Endogenous Substrates of Sphingosine-Dependent Kinases (SDKs) Are Chaperone Proteins: Heat Shock Proteins, Glucose-Regulated Proteins, Protein Disulfide Isomerase, and Calreticulin†

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ABSTRACT: Protein kinases whose activity is detectable only in the presence of sphingosine (Sph) or *N*,*N*′-dimethyl-Sph (DMS), but not in the presence of 15 other sphingolipids, phospholipids, and glycerolipids tested (Megidish, T., et al. (1995) *Biochem. Biophys. Res. Commun. 216*, 739−747), have been termed "sphingosine-dependent kinases" (SDKs). We showed previously that a purified SDK (termed "SDK1") phosphorylates a specific Ser position of adapter/chaperone protein 14-3-3 isoforms β, η, and ζ but not τ or σ (Megidish, T., et al. (1998) *J. Biol. Chem. 273*, 21834−45). In this study we found the following: (i) other SDKs with different substrate specificities are present in cytosolic and membrane extracts of mouse Balb/c 3T3 (A31) fibroblasts. (ii) The activation of these SDKs is specific to D-*erythro*-Sph and its *N*-methyl derivatives, the effect of L-*threo*-Sph or its *N*-methyl derivatives is minimal, and nonspecific cationic amphiphiles have no effect at all. An SDK separated as fractions "TN31−33" phosphorylated a 50 kDa substrate which was identified as calreticulin, as well as two endogenous substrates with molecular mass 58 and 55 kDa, both identified as protein disulfide isomerase (PDI). This SDK, which specifically phosphorylates calreticulin and PDI, both molecular chaperones found at high levels in endoplasmic reticulum, is tentatively termed "SDK2". Another SDK activity was copurified with glucose-regulated protein (GRP) and heat shock proteins (HSP). One GRP substrate had the same amino acid sequence as GRP94 (synonym: endoplasmin); another HSP substrate had the same amino acid sequence as mouse HSP86 or HSP84, the analogues of human HSP90. An SDK activity separated and present in "fraction 42" from Q-Sepharose chromatography specifically phosphorylated GRP105 (or GRP94) and HSP68 but did not phosphorylate PDI or 14-3-3. This SDK is clearly different from other SDKs in its substrate specificity and is tentatively termed "SDK3". Interestingly, substrates of all these SDKs so far identified are molecular chaperones or adapters capable of binding to enzymes and key molecules involved in signal transduction, maintaining tertiary structure of bioactive molecules, or maintaining cellular homeostasis in response to environmental stress. Thus, the essential role of Sph and DMS is to activate molecular chaperones, thereby providing a link to the mechanism by which SDK activity regulates cellular homeostasis and signal transduction.

Sphingolipids are structural components of cell membranes that can be metabolized to generate catabolites such as ceramide (Cer),[1] sphingosine (Sph), and sphingosine-1-phosphate (Sph-1-P). These catabolites are modulators of transducer molecules involved in signal transduction and play essential roles in regulation of mitogenesis, differentiation, cell death, cell cycle arrest, and senescence (*1*−*7*).

Sph, the backbone component of all sphingolipids, was originally discovered as an inhibitor of protein kinase C (PKC), in contrast to the stimulatory effect of diacylglycerol

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[1] Abbreviations: Cer, ceramide; CKII, casein kinase II; DMS, *N*,*N*-dimethylsphingosine; ER, endoplasmic reticulum; GRP, glucose-regulated protein; HSP, heat shock protein; LPA, lysophosphatidic acid; PDVF, poly(vinylidene difluoride); PKC, protein kinase C; QS, Q-Sepharose; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SDK, sphingosine-dependent protein kinase; Sph, sphingosine; Sph-1-P, sphingosine-1-phosphate; TMS, *N*,*N*,*N*-trimethylsphingosine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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(*8*). The inhibitory effect of D-*erythro*-Sph and its methylated form *N*,*N*′-dimethyl-Sph (DMS) on PKC was stronger than that of other Sph stereoisomers and Sph derivatives (*9*). DMS is generated from Sph by an enzyme found in mouse brain (*10*), and mass spectrometric analysis indicated that DMS is a minor component relative to Sph in HL60 cell extract (*11*). These findings suggested that both Sph and DMS are physiologically significant. Many subsequent studies demonstrated that Sph and DMS modulate activity of various protein kinases, e.g. calmodulin-dependent kinase (*12*), insulin receptor tyrosine kinase (*13*), casein kinase II (CKII) (*14*), epidermal growth factor receptor kinase (*15*), and Src kinase (*16*).

Sph has been implicated as a second messenger analogous to Cer and Sph-1-P, since its low level is increased upon stimulation of cells. Agonists such as platelet-derived growth factor (PDGF) and other growth factors cause activation of ceramidase, leading to increase of Sph level (*2*, *17*). TNFα treatment of human neutrophils (*18*) or phorbol ester treatment of HL60 cells (*19*) causes a 3- to 4-fold increase in Sph levels, leading to apoptosis. Sph and DMS induce protein phosphorylation in intact cells.

Addition of Sph to Jurkat cell extracts induces prominent phosphorylation of multiple endogenous proteins (18−165 kDa), suggesting the existence of multiple substrates of protein kinases activated by Sph (*20*). We demonstrated previously a group of protein kinases specifically activated by Sph and DMS but not Cer, Sph-1-P, or various other sphingolipids, phospholipids, and glycerolipids, termed "sphingosine-dependent protein kinases" (SDKs). The adapter/chaperone protein 14-3-3 was identified as a substrate of these kinases (*21*). Recently, we characterized one member of the SDKs, termed SDK1, a novel serine kinase that specifically phosphorylates only certain 14-3-3 isoforms (*β*, *ζ*, *η*), and identified the phosphorylation site on 14-3-3 protein (*22*).

Here we report isolation, purification, and identification of several cellular proteins that are phosphorylated in vitro by SDKs from extracts of Balb/c 3T3 (A31) fibroblasts. Interestingly, these substrate proteins were identified as calreticulin, protein disulfide isomerase, and members of the heat shock protein family, all of which are chaperone proteins. Thus, Sph and DMS may have specific roles in maintaining homeostasis of cellular functions through modulation of these molecular chaperones.

EXPERIMENTAL PROCEDURES

Materials and Reagents. D-*erythro*-Sph (*23*, *24*), *N*,*N*-dimethyl-D-*erythro*-Sph (*9*), and Sph-1-P (*25*) were synthesized chemically as described. L-*threo*-Sph, C2-Cer, C6-Cer, and C18-Cer were from Matreya Inc. (Pleasant Gap, PA). *N*,*N*-Dimethyl-D-*threo*-Sph was prepared from L-*threo*-Sph with formaldehyde and sodium borohydride (*9*). Stearylamine, hexadecyltrimethylammonium bromide, lysophosphatidic acid, and various other lipids used to test SDK activation and for lipid specificity were from Sigma Chemical Co. (St. Louis, MO). Protein disulfide isomerase, TPA, and DTT were from Calbiochem (San Diego, CA). Columns with Q-Sepharose Fast Flow HR 10/10, Mono-S (HR 5/5), Mono-Q (HR 5/5), and Phenyl-Sepharose HR 5/5 were from Pharmacia LKB (Pleasant Hill, CA). Leupeptin, aprotinin, pepstatin, trypsin inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), *n*-octyl-*β*-glucoside, Triton X-100, and NP-40 were from Boehringer Mannheim Biochemicals (Indianapolis, IN). (γ-^{32}P)ATP (specific activity 3000 mCi/mmol) was from Dupont-NEN (Boston, MA). Prestained standards were from Gibco-BRL (Grand Island, NY). Reagents for SDS−PAGE and its silver staining and poly(vinylidene difluoride) (PDVF) membrane were from Bio-Rad (Richmond, CA). Dulbecco modified Eagle's medium (DMEM) was from Irvine Scientific (Santa Ana, CA). FBS was from HyClone (Logan, UT). T-Mat film was from Kodak (Tukwila, WA).

Cell Culture and Stimulation with TPA. Confluent fibroblasts treated with TPA under serum-free conditions were used for purification of SDKs and their substrates, since intracellular Sph level was increased while PKC activity was reduced under these growth conditions (*26*, *27*). Mouse Balb/c 3T3 (A31) cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM supplemented with 10% FBS, in a humidified atmosphere containing 10% CO_2 and 90% air, at 37 °C. The number of passages was limited to five. Cells (5×10^5) were plated on 150 mm dishes, cultured for 6−8 days until confluence, washed 3× with serum-free DMEM, and incubated in the same medium for 3 h and then in DMEM containing 200 nM TPA in 0.01% DMSO for 3−4 h. Next, cells were washed with ice-cold PBS without Ca^{2+} and Mg^{2+}, harvested, and kept frozen at −80 °C until utilized for further purification of SDKs and their substrates.

Preparation of Cell Extracts from Balb/c 3T3 (A31) Fibroblasts. Step 1: Preparation. Aliquots (7 mL, 400 mg) of frozen packed cells were thawed in ice water and suspended in homogenizing buffer consisting of 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 2 mM Na_3VO_4, 50 mM NaF, 10 mM *β*-mercaptoethanol, 25 *μ*g/mL leupeptin, 25 *μ*g/mL aprotinin, 25 *μ*g/mL pepstatin, 1 mg/mL AEBSF, and 5% glycerol. Cells were placed in a tight-fitting Dounce homogenizer and given 25 strokes. The homogenate was centrifuged at 500*g* for 10 min to remove cell debris. The microsomal fraction was obtained by centrifugation at 100 000*g* for 60 min. The microsomal pellet was resuspended in lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 100 mM NaCl, and 50 mM NaF) plus 30 *μ*g/mL leupeptin, 30 *μ*g/mL aprotinin, 30 *μ*g/mL trypsin inhibitor, 30 *μ*g/mL pepstatin, 50 *μ*g/mL AEBSF, and 0.5% Triton X-100 and combined with the cell debris pellet. These combined pellets were used for partial purification of membrane SDKs and 50 kDa substrate. The supernatant (cytosol fraction) and particulate fractions were subjected to further purification of SDKs and their substrate proteins through ion-exchange and hydrophobic interaction.

Purification of the Endogenous Substrate of SDK (Steps II−IV). All procedures were carried out at 0−4 °C unless otherwise indicated.

Step II: Q-Sepharose Column Chromatography. This purification step was performed immediately after cell extraction. pH of the cytosolic extract was corrected to 8.5. This extract was applied at flow rate of 1 mL/min to Q-Sepharose (QS) ion-exchange column (2 × 10 cm) equilibrated with 20 mM Tris buffer, pH 8.5, 1 mM EDTA, and 10 mM *β*-mercaptoethanol (buffer A) containing 10% glycerol, 10 mM NaF, 0.5 mg/mL AEBSF, and 0.2% octyl-

β-glucoside. The column was washed with 5 volumes of the same buffer and eluted with a gradient of NaCl (0−1.0 M). Fractions were subjected to SDK assay. Phosphorylation of proteins in a DMS-dependent manner was used as a guide for substrate protein purification. Fractions containing phosphorylated proteins with the same size, as determined by their migration on reduced SDS−PAGE, were pooled and dialyzed overnight against buffer A.

Step III: Phenyl-Sepharose Column Chromatography. The NaCl concentration in the pooled fractions was brought to 1.5 M. The sample was applied to a Phenyl-Sepharose column equilibrated with buffer A containing 1.5 M NaCl at a flow rate of 1 mL/ min, utilizing the SMART system (Pharmacia). The column was washed with the same buffer, proteins were eluted at a flow rate of 200 μL/min by decreasing the NaCl gradient from 1.5 to 0 M in buffer A, and 0.5 mL fractions were collected. Hydrophobic proteins were eluted with 50% ethylene glycol. Fractions containing endogenous substrates were pooled and dialyzed against buffer A for at least 6 h.

Step IV: Mono-Q Column Chromatography. The dialysate from Phenyl-Sepharose was applied to a mini Mono-Q 3.23/3 (3.2 × 30 mm) ion-exchange column equilibrated with buffer A containing 10 mM NaF and 0.2% octyl-β-glucoside at a flow rate of 0.2 mL/min, utilizing the SMART system. Proteins were eluted by applying a linear gradient from 0 to 1.0 M NaCl in the above buffer at a flow rate of 0.05 mL/ min. Fractions of 0.2 mL were collected and analyzed.

Purification of the Endogenous Substrates of SDKs with Molecular Mass 105, 68, 58, 55, and 50 kDa. The overall purification of the 55 and 58 kDa proteins isolated from cytosol included separation on QS ion-exchange and Phenyl-Sepharose followed by Mono-Q ion-exchange chromatography. Purification of the 68 and 105 kDa proteins isolated from cytosol included QS ion-exchange followed by Mono-Q ion-exchange chromatography. Purification of a membrane SDK and the 50 kDa protein included QS ion-exchange, Mono-Q ion-exchange, and Phenyl-Sepharose followed by Mono-Q ion-exchange chromatography.

Sphingosine-Dependent Kinase Assay. DMS was prepared as 10× solution in 10% ethanol and 0.5 M NaCl by sonication in a bath sonicator, three times for 1 min at room temperature. Other lipids were used as indicated. The assay (total 30 μL solution) contained 5 μL (0.2−5.0 μg) of crude cytosol or fractions from column chromatography, 3 μL of DMS (1 μg in 3 μL of vehicle), and 12 μL of 50 mM Tris-HCl buffer (pH 7.5). The reaction was initiated by addition of 10 μL of 150 μM ATP, 45 mM magnesium acetate in 50 mM Tris-HCl (pH 7.5), and 4 μCi (γ-^{32}P)ATP, continued for 10 min at 30 °C, and terminated by addition of 4× concentrated reduced Laemmli sample buffer and heating to 100 °C for 3 min. The reaction mixture was analyzed by SDS−PAGE (12% or 10%). Gels were stained with Coomassie Brilliant Blue R-250 (0.1% in 50% methanol and 10% acetic acid), destained, dried, and subjected to autoradiography with T-MAT film (Kodak) using intensifier screens (exposure lasted 2−48 h).

Phosphorylation of authentic PDI and calreticulin (purified from liver) was performed as above, except that 5 μL (0.1 μg) of substrates and 1 μL of kinase (partially purified from A31 fibroblasts membrane) were added to the reaction. Phosphorylated PDI was excised from SDS−PAGE and subjected to scintillation counting. Values obtained from gel without phosphorylated protein (blank) were subtracted from each determination.

Amino Acid Sequencing of Purified SDKs Substrates. For microsequencing, fractions containing relatively pure substrate were separated on SDS−PAGE and transferred to PDVF membrane, and proteins were visualized by staining with Coomassie Blue, according to manufacturer's instructions. The phosphorylated bands were excised and subjected to amino-terminal sequence analysis on a protein sequencer (Applied Biosystems model 477A) equipped with an on-line phenylthiohydantoin analyzer (Applied Biosystems model 120A).

For internal peptide sequences, protein was digested on the PVDF membrane (*28*) with *Achromobacter* protease I (*29*) (a generous gift from Dr. T. Masaki, Ibaraki University, Ami, Inashiki, Japan) in 50 mM Tris-HCl, pH 8.5, at 37 °C for 15 h. Peptides generated were separated by reversed-phase HPLC on a column of Supersper RP-SelectB (0.21 × 120 mm, Merck) at a flow rate of 0.2 mL/min, with a linear gradient of acetonitrile (0−60% in 40 min) in 0.1% trifluoroacetic acid (TFA), using a Hewlett-Packard model 1090M liquid chromatograph equipped with a diode array detector.

Other Procedures. Protein concentration was measured by the method of Bradford (*30*), using reagents and kit purchased from Bio-Rad (Richmond, CA).

RESULTS

Activation of Sphingosine-Dependent Kinases in Vitro Is Not Due to Detergent Effects. To demonstrate the presence of sphingosine-dependent protein kinases (SDKs) and characterize their nature, we examined the effect of Sph and other sphingolipids on the phosphorylation of endogenous proteins from cytosol and membrane fractions obtained from Balb/c 3T3 (A31) fibroblasts. Activation of SDKs and consequent phosphorylation of their endogenous substrates was demonstrated in both cytosol (Figure 1A) and membrane extracts (Figure 1B) only after addition of DMS or Sph. These sphingolipids at concentration 5−100 μM delivered in ethanol (data not shown) activate SDKs, similar to their effect on SDK1, a kinase that phosphorylates 14-3-3 (*22*). These findings suggest that Sph and DMS activate SDKs found in both cytosolic and membrane compartments of the cell. Furthermore, activation of these kinases by DMS was enhanced in the presence of detergent 0.3% *n*-octyl-β-glucoside or 0.3% Triton X-100 (Figure 1A,B). The concentration of *n*-octyl-β-glucoside used was below its critical micellar concentration (CMC) of 0.7%, whereas Triton X-100 concentration was above its CMC of 0.2%. These observations indicate that Sph/DMS-dependent activation of SDKs is not a detergent effect.

Only Sph and Its N-Methyl Derivatives, but Not Cationic Amphiphiles nor Other Sphingolipids, Activate SDKs in the Presence of Detergent. To further confirm the lipid specificity of SDK activation, we compared the effects of different sphingolipids on protein phosphorylation in the absence or presence of *n*-octyl-β-glucoside. Several endogenous proteins (molecular mass = 215, 105, 98, 75, 45, 33, and 20 kDa) were phosphorylated by SDKs activated by DMS (Figure 2A, lane 3). A similar phosphorylation pattern was observed in the presence of Sph (lane 2). The water-soluble analogue

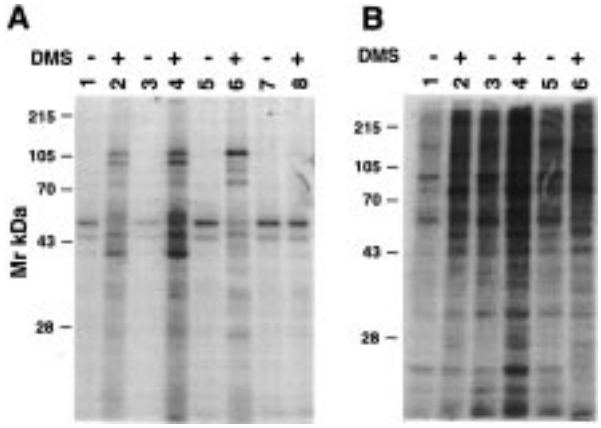

FIGURE 1: Effect of detergent on activation of SDK and phosphorylation of endogenous substrates extracted from cytosolic fraction (panel A) and membrane fraction (panel B). Cytosolic or particulate fractions extracted from membrane were prepared from confluent Balb/c 3T3 cells treated with TPA under serum-free conditions as described in Experimental Procedures. These fractions (20 μg) were incubated in the absence (−) or presence (+) of 300 μM DMS delivered in 1% ethanol (lanes 1 and 2), 0.3% Triton X-100 (lanes 3 and 4), 0.3% *n*-octyl-β-glucoside (lanes 5 and 6), or 0.3% NP-40 (lanes 7 and 8). Phosphorylation followed by SDS−PAGE was performed as described in the text. Positions of molecular weight markers are indicated at left.

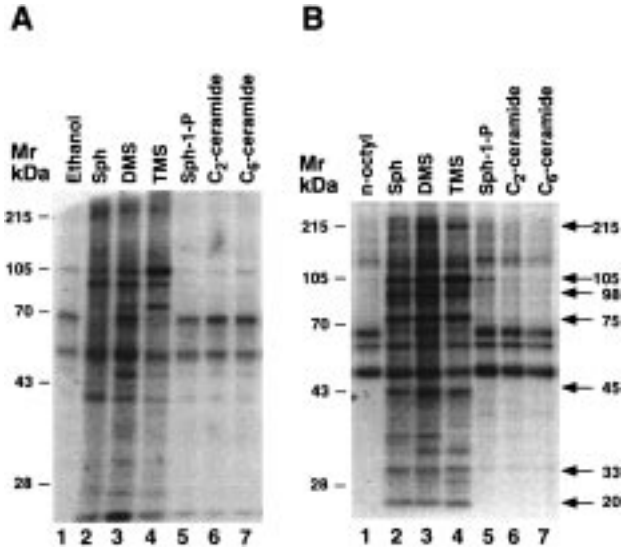

FIGURE 2: Sph and its *N*,*N*-dimethyl and *N*,*N*,*N*-trimethyl derivatives (DMS and TMS, respectively), but not Cer or Sph-1-P, activate SDKs in the presence of detergent. Panel A: Cytosolic extracts (15 μg) were incubated in the absence (lane 1) or presence of 100 μM of various lipids (Sph, DMS, TMS, Sph-1-P, C2−Cer, C6−Cer; lanes 2−7, respectively). Lipids were delivered in 1% ethanol and 50 mM NaCl. Panel B: Lipids were delivered in 0.3% *n*-octyl-β-glucoside (lane 1, detergent control). Positions of endogenous substrates are indicated by arrows. Positions of molecular weight markers are indicated at left.

of DMS, *N*,*N*,*N*-trimethyl-Sph (TMS), also activates SDK and induces phosphorylation of 105, 98, and 75 kDa proteins (lane 4). This activating effect is not due to cationic amphiphilic properties of the factors, since the effect is specific to D-*erythro* stereoisomer, and simple cationic amphiphiles have no effect (see the section Lipid Specificity of Substrate Phosphorylation). In contrast, these phosphorylated bands were not observed in the presence of Sph-1-P, C2-Cer, C6-Cer (lanes 5−7), or ethanol vehicle alone (lane 1). A similar but slightly enhanced phosphorylation pattern was observed when the sphingolipids were delivered in *n*-octyl-β-glucoside (Figure 2B). These findings exclude the possibility that sphingolipids produce detergent effects that induce nonspecific lipid−lipid or lipid−protein interaction under these conditions. The effect of Sph, DMS, and TMS on SDK activity is specific, since Cer and Sph-1-P, even in the presence of detergent, had no effect on SDK activity and phosphorylation of these substrates.

Interestingly, growth factors or mitogenic signals induced a 2-fold increase in phosphorylation of these endogenous SDK substrates, whereas phosphorylation of a unique substrate (45 kDa) was observed only in confluent cells but not in exponentially growing cells that were 50% confluent (data not shown).

Purification of 105 and 68 kDa Substrates of SDK and Their Identification as Members of the HSP Family. SDKs and their substrates were purified from extracts of confluent fibroblasts treated with TPA under serum-free conditions by a series of chromatographies as described in Experimental Procedures. Under these conditions, the intracellular level of Sph increased while PKC activity decreased (*26*, *27*). Sph and DMS both activate SDKs; however, DMS was used for detection of SDK activity during purification, since it cannot be metabolized to either Cer or Sph-1-P. Only 1 out of the 7 endogenous substrates detected in crude cytosolic extracts was purified and identified. This 105 kDa protein is shown to have amino acid sequence homology to GRP94 (a synonym for endoplasmin). The slight difference between assigned molecular mass (94 kDa) and originally observed molecular mass (105 kDa) may be ascribable to a difference in glycosylation. During purification, another SDK substrate with molecular mass 68 kDa was isolated, which has homology to two HSPs, mouse HSP86 and HSP84 (Table 1). The original molecular mass observed in crude extract was 98 kDa, whereas the substrate recovered during purification, on which amino acid sequence analysis was performed, had molecular mass 68 kDa. This discrepancy presumably resulted from proteolysis of mouse HSP86 or HSP84, since their sequence was clearly identified. Proteolysis during purification of cellular proteins is often observed. Mouse HSP86 is the analogue of human HSP90.

Purification of 58 and 55 kDa Substrates of SDK and Their Identification as PDI. Cytosolic endogenous substrates of SDKs were purified by sequential column chromatography on Q-Sepharose (QS), Phenyl-Sepharose, and Mono-Q. SDK activity and its 58 and 55 kDa substrates were coeluted between 140 and 180 mM NaCl from QS ion-exchange (purification step II). These fractions include CKII, a kinase whose activity is enhanced by Sph. Purification of the QS active fractions on Phenyl-Sepharose (purification step III) resulted in separation of the 58 kDa substrate from SDK, the 55 kDa substrate, and hydrophobic proteins, which were eluted by 50% ethylene glycol. Thus, purification of the 58 kDa protein on Mono-Q (step IV) required addition of semipurified kinase. Both the 58 and 55 kDa substrates were further purified on Mono-Q and eluted at 150 mM NaCl and 250 mM NaCl, respectively. The 55 kDa protein was coeluted with SDK from Mono-Q ion-exchange chromatography as a single peak (fractions 13 and 14) is shown in the chromatogram with UV detector system (Figure 3) or Coomassie stained SDS−PAGE (Figure 3, inset A). How-

Table 1: Identification of the Endogenous Substrates of SDKs (Isolated from Mouse Balb/c 3T3 A31 Cells) as Calreticulin, PDI, HSP86, HSP84, and GRP94 with Amino Acid Sequences Determined by N-Terminal Sequencing of Proteins Blotted by PDVF, or Sequencing of Peptides Recovered after Digestion of PDVF-Bound Proteins with *Achromobacter* Protease I, Which Digests the Lysyl Bonds in Proteins

fraction	elution from QS[a]	elution from Mono-Q[a]	molec mass (kDa)	no. of peptides identified	sequence determined	homology
TN25	350	200−250	50	1	XPAIYFKEQ	calreticulin
C7−8	140	150	58	1	SDVLELTDENFESRVSDTGSAGLML	protein disulfide isomerase (PDI)
D14	180	250	55	1	DALEEEDNVLVLKKXN	PDI
F18−19	250−325	240	68	3	DQPMEEEEVETFAFQAEIAQ	heat shock protein 86
					PEETQTQDQPMEEEEVET	heat shock protein 86
					PEEVHHGEEEVETFAFQA	heat shock protein 84
H38	325−380	340	105	3	DDEVDVDGTVEEDLGK	endoplasmin
					DISTNYYASQK	(glucose regulated
					YNDTFWK	protein 94)

[a] mM NaCl. X = unidentified amino acid.

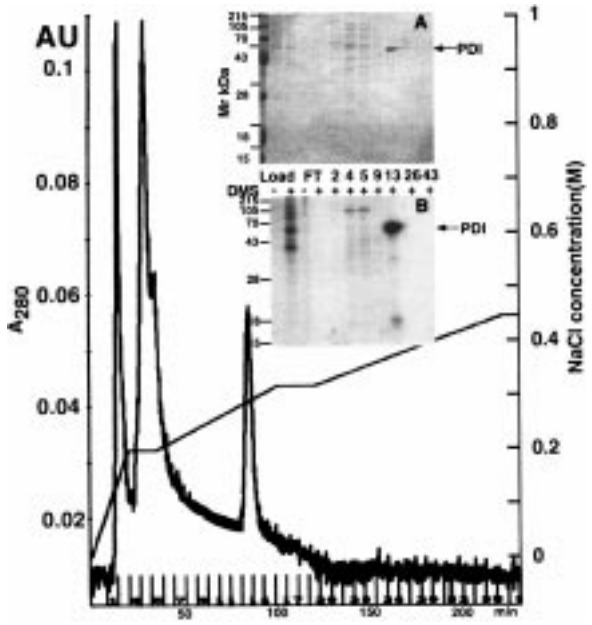

FIGURE 3: Purification of a 55 kDa endogenous substrate of an SDK, PDI, from cytosol on Mono-Q chromatography (step IV). The cytosol extract (step I) was purified through QS (step II). Fractions containing Sph-induced phosphorylation of the 55 kDa substrate were pooled and further purified on Phenyl-Sepharose (step III), followed by Mono-Q chromatography (step IV). Proteins which bound to Phenyl-Sepharose at step III (load) were fractionated on Mono-Q ion-exchange chromatography. Proteins were eluted by a combination of linear gradient changes and stepwise changes (solid line) of NaCl concentration, indicated on the right ordinate. Left ordinate indicates protein concentration eluted and monitored with an on-line UV monitor at 280 nm. Inset B: autoradiogram of 12% SDS−PAGE generated from phosphorylation reactions of the indicated fractions in the absence (−) or presence (+) of 100 μM DMS (FT, flow-through fraction). Inset A: Coomassie Blue staining pattern of the same SDS−PAGE as in inset B. Arrow indicates position of the 55 kDa endogenous substrate. Positions of molecular weight markers are indicated at left. This substrate was clearly purified as indicated by the UV chromatogram and Coomassie Blue staining. The 55 kDa substrate was eluted together with an SDK and was identified as PDI.

ever, SDK activity that phosphorylates this 55 kDa protein was also detected in fractions 11 and 12 (data not shown). This SDK activity also phosphorylates the 58 kDa protein extensively purified on Mono-Q, at purification step IV (data not shown). The final isolation of endogenous SDK substrates purified in steps I−IV was accomplished by separation on SDS−PAGE, where the 58 and 55 kDa substrates were purified from minor contaminants.

The N-terminal amino acid sequences of the 55 and 58 kDa proteins (Table 1) indicate that these are two different forms of PDI. The sequence of the 55 kDa protein shows high homology with mouse PDI, and that of the 58 kDa protein shows complete homology (identity) with mouse PDI. This type of SDK substrate is associated with ER, since PDI has ER retention signal (KEDL or QEDL) at the C-terminal region.

The 50 kDa SDK Substrate Isolated from the Membrane Is Calreticulin. SDKs and their endogenous substrates were extracted from membrane of Balb/c 3T3 (A31) fibroblasts and purified by sequential column chromatography on QS, Mono-Q, Phenyl-Sepharose, and Mono-Q. SDK activity and its 50 kDa substrate were coeluted by 350 mM NaCl from QS ion-exchange (purification step II). Membrane SDK and its 50 kDa substrate did not bind Phenyl-Sepharose, suggesting that these proteins were relatively hydrophilic. Both proteins were further purified on Mono-Q and eluted with 350 mM NaCl. Active fractions containing membrane SDK and 50 kDa endogenous substrates of SDK had CKII activity. Upon further purification on Mono-Q, the 50 kDa substrate (fraction 28) was partially separated from a 20 kDa SDK substrate (fraction 36), as shown in Figure 4.

The final isolation of the purified 50 kDa protein was accomplished by separation on SDS−PAGE, where the 50 kDa protein was purified from minor contaminants. The amino acid sequence alignment of the generated peptide revealed complete homology with calreticulin (Table 1).

A Novel SDK, "SDK2", from Membrane Fractions "TN31−33". The purification of the 50 kDa substrate calreticulin from membrane resulted in separation of SDKs from their endogenous substrates. Fraction 33, eluted by 330 mM NaCl (Figure 4), did not phosphorylate any endogenous proteins (Figure 4, inset B). Fractions "TN31−33" phosphorylated the 50 kDa protein, indicating that these fractions contain an SDK (Figure 4, inset C). This SDK was not clearly defined in its homogeneity but is hereby tentatively termed "SDK2" on the basis of its substrate specificity. We confirmed that SDK2 was separated from the SDK substrates as follows: (i) Incubation of SDK2 (fractions "TN31−33") by itself or 50 kDa substrate by itself, in the presence or absence of DMS, did not induce phosphorylation of any protein (Figure 5, lanes 1−4). (ii) Phosphorylation of the 50 kDa protein was induced only upon addition of SDK2 fraction (Figure 5, lanes 5−6). (iii) Heat treatment of the 50 kDa protein, calreticulin, did not inhibit its phosphorylation (lanes 13 and 14), whereas heat inactivation of the kinase fractions

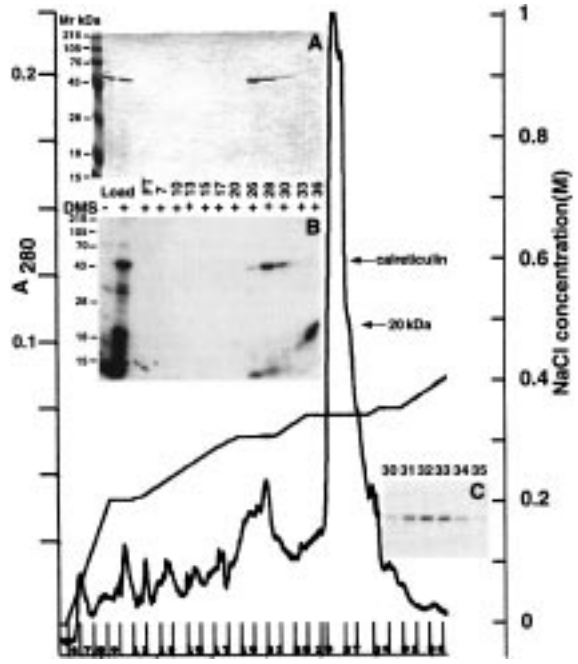

FIGURE 4: Purification of a 50 kDa endogenous substrate of an SDK from membrane fraction on Mono-Q ion-exchange chromatography (step V) and its identification as calreticulin. Membrane proteins were extracted with 2% Triton X-100 (step I), and the 50 kDa substrate was purified through QS (step II), Mono-Q (step III), Phenyl-Sepharose (step IV), and finally a second run of Mono-Q chromatography (step V). Pooled fractions containing 50 kDa SDK substrate were eluted from Mono-Q by a combination of linear gradient changes and stepwise changes of NaCl concentration, indicated on the right ordinate. Protein elution was monitored with an on-line UV monitor at 280 nm (left ordinate). Inset B: autoradiogram of 12% SDS−PAGE generated from phosphorylation reactions in the absence (−) or presence (+) of 100 μM DMS of indicated fractions. The existence of 50 kDa SDK substrate before fractionation (load) is indicated. Inset A: Coomassie Blue staining pattern of the same SDS−PAGE as in inset B. Arrows indicate positions of the 50 kDa endogenous substrate and 20 kDa substrate. Positions of molecular weight markers are indicated at left. This substrate was clearly purified as indicated by the UV chromatogram and Coomassie Blue staining. Inset C: SDK activity found in fractions 31−33 was separated from the 50 kDa endogenous substrate, calreticulin, and detected only after addition of substrate-containing fraction.

abolished phosphorylation (lanes 7 and 8 and 11 and 12). This kinase fraction was found to phosphorylate substrates PDI and calreticulin. The purified SDK activity in fractions TN31−33 is distinct from CKII activity detected in the same fractions, since one of the SDK substrates, namely PDI, is not a CKII substrate.

PDI and Calreticulin Are Substrates of SDK2. To further confirm the finding that the 58 and 55 kDa endogenous substrates of SDKs were PDI, and the 50 kDa substrate was calreticulin, phosphorylation of purified bovine PDI and rat calreticulin utilizing the SDK2 fraction were examined. Both PDI and calreticulin were phosphorylated by semipurified SDK2 fraction. This phosphorylation was dependent on DMS (Figure 6A).

A comparison of the effects of various concentrations of Sph and DMS, delivered in ethanol, on phosphorylation of purified PDI by membrane SDK2 fraction is shown in Figure 6B,C. Low concentrations (5 μM DMS or 25 μM Sph) were required for a strong phosphorylation of PDI. The reaction

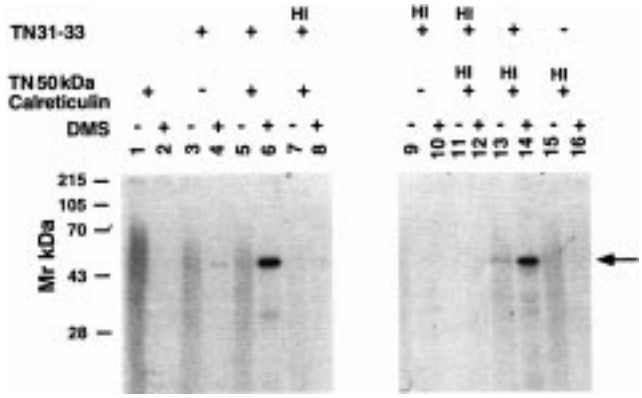

FIGURE 5: Membrane fractions 31−33 ("TN31−33") containing an SDK ("SDK2") that phosphorylates a purified 50 kDa substrate identified as calreticulin. The effect of heat inactivation (HI) on phosphorylation of calreticulin by semipurified membrane kinase in the absence (−) or presence (+) of DMS was tested. The reactions were as indicated in the lanes numbered 1−16. The arrows indicate predominant phosphorylation of the 50 kDa substrate calreticulin before and after heat inactivation. The autoradiogram confirms that SDK activity associated with fractions 31−33 (TN31−33) is sensitive to heat inactivation (lanes 7 and 8), while the 50 kDa protein is a substrate not sensitive to heat inactivation (lanes 13 and 14).

reached plateau with 25 μM DMS or Sph, and a small decline was observed when Sph or DMS concentrations were above 100 μM.

Lipid Specificity of Substrate Phosphorylation. In view of the finding that phosphorylation of 105 kDa substrate, PDI, calreticulin, and other substrates was activated by Sph and DMS, we studied whether the effect of Sph and DMS is due to their cationic amphiphilic property and whether various other related sphingolipids and phospholipids would affect the activity of SDKs for these substrates. DMS enhanced phosphorylation of 70 kDa protein (Figure 7A), whereas surprisingly LPA, as well as DMS, enhanced phosphorylation of the 105 kDa endogenous substrate of SDKs (Figure 7B).

Dose-dependent effects of stereoisomers of Sph, its *N*-methyl derivatives (DMS and TMS), other cationic amphiphiles, Sph-1-P, and Cer on SDK-dependent phosphorylation of GRP94 and PDI as substrates were compared systematically. Phosphorylation of GRP94, dependent on copurified SDK (SDK3), was promoted strongly by D-*erythro*-DMS and -TMS and to a lesser extent by D-*erythro*-Sph. However, L-*threo*-Sph, stearylamine, and hexadecyltrimethylammonium bromide had no effect (Figure 8A). Thus, the activating effect of D-*erythro*-Sph and its *N*-methyl derivatives is stereospecific and excludes the effect of nonspecific cationic amphiphiles. This trend was seen more clearly for PDI, whose phosphorylation, catalyzed by membrane-bound SDK (SDK2), was enhanced strongly by D-*erythro*-Sph and D-*erythro*-DMS but minimally affected by L-*threo*-Sph, L-*threo*-DMS, and D-*erythro*-TMS. Stearylamine, hexadecyltrimethylammonium bromide, Sph-1-P, and Cer had no effect (Figure 8B). For both GRP94 and PDI, only D-*erythro*-Sph and D-*erythro*-DMS showed strong activation at low concentration (5−10 μM); this can be regarded as a highly specific effect.

Presence of Multiple Forms of SDK. In view of the finding that cytosolic and membrane SDKs phosphorylate several

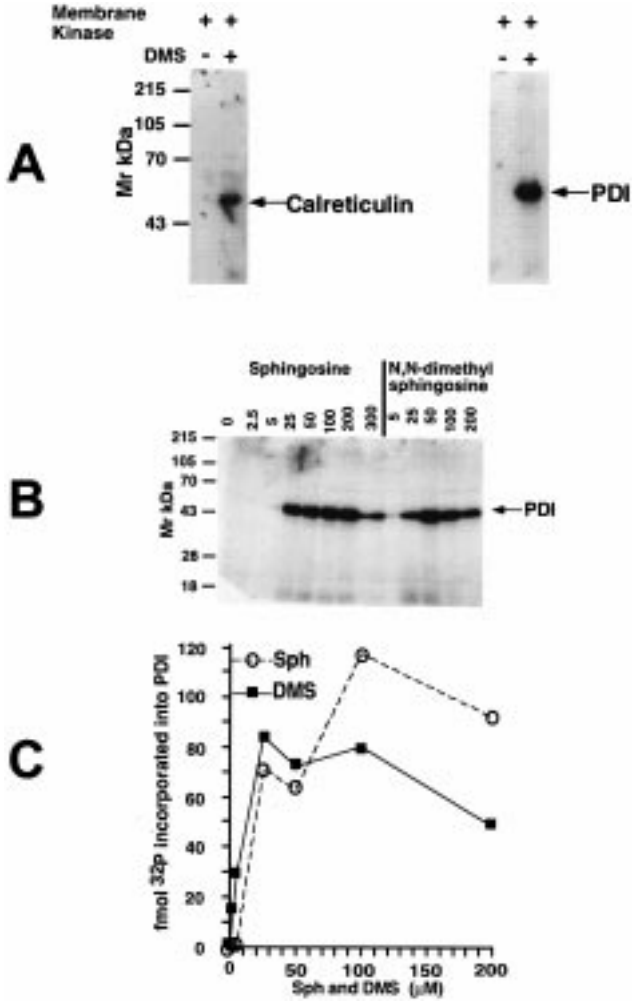

FIGURE 6: SDK-dependent phosphorylation of PDI and calreticulin. Panel A: Authentic PDI and calreticulin purified from liver were phosphorylated by semipurified membrane kinase in the absence (−) or presence (+) of DMS. An autoradiogram confirms that these proteins are indeed substrates of SDK. The arrows indicate predominant phosphorylated substrates PDI and calreticulin. Panel B: The degree of PDI phosphorylation is dependent on sphingolipid concentrations. Phosphorylation of authentic PDI (0.1 μg) by semipurified membrane kinase (75 ng) was induced by the indicated concentration (in μM) of Sph or DMS, solubilized in 1% ethanol. Phosphorylation was resolved on 12% SDS−PAGE and autoradiographed. An arrow indicates the predominant phosphorylated substrate PDI. Panel C: Dose-dependent effects of Sph (○) and DMS (■) on phosphorylation of PDI. The amount of ^{32}P incorporated into PDI was quantified as described in Experimental Procedures.

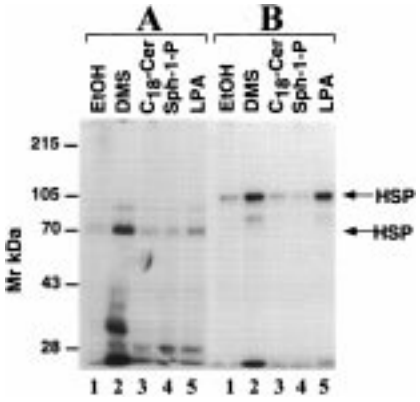

FIGURE 7: Effects of DMS, C_{18}-Cer, Sph-1-P, and LPA on SDK-dependent phosphorylation of semipurified 78 kDa HSP (panel A) and semipurified 105 kDa GRP (panel B). SDK activity was copurified with HSP. Endogenous substrates copurified with SDK were incubated in the absence (lane 1) or presence of 20 μM DMS, C_{18}-Cer, Sph-1-P, or oleoyl-LPA (lanes 2−5, respectively). Arrows indicate the position of HSP.

substrates (PDI, calreticulin, HSP, GRP), we studied the specificity of SDKs for these substrates. SDK in fraction 42 separated from Q-Sepharose chromatography, which specifically phosphorylates HSPs with 105 and 68 kDa, did not phosphorylate PDI or 14-3-3 (Figure 9A). Membrane-bound SDKs, isolated from membrane fractions, phosphorylated several substrates having molecular mass 100, 90, 40, and 34 kDa but did not phosphorylate recombinant 14-3-3 ζ (Figure 9B). These results suggest that an SDK specific to HSP is distinct from SDKs specific to PDI or to 14-3-3; this SDK is tentatively termed "SDK3" on the basis of its substrate specificity.

Membrane-bound SDK was copurified with calreticulin and phosphorylates calreticulin. The SDK that was separated from calreticulin phosphorylates both calreticulin and PDI (Figure 6). The SDK present in fraction 30 phosphorylates calreticulin (Figure 4, inset C). This kinase is defined as SDK2.

DISCUSSION

Protein kinases activated only in the presence of Sph and its *N*-methylated derivative DMS, but not in the presence of Cer, Sph-1-P, or various other sphingolipids, phospholipids, and glycerolipids (in total 17 lipids tested), were found in extract of mouse Balb/c 3T3/A31 fibroblasts and termed "sphingosine-dependent protein kinases" (SDKs) (*21*, *22*). SDKs are distinct from Cer-activated kinase (*31*), since they are not activated to phosphorylate their substrates by Cer or Sph-1-P. Furthermore, requirement of SDKs for D-*erythro* but not L-*threo* stereoisomers and lack of effect by nonspecific cationic amphiphiles are now clearly demonstrated. Together, these findings indicate the physiological significance of specific sphingolipids in activation of SDKs. SDKs are also distinguishable from some other protein kinases whose activities are detectable in the absence of Sph, DMS, and other lipids but are modulated (activated or inhibited) by these lipids, e.g. PKC (*8*, *9*), EGF receptor tyrosine kinase (*15*), Src, CKII (*16*), focal adhesion kinase, and paxillin (*32*).

Our previous studies indicate that activated SDKs phosphorylate several target molecules. One target of SDKs was identified previously as the adapter/chaperone molecule 14-3-3 (*21*). The SDK that phosphorylates 14-3-3 (termed SDK1) was purified and found to be highly specific to 14-3-3 isoforms β, ζ, and η. SDK1 phosphorylates 14-3-3 on Ser (S*) next to Trp (W) in the sequence RSS*WR located in helix 3 but does not phosphorylate PDI or calreticulin (*22*).

In this study, we found other types of SDK with substrate specificity entirely different from that of SDK1 in extract of 3T3/A31 fibroblasts. The substrates phosphorylated in the presence of Sph/DMS and the fraction containing SDK activity were separated from cytosolic and membrane fractions by a series of chromatographies. Several substrates (PDI, calreticulin, HSP84, HSP86, GRP94) were identified by the following criteria: (i) phosphorylation by endogenous kinase only in the presence of Sph or DMS; (ii) partial amino

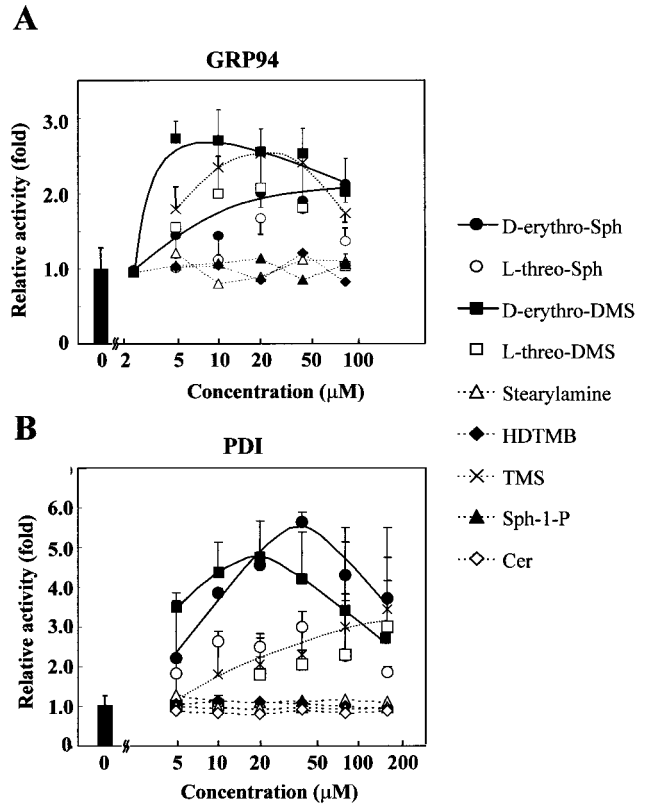

FIGURE 8: Comparative dose-dependent effect of Sph stereoisomers, *N*-methyl derivatives, and other cationic amphiphiles on SDK-dependent phosphorylation of GRP94 and PDI. Panel A: SDK-dependent GRP94 phosphorylation. A 5 μL volume of test solution containing both GRP94 and copurified SDK was added with 3 μL of lipids at various concentrations dissolved in 5% octyl-β-glucoside, 3 μL of 30 mM dithiothreitol, and 9 μL of 50 mM Tris buffer (pH 7.5). The reaction was initiated by addition of 10 μL of ATP (75 μM containing 2.5 μCi (γ-^{32}P)ATP), giving a total volume of 30 μL, and incubated at 30 °C for 10 min. The final concentration of octyl-β-glucoside was 0.5%. The reaction was terminated by addition of 2× concentrated Laemmli's sample buffer containing β-mercaptoethanol and 2 mM $NaVO_4$ and boiling for 5 min. The reaction mixture was separated on 8.5% SDS−PAGE, and phosphorylation activities of GRP94, detected by autoradiography, were probed by rabbit anti-GRP94 (Santa Cruz Biotechnology). Band intensity was determined by Scion Image program (Scion Corp., Frederick, MD). Panel B: SDK-dependent PDI phosphorylation. Partially purified membrane fraction (see text) containing 75 ng of protein was mixed with authentic PDI (0.3 μg) per tube. The mixture was added with various concentrations of lipids and other compounds as described for panel A. Reaction was initiated, followed by SDS−PAGE, and activity associated with PDI was determined as described above. In both panels A and B, activity (ordinate) is expressed relative to control sample without addition of lipids (but containing 0.5% octyl-β-glucoside), and concentration of lipid added to reaction mixture (in μM) is indicated on the abscissa. Symbols at right indicate lipids added.

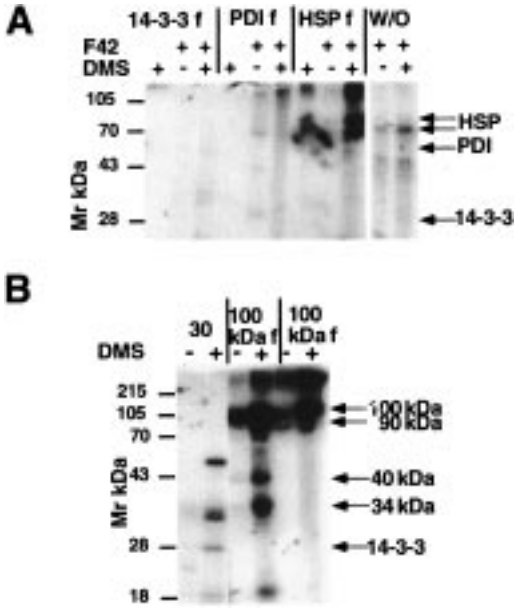

FIGURE 9: SDK3 isolated from the cytosol or membrane specifically phosphorylate HSP but not 14-3-3- or PDI-containing fractions. Panel A: SDKs recovered from cytosolic extract eluted from Q-Sepharose chromatography (250−325 mM NaCl) were purified on Mono-Q, where the SDK substrate (68 kDa/HSP84) was separated from an SDK fraction (fraction 42). 14-3-3-, PDI-, and HSP-containing fractions (0.2−0.5 μg/30 μL per tube) were subjected to phosphorylation in the absence or presence of SDK (fraction 42, 50 ng/30 μL) and in the absence or presence of DMS. Autoradiography confirmed that this substrate did not contain an SDK, as indicated by reaction in the presence of DMS. Note that cytosolic SDK (fraction 42) specifically phosphorylates only fractions containing 70 and 84 kDa substrate but not fractions containing PDI or calreticulin. "W/O" indicates without addition of fraction containing known substrates (weak band shown is due to presence of endogenous substrate). Panel B: Autoradiogram of phosphorylation reaction of "fraction 30" (SDK2, left two lanes) and major membrane-bound SDK3 (middle and right two lanes) purified from membrane in the absence or presence of DMS. These fractions contain an SDK or SDKs that are copurified with their substrate.

acid sequence of the isolated protein; (iii) confirmation that purified substrates isolated from other sources are phosphorylated by semipurified SDK in a Sph- or DMS-dependent manner. PDI and calreticulin were identified by all three criteria, whereas HSP84, HSP86, and/or GRP94 were identified based on criteria i and ii. Thus, additional cellular targets of SDKs were discovered as chaperone proteins which associate with other proteins, facilitate their folding, and maintain their tertiary structure.

PDI contains ER retention signal (KEDL or QEDL) at the C-terminal region and is therefore present in high concentration in ER. It is a multifunctional protein which binds nonspecifically to various proteins as a molecular chaperone and also catalyzes joining of thiol groups to form disulfide-dependent tertiary structure (for review see ref *33*). Thus, PDI function is essential to convert nonfunctional nascent polypeptides to functional mature proteins with correct folding and to maintain their secondary or tertiary structure (*34*). In this sense, PDI may function to prevent apoptosis, similarly to thiol reducing reagents (*35*). However, the functional effect of phosphorylation of PDI by SDK has yet to be elucidated.

Calreticulin, another multifunctional ER protein, binds Ca^{2+}, interacts with Ca^{2+} pump (Ca^{2+}−ATPase) and IP3 receptor and regulates intracellular Ca^{2+} level (*36*). Its chaperone effect on glycoprotein folding is based on its novel binding activity to mono-α-glucosyl residue linked to high-mannose core of N-linked structure that appears during glycoprotein processing in ER membrane. Calreticulin together with another chaperone protein, calnexin, is essential in converting nascent glycosylated structure into mature, functional glycoprotein (*37*−*39*). Both PDI and calreticulin, as SDK substrates, interact with each other, and this interaction inhibits their activities (*40*). Although the biological signifi-

cance of SDK-dependent phosphorylation of PDI or calreticulin in intact cells remains to be elucidated, it is reasonable to assume that this process may modulate their mutual interaction. The inhibitory effect of Sph on calcium mobilization (*41*) may be explained by increased association between PDI and calreticulin upon their phosphorylation in intact cells.

Many members of the HSP and GRP families are closely related in their function and have been identified as major ER components. The expression mechanism and the pathobiological function of HSPs and GRPs are subjects of a great number of recent studies (for reviews see refs *42*−*44*). Their expression is strongly activated by adverse environmental factors or stress (e.g. temperature shift, exposure to nonphysiological reagents, glucose starvation). Heat shock induces expression of HSPs, which promote survival and protect cells from apoptosis (*45*). HSP27 blocks apoptosis induced by Fas or staurosporine (*46*). TNFα and IL-1, two agonists that increase Cer levels, induce phosphorylation of HSP27 (*47*). HSPs form complexes with nuclear receptors and protein kinases, such as Raf, Src, p21 *ras*, p53, and Rb. HSP90 controls v-src, but not c-src, activity (*48*). Mouse HSP86 found in cytoplasm has a sequence nearly identical to that of human HSP90, which binds to various signal transducer molecules (e.g. Raf, CDK4), other HSPs (HSP70, HSP40), and steroid hormone receptors (androgens, estrogens, glucocorticoids) (for review see refs *42*, *49*, and *50*). GRP94, also termed endoplasmin, is a major glycoprotein in ER, with high Ca^{2+} binding capability, that binds ATP similarly to HSP70 and GRP78 (for review see ref *42*). GRP94 has been shown to have serine kinase activity ("94-kinase") which is enhanced by association with GRP78 (*51*). A 105 kDa protein SDK substrate was identified as GRP94 and copurified with SDK, in the present study; however, it is not clear whether the 94-kinase per se is an SDK.

Several classes of HSP, with molecular mass around 27, 60, 70, and 90 kDa, exist and show homology with GRP. The fact that crude cytosolic extract of serum-starved fibroblasts contains several SDK substrates (215, 105, 98, 75, 45, 33, 20 kDa) suggests that these substrate are HSPs which are up-regulated upon serum starvation. GRP94 (105 kDa protein) is the only identified SDK substrate in which phosphorylation was observed in crude extract reaction. The 68 kDa substrate recovered during purification could be a degradation product of the 98 kDa substrate (HSP86). Future study on in vitro phosphorylation by SDK of purified proteins representing all classes of HSP would confirm this possibility. The biological significance of HSP and/or GRP phosphorylation by SDKs is unknown. It is possible that binding capability of GRP to ATP, Ca^{2+}, and the activity of the associated kinase, as well as binding of HSP to ATP, are modulated upon phosphorylation by an SDK.

Target molecules of Sph and DMS through which they control cellular function are now identified as SDKs. SDKs are insusceptible to Cer and Sph-1-P, and control mechanisms of cellular function through Cer and Sph-1-P are therefore basically different from those through Sph and DMS. Results from this and previous studies (*22*) indicate that a common function of SDK activity is modulation of molecular chaperones essential for maintenance of homeostasis and protection from environmental stress. Further studies will reveal the cellular mechanisms controlled by sphingolipids.

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