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Changes of Gene Expression by Lysophosphatidylcholine in Vascular Endothelial Cells: 12 Up-Regulated Distinct Genes Including 5 Cell Growth-Related, 3 Thrombosis-Related, and 4 Others[1]

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Lysophosphatidylcholine (lysoPC), a component of oxidatively modified lipoproteins, is present in atherosclerotic lesions, and its proatherogenic properties have been demonstrated. To gain an insight into lysoPC-mediated endothelial gene expression, we applied nonradioactive differential display analysis of mRNA from lysoPC-treated and untreated human umbilical vein endothelial cells. We identified 12 up-regulated distinct genes including 5 cell growth-related genes (two phosphatases CL100 and B23/hVH-3, gravin, activating transcription factor-4, and heparin-binding epidermal growth factor-like growth factor), 3 thrombosis-related genes (plasminogen activator inhibitor-1, tissue plasminogen activator, and thrombomodulin), and 4 others (stanniocalcin, NAD-dependent methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase, BENE, and reducing agents and tunicamycin-responsive protein). We isolated a full-length cDNA of human gravin. The cDNA sequence of gravin was homologous with rat mitogenic regulatory gene or rat protein kinase C binding protein and substrate, suggesting that gravin would regulate cell growth. Thus, lysoPC apparently accelerates atherosclerosis by regulating the expression of a wide variety of genes. Our data suggest the involvement in atherogenesis of the genes hitherto regarded as atherosclerosis-unrelated.

Key words: atherosclerosis, gravin, growth regulation, MAP kinase, oxidized LDL.

Oxidative modification of LDL occurring in the subendothelial space is a crucial step in the pathogenesis of atherosclerosis (*1, 2*). It is associated with generation of lipid peroxides, lysophospholipids, aldehydes, and oxysterols. Lysophosphatidylcholine (lysoPC), which is generated in oxidized LDL and present in atherosclerotic plaques, is a major factor in the atherogenic effects of oxidized LDL (*3*). LysoPC has been shown to possess a wide variety of proatherogenic properties: chemotactic activity for circulating monocytes and T-lymphocytes (*4, 5*), impairment of endothelium-dependent arterial relaxation (*6*), mitogenic effect on macrophages and smooth muscle cells (*7, 8*), promotion of plasminogen activator inhibitor-1 (PAI-1) release (*9*), and induction of proatherogenic endothelial gene expression including vascular adhesion molecule-1, intracellular adhesion molecule-1 (*10*), platelet derived growth factor, and heparin-binding epidermal growth factor-like growth factor (*11, 12*). In contrast, several studies have demonstrated that lysoPC augments the endothelial gene expression of potentially antiatherogenic or vasoprotective molecules such as nitric oxide synthase (*13, 14*) and cyclooxygenase-2 (*15*). These properties counter the proatherogenic profile of lysoPC. Therefore, the functional consequence of endothelial cell response to lysoPC stimulation is likely to be a complex process, and some aspects would be mediated by a wide spectrum of gene expression.

Endothelial dysfunction caused by various types of vascular injury is the initial step in atherosclerosis. LysoPC is considered to provide an atherogenic stimulus affecting endothelial cells (*1*). In the present study, we performed differential display analysis of mRNA on lysoPC-treated and untreated human umbilical vein endothelial cells (HUVEC) to identify potential mediators in atherogenesis. We identified 12 lysoPC up-regulated genes, including 5 cell growth-related genes, 3 thrombosis-related genes, and 4 others.

EXPERIMENTAL PROCEDURES

Cell Culture—HUVEC (Clonetics) were seeded on plastic plates precoated with bovine type I collagen (Sumitomo)

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Abbreviations: lysoPC, lysophosphatidylcholine; PAI-1, plasminogen activator inhibitor-1; HUVEC, human umbilical vein endothelial cells; 5′-RACE, rapid amplification of 5′-end of cDNA; PCR, polymerase chain reaction; PKCBP, protein kinase C-binding protein and substrate; 3′-UTR, 3′-untranslated region; ATF4, activating transcription factor-4; tPA, tissue plasminogen activator; NMDMC, NAD-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase; RTP, reducing agents and tunicamycin-responsive protein.

and cultured in modified MCDB131 medium (Sigma) supplemented with 2% fetal bovine serum (GIBCO BRL), 10 ng/ml human basic fibroblast growth factor (R&D Systems), and heparin at 10 units/ml (Novo Nordisk). All experiments were performed using confluent second to third passage cells. At 24 h before the addition of lysoPC (L-α-lysoPC, palmitoyl, Sigma), the medium was replaced with fresh medium. Cells cultured in the presence or absence of 50 μM lysoPC for 4 h were used for the differential display analysis. In the time-course experiments, cells were treated with 50 μM lysoPC for 0, 1, 2, 4, 6, 12, and 24 h. In the dose-response experiments, cells were treated with 0, 15, 30, or 50 μM lysoPC. In this set of experiments, the incubation time was adjusted to attain the maximum intensity of each mRNA level. In the culture medium containing 2% fetal bovine serum, lysoPC at concentrations of 50 μM and below, as estimated by trypan blue exclusion and cell counting, did not affect the viability of HUVEC incubated up to 24 h.

RNA Preparation—Total RNA was isolated from lysoPC-treated or untreated cells by a modified acid-guanidine thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene). For the differential display analysis, total RNA was further treated with MessageClean Kit (GenHunter) to remove residual DNA contamination, if present. For rapid amplification of 5′-ends of cDNA (5′-RACE), poly(A)$^+$ RNA was isolated from the total RNA by using Oligotex-dT 30 〈Super〉 (Takara Shuzo).

Differential Display Analysis—Differential display analysis (*16*) was performed as previously described (*17*). Briefly, purified total RNA (0.4 μg) was reverse-transcribed with 200 units of Superscript II reverse transcriptase (GIBCO BRL) in the presence of 0.5 μM degenerate oligo(dT) T11VN (where V represents either dG, dA, or dC, and N represents either dG, dA, dT, or dC) anchor primer and 0.5 mM dNTP. By this process, 12 different single-stranded cDNA pools were obtained. Each cDNA pool was then subjected to the polymerase chain reaction (PCR) for differential display analysis. We used 31 distinct decanucleotide primers. The PCR amplification products were separated on a non-denaturing 6% polyacrylamide gel ($126\times137\times1$ mm) and visualized with SYBR Green I (Molecular Probes). The differentially expressed bands ($\sim$150 to $\sim$500 bp) were extracted from the gels and reamplified using the corresponding primer sets.

TA Cloning and Sequence Analysis—The reamplified products were subcloned into the TA cloning vector pCRII (Invitrogen). The sequence reaction was performed using a Dye Primer Cycle Sequencing Kit (Perkin Elmer) and a Molecular Biology Labostation Catalyst model 8000 (Perkin Elmer). The reaction products were analyzed with a DNA sequencer model 373A (Perkin Elmer). The nucleotide sequences were compared with those stored in GenBank and EMBL databases by using BLAST (*18*) and FASTA (*19*). The PCR-amplified insert DNA fragments described here were also used for preparation of probes in the Northern blot analysis described below.

Northern Blot Analysis—Samples of total RNA (5 μg/lane) were separated on a 1% agarose gel containing 0.66 mM formaldehyde, 40 mM morpholinopropanesulfonic acid-NaOH (pH 7.2), 10 mM sodium acetate, 1 mM EDTA, and 0.01% SYBR Green II (Molecular Probes). After electrophoresis, the gel was excited at 254 nm with a transilluminator to verify the amount of RNA in each lane. RNA was transferred by capillary technique to a GeneScreen membrane (DuPont NEN), which was then UV-crosslinked using UV Stratalinker 2400 (Stratagene). Specific fluorescein-labeled DNA probes were generated by PCR using two primers (5′-AGTGTGCTGGAATTCGGC-3′ and 5′-ATATCTGCAGAATTCGGC-3′) that flank insert DNA in the pCRII vector (*17*). The PCR was performed using fluorescein-12-dUTP (DuPont NEN) and the PCR product described above. A fluorescein-labeled probe specific to human PAI-1 was also generated by the PCR using HUVEC cDNA as a template. Sense primer (5′-TTCATGCCCCACTTCTTCAG-3′) and antisense primer (5′-CAGGATGTCGTAGTAATGGC-3′), each corresponding to nucleotide position 337-356 and 665-684 of human PAI-1 cDNA (*20*), were used. The resultant PCR fragments were directly sequenced from both ends using a Dye Terminator Cycle Sequencing Kit (Perkin Elmer), yielding a predicted sequence of PAI-1. Hybridization and chemiluminescence detection were performed according to the manufacturer's instructions supplied with the RENAISSANCE Random Primer Fluorescein dUTP Labeling Kit (DuPont NEN).

cDNA Cloning of Gravin from HUVEC cDNA Library—A cDNA library constructed from homocysteine-treated HUVEC by using lambda ZAP vector (*17*) was screened for the isolation of human gravin cDNA. Positive phages were cloned by the PCR-based screening method as described previously (*17*). Approximately 2.3×10^5 phages were screened by the PCR using the gravin-specific primers (5′-GGATACCTCAGTATCTTGGG-3′ and 5′-AATGACTCCCAGGTGGAAAC-3′), which are based on the 5′-region sequences of fragment GG11 obtained by the differential display analysis. Finally, two positive phages (#10-1-32 and #11-5-13) were cloned. The pBluescript phagemids were excised by the *in vivo* excision protocol using ExAssist helper phage and *Escherichia coli* SOLR strain (Stratagene). The insert DNAs were sequenced using the dye-primer methods and the dye-terminator methods with 30 synthesized specific primers. The plasmids, pGRA-a and pGRA-b, were 4,426 and 6,072 bp in size, respectively. From their alignment with the nucleotide sequence of rat mitogenic regulatory protein gene and rat protein kinase C-binding protein and substrate (PKCBP) gene, both plasmids containing human gravin were considered to be partial. Therefore, we attempted to isolate the plasmids with the longer insert DNA using the same HUVEC library. We isolated six additional clones, but all of them showed the same 5′-sequence as pGRA-b. In order to obtain the 5′-nucleotide sequence of the gravin cDNA, we carried out the 5′-RACE assay using Marathon cDNA Amplification Kit (Clontech) and TaKaRa LA PCR Kit Ver.2 (Takara Shuzo). The amplified DNA fragments were cloned into the plasmid vector pCR II and sequenced.

RESULTS

Differential Display and Sequence Analyses—We compared the mRNA expression patterns of lysoPC-treated HUVEC with those of untreated cells by using differential display analysis. Figure 1 shows the representative patterns of amplified PCR products. We modified the differential display analysis using highly sensitive DNA staining SYBR Green I instead of radioactive ^{35}S and obtained 13

PCR fragments, all of which were expressed at higher levels in lysoPC-treated cells than in untreated cells. They are listed with their sizes in Table I. Except for GG11 and GT15, each sequence contained a potential polyadenylation signal (AATAAA).

The nucleotide sequences of the insert DNA were searched for homology with the GenBank and EMBL databases. The fragments CA14, CC19, AA2, GG11, CC1, and AT30 were respectively identical to or highly homologous with the 3′-untranslated region (3′-UTR) of the following cell growth-related genes: dual specificity phosphatase CL100 (*21*), dual specificity phosphatase B23/hVH-3 (*22, 23*), gravin (*24*), rat mitogenic regulatory gene (*25*)/rat PKCBP (*26*), activating transcription factor-4 (ATF4) (*27, 28*), and heparin-binding epidermal growth factor-like growth factor (*29*). The fragment AA2 showed 99.5 and 69% identity to 3′-UTR of human gravin and rat mitogenic regulatory gene, respectively. As described in a later section, we cloned and characterized the human gravin cDNA. The nucleotide sequencing of human gravin cDNA demonstrated that rat mitogenic regulatory gene or rat PKCBP gene was a rat homologue of gravin or a rat gravin-related gene. The fragment GG11 showed 78.9% identity to the open reading frame of rat mitogenic regulatory gene and 100% identity with the human gravin that was cloned in the present study. Therefore, both the AA2 and GG11 fragments were apparently derived from the identical gene, gravin.

The fragments GT31, GG17, and CA28 encoded thrombosis-related genes. Fragment GT31 showed 100% identity with PAI-1 (*20, 30*) and 95% identity with α-actinin (*31*), both of which share a highly homologous DNA sequence at the 3′-terminal portion. The fragments GG17 and CA28 were nearly identical to the 3′-UTR of tissue plasminogen activator (tPA) (*32*) and thrombomodulin (*33*), respectively.

The fragments GT15, AC27, CG22, and GC4 were respectively identical to or highly homologous with the 3′-

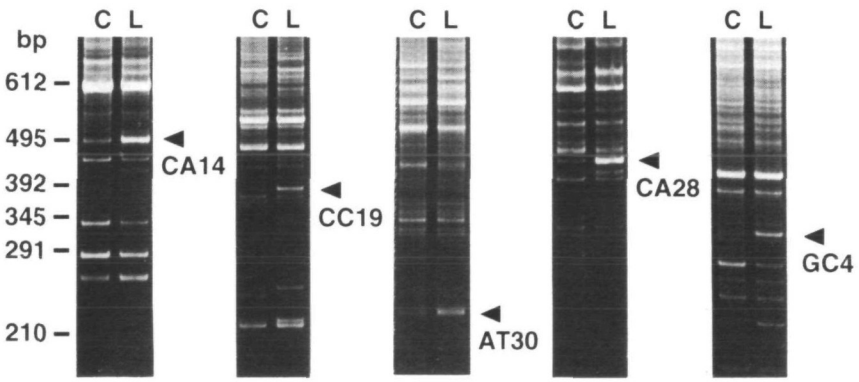

Fig. 1. **Representative band patterns on differential display analysis showing up-regulated PCR fragments in lysoPC-treated HUVEC.** Confluent monolayers of HUVEC were treated with or without 50 μM lysoPC in modified MCDB131 medium with 2% fetal bovine serum for 4 h. Total RNA was extracted and subjected to differential display analysis. Non-denaturing 6% polyacrylamide gels stained with SYBR Green I are shown for 5 different primer combinations that identified 5 distinct fragments (arrows) up-regulated in the lysoPC-treated HUVEC. The primer combinations used are as follows: (from left to right) T11CA and 5′-GATCAAGTCC-3′; T11CC and 5′-GATCATAGCC-3′; T11AT and 5′-ATTACCTGCC-3′; T11CA and 5′-CAACCATTCC-3′; T11GC and 5′-TTTTGGCTCC-3′. DNA size standards derived from ϕX174 DNA digested with *Hin*cII are shown in bp on the left.

TABLE I. **Differentially up-regulated fragments identified in lysoPC-stimulated HUVEC.**

cDNA fragment	T_{11}VN primer	Primers for PCR	Fragment size (bp)[a]	mRNA size (kb)[b]	Known gene with sequence similarity	% identity/bp[c]	GenBank accession No.
Cell growth-related proteins							
CA14	T_{11}CA	GATCAAGTCC, