly altered (Figure 6A). However, expression of cell surface DR4, but not DR5, was once again dramatically reduced in cells lacking expression of SRP54 or SRP72 (Figure 6B). The reduction of DR4 from the cell surface of SRP-depleted clones correlated with their diminished sensitivity to DR4-induced apoptosis, whereas these same cell lines remained sensitive to DR5 agonistic antibodies (Figure 6C). Thus, with either acutely or chronically diminished expression of the SRP complex, DR4 cell surface localization and DR4-mediated apoptosis was blocked. Notably, these SRP-depleted subclones exhibit similar morphology and growth rates as control HeLa cells (unpublished data).

The SRP complex directs cotranslational import into the ER. Proteins destined for the plasma membrane are then trafficked from the ER and through the Golgi before insertion into the plasma membrane. Because DR4 mRNA levels were not significantly influenced by depleting SRP subunits, but DR4 cell surface receptor levels were (Figure 6, A and B), we used protein localization methods to address the fate of DR4 receptors after SRP depletion. Extracts made from control or stable cell lines with depleted SRP54 were fractionated using discontinuous sucrose gradients followed by immunoprecipitation of endogenous DR4 or DR5 from each fraction. Control cells exhibited DR4 and DR5 distribution across the gradient with slight accumulation in both the Golgi- and ER-containing fractions (Figure 7A). In SRP depleted cells, DR4 is comparatively reduced in fractions containing resident ER proteins and in the bottom of the gradient where plasma membrane DR4 would be expected to sediment, but remains similar or slightly enhanced in Golgi

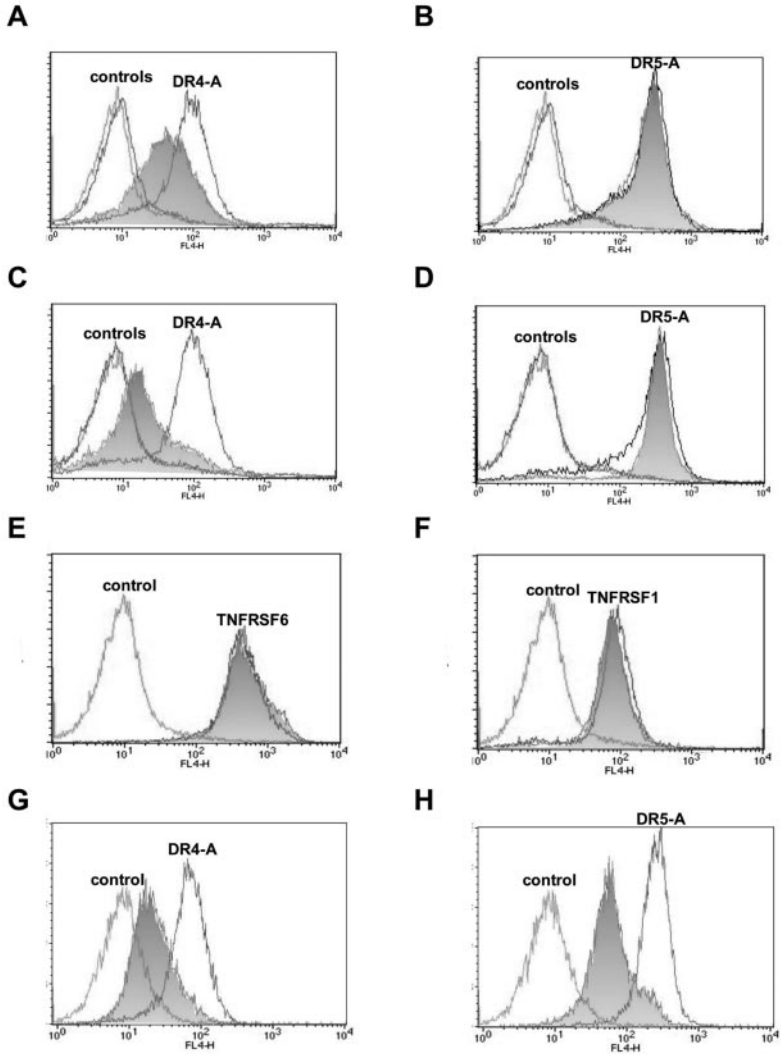

Figure 4. DR4 cell surface expression is preferentially suppressed by silencing expression of SRP subunits. HeLa cell surface DR4, DR5, Fas (TNFRSF6), or TNF-α (TNFRSF1) receptor levels were determined by flow cytometry using no primary antibody or control IgG (controls) or DR4, DR5 TNFRSF6, or TNFRSF1 primary antibodies followed by allophcocyanin-conjugated secondary antibody in each case. (A) DR4 or (B) DR5 cell surface expression 48 (A and B) or 72 h (C and D) after transfection with control siRNA or siSRP72 (shaded plots). (E) TNFRSF6 or (F) TNFRSF1 cell surface receptor levels 48 h after transfection with control siRNA or siSRP72 (shaded plots). (G) DR4 or (H) DR5 levels 48 h after transfection with control siRNA or siDR4 (shaded plot in G) or siDR5 (shaded plot in H). Note that siDR4 did not influence cell surface DR5 and siDR5 did not influence DR4 cell surface levels (unpublished data). Data represent an average of three independent experiments. Similar results were obtained using siSRP54 (unpublished data). An increase in receptor level in indicated by an increase in fluorescence intensity (right shift) and a decrease in detectable receptors is indicated by a decrease in fluorescence intensity (left shift) on a logarithmic scale.

containing samples (Figure 7A). Notably, all detectable DR4 protein in control and SRP-depleted cells resides in membrane containing fractions. Thus, SRP depletion appears to primarily influence DR4 plasma membrane localization, although apparent ER concentrations of DR4 are also diminished.

Control cells and stable shSRP54 or shSRP72 that were transiently tranfected with DR4-GFP or DR5-GFP expression constructs did not reveal significant differences by gross miscroscopic examination over a 72-h period (Figure 7B and unpublished data). On close inspection loss of DR4-GFP localization to the plasma membrane, with apparent accumulation in the Golgi, can be observed in SRP-depleted cells relative to control cells (Figure 7B). In addition, we consistently noted that total DR4-GFP fluorescence is also decreased in cells with stably diminished SRP. Although we did not find significant differences in total DR4 protein levels in cells transiently transfected with siSRP54 or siSRP72 (Figure 5), all of our clones stably expressing shSRP54 or shSRP72 contain less ectopically expressed or endogenous total DR4 protein, ranging from 50 to 80% of that found in control cells (Figure 7 and unpublished data). Because DR4 mRNA levels are unaffected by SRP depletion (Figure 6A), we suspect the decreased abundance of total DR4 protein in

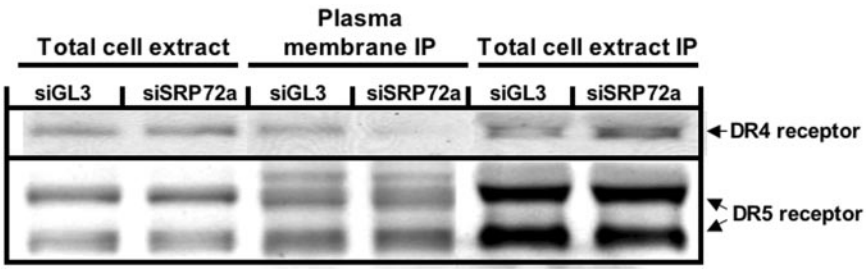

Figure 5. Silencing components of the SRP complex inhibits DR4 cell surface localization but not its expression. After transfection with siGL3 (control) or siSRP72, DR4 and DR5 were visualized by Western blot analysis in total protein extracts or after immunoprecipitation (IP) from the cell surface (Plasma membrane IP) or after IP from total cell extracts (see *Materials and Methods*). Notably, both DR4 and DR5 receptors are found in the membrane or nonionic detergent soluble fraction of cell lysates regardless of SRP depletion.

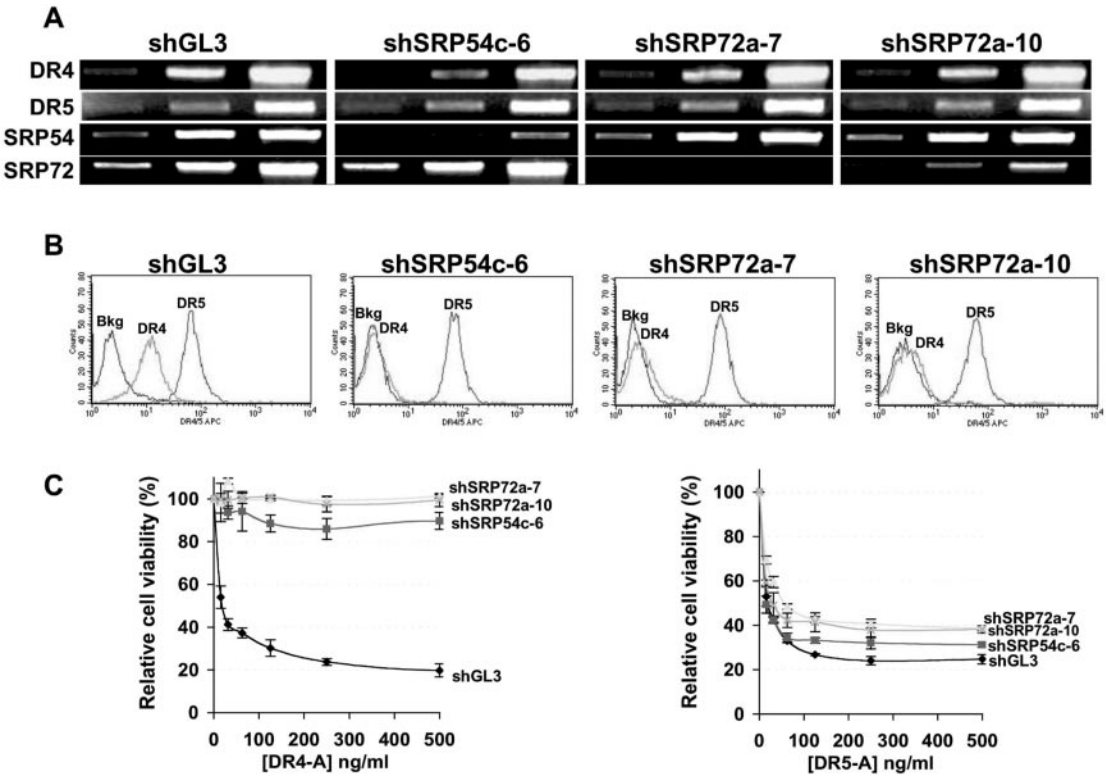

Figure 6. DR4 cell surface localization and function are suppressed in cell lines with stably silenced SRP54 or SRP72 (A) HeLa cells stably expressing short hairpin RNA (shRNA) specific for SRP54 (shSRP54c6), SRP72 (shSRP72a-7 and shSRP72a-10), or control shRNA vectors (shGL3) were analyzed by semiquantitative PCR for DR4, DR5, SRP54, or SRP72 mRNA levels. Samples pictured were removed and resolved on agarose gels after 27, 29, and 31 cycles. Note that although we were unable to obtain antibodies to SRP72, antibodies to SRP54 revealed a corresponding decrease in SRP54 protein (unpublished data). (B) Cell surface exposure of DR4 and DR5 was assessed by FACS analysis in cell lines corresponding those pictured in A. (C) Sensitivity of the SRP-depleted cell lines to DR4 or DR5 agonistic antibodies compared with control cells was measured using viability assays. Experiments were performed in triplicate and are represented as average and SD from the average.

stable SRP-depleted cells is due to increased degradation of the DR4 receptor. However, we have not ruled out the possibility that translational defects contribute to lower levels of both ectopically expressed or endogenous DR4 in cells with stably diminished SRP. Total DR5 protein levels remained unchanged in SRP depleted clones relative to control cells.

DISCUSSION

TNF family biology and their association with disease have been intensely studied because the discovery of TNF in 1975 (Carswell *et al.,* 1975; Orlinick and Chao, 1998). However, there is surprisingly little known about the role of protein trafficking in the regulation of TNF-related receptors and apoptosis. The advent of RNAi technologies allowed us to conduct a relatively large-scale transient gene-silencing experiment in mammalian tumor cell lines to probe the DR4 and DR5 apoptosis pathways, thus uncovering the necessity of the SRP complex in the DR4 death-receptor pathway. Silencing SRP72 or SRP54 revealed unexpected specificity for the regulation of DR4 over DR5 and other TNF receptors we observed. The molecular basis of this specificity may reside in the ~ 60 amino acid N-terminus of DR4, which is unique among TNF family members. However, initial studies deleting this 60 amino acid region did not influence cell-surface expression or SRP dependence of this DR4 deletion product (unpublished data). Thus, further structure-function studies will be necessary to delineate the precise sequences necessary for DR4 cell surface localization. In this regard, how DR5 and other TNF family receptors find their way to the plasma membrane remains an intriguing, yet unexplored question.

Based on FACS, immunoprecipitation, and visualization of ectopically expressed GFP-tagged versions of DR4 and DR5, cell surface localization of these receptors appears to represent only a fraction of their total cellular concentration. Although qualitative in nature, these data may suggest that sorting to the plasma membrane represents a rate-limiting step in the TRAIL pathway. In this regard, recent studies of TRAIL resistant colon carcinoma cell lines revealed that these cells down-regulated DR4 from the cell surface although total DR4 mRNA, and protein levels remained similar to their TRAIL sensitive counterparts (Jin *et al.,* 2004). These authors suggested that defects in the regulation of death receptor trafficking might play an important role in tumor cell sensitivity to TRAIL. In a separate study, normal prostate cells were reported to express similar levels of DR4 as malignant prostate cancer cells; however, only the malignant cells exhibited DR4 cell surface expression (Voelkel-Johnson, 2003). Correspondingly, malignant prostate cancer cells were sensitized to DR4 agonistic antibodies, whereas the normal prostate cells were not. These data indicate that regulation of DR4 cell surface sorting may occur during the

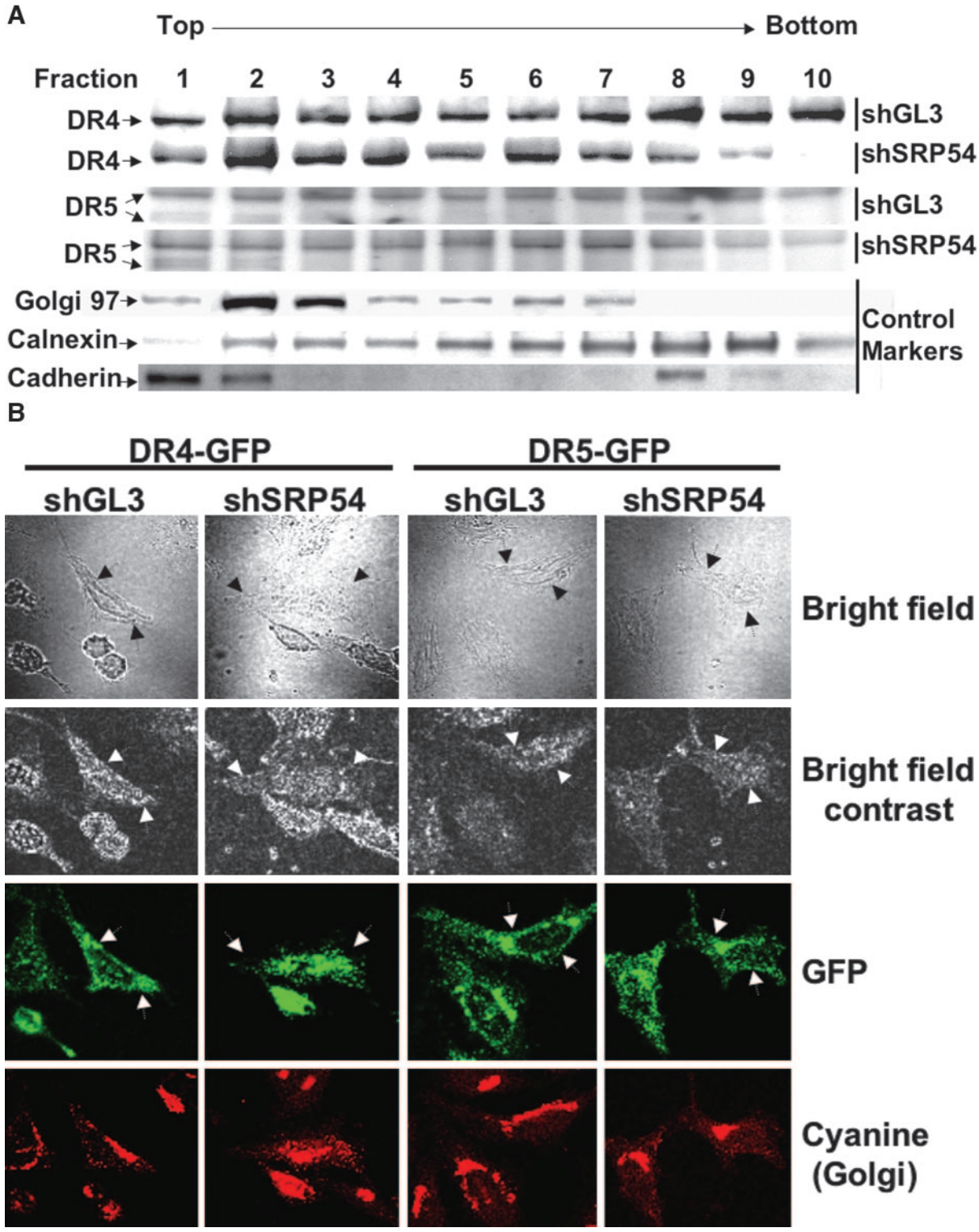

Figure 7. DR4 and DR5 localization in stable SRP depleted cells. (A) Sucrose gradient fractionation. Cell lysates made from HeLa cells stably expressing shRNAs specific for SRP54 (clone 6) or control shRNA vectors were fractionated on discontinuous sucrose gradients. DR4 and DR5 receptors were immunoprecipitated from each fraction and analyzed on Western blots. Antibodies specific for Golgi 97, calnexin, and cadherin were used to identify Golgi-, ER-, and plasma membrane–containing fractions. Note that cadherin resides in the ER and plasma membrane fractions (bottom of the gradient) and the light vesicle fraction (top of the gradient). (B) cDNA constructs containing DR4-GFP or DR5-GFP were transiently transfected into stable shGL3 or shSRP54 expressing HeLa cell lines. Cell were examined by confocal microscopy over a 72-h period (36-h time point shown) and pictured by bright field, bright field high contrast (cell outline), and GFP fluorescence (DR4 and DR5 receptor localization [green]). Arrows denote the plasma membrane. Cyanine (Red) depicts the Golgi as assessed by antibodies specific for the Golgi marker GM130. Pictures are representative of the indicated populations and multiple experiments in both SRP72 and SRP54 depleted stable cell lines.

transformation process, facilitating the observed tumor specificity of DR4-induced apoptosis. Regardless, DR4 protein trafficking appears to be a significant, yet relatively unexplored mechanism for regulating the TRAIL apoptosis pathway.

The SRP complex is the molecular machinery of the "signal hypothesis" for protein sorting through the secretory pathway (Anderson and Walter, 1999; Keenan *et al.*, 2001). This complex has been conserved throughout evolution and its function is essential for viability in bacteria (Phillips and Silhavy, 1992) and for normal growth in yeast (Hann and Walter, 1991) and protozoan parasites (Liu *et al.*, 2002). Given that the SRP complex is thought to play a much larger role in protein targeting in mammalian cells (Bernstein and Hyndman, 2000), it is surprising that depleting SRP72 or SRP54 in several tumor cell lines did not produce overt cellular phenotypes, such as growth arrest or death—even in cells selected for stable reduction of SRP components. However, we cannot rule out the possibility of incomplete silencing of SRP function as an explanation for these results and the observed differential regulation of TRAIL receptors by SRP depletion. In this regard, DR4 may bind less efficiently to the SRP complex than DR5 and the other receptors we tested. Thus, when SRP becomes rate limiting, sequences with more optimal SRP-binding properties may simply out-compete those with weaker affinities and are therefore preferentially sorted. An alternative explanation may be an underappreciated role of SRP-independent protein-trafficking mechanisms in mammalian cell biology.

Inhibition of the SRP pathway in the yeast *Saccharomyces cerevisiae* results in sick cells with severely compromised growth rates; nevertheless, these cells do not display dramatic translocation defects in SRP-dependent protein translocation (Ogg *et al.*, 1992). Walter and coworkers provided a molecular explanation for this "adaptation response," reporting that heat shock protein induction protects cells from mislocalized precursor proteins in the cytosol (Mutka and Walter, 2001). A subsequent reduction in protein synthesis appears to aid protein sorting by reducing the load on the protein translocation apparatus. On the basis of those studies, the authors proposed that "cells trade speed in cell growth for fidelity in protein sorting to adjust to life without SRP" (Mutka and Walter, 2001). In the context of heat-shock–mediated compensation or protection after loss of SRP function, it is interesting to compare the similarities in protein recognition and insertion into barrel-like chaparonin or proteasome complexes in the cytosol and SRP-mediated "feeding" of a linear polypeptide chain into the ER for proper folding and translocation.

In trypanosomes where SRP54 was depleted using RNAi—resulting in severe growth defects, all the signal peptide containing proteins examined in this study were nevertheless translocated to the ER, leading the authors to propose that an alternative protein translocation pathways exists in Trypanosomes (Liu *et al.*, 2002). Several of the investigated proteins were subsequently mislocalized to post-ER membranous structures, which was suggested to arise from defects secondary to SRP depletion, such as defects in the translocation of ER or Golgi membrane proteins, thus indirectly influencing intracellular trafficking (Liu *et al.*, 2002). Likewise, after loss of DR4 from the cell surface in SRP-depleted human cancer cells; we observed that DR4 appears to partition to Golgi containing sucrose gradient fractions, whereas DR5 localization and function was unaffected. Thus, we also cannot exclude the possibility that the relationship between SRP function and DR4 cell surface localization is indirect. Clearly, further studies will be necessary to determine whether SRP depletion directly or indirectly influences DR4 localization. If DR4 is indeed translocated to the Golgi and the failure to properly traffic to the plasma membrane is a secondary effect of SRP depletion, it will be important to determine how DR4 and other proteins are translocated to the Golgi and whether these events are dependent on chaperones or the ER. Intriguingly, the trafficking of DR5, and possibly other TNF-receptors, appears to occur by distinct mechanisms.

The function of the SRP complex in protein translocation has been well documented, and recent studies have begun to report the molecular details of the SRP structure-function relationship (Koch *et al.*, 2003; Nagai *et al.*, 2003; Schwartz and Blobel, 2003; Egea *et al.*, 2004; Focia *et al.*, 2004). However, the role of SRP complex in cellular events such as growth, differentiation and cell-death remain largely unexplored in mammalian cells. Surprisingly, at least some human cancer-derived cell lines, which exploit cell growth as an essential phenotype, appear not to sacrifice growth rates in adaptation to SRP depletion, exhibiting both clonal expansion and growth rates similar to that of control cells. The lack of lethality or severe growth defects after SRP depletion allowed us to uncover an essential role of the SRP complex in localization of DR4 to the cell surface expression, revealing a mechanistic relationship between protein sorting machinery and an apoptosis pathway. These studies highlight the potential importance of protein trafficking in the regulation of DR4-mediated apoptosis and should facilitate the investigation of SRP function in other aspects of mammalian cell biology. The unexpected specificity for the necessity of the SRP complex in the DR4, but not DR5 apoptosis pathway, reveals an intriguing and yet relatively unexplored aspect of the TRAIL death-receptor pathway.

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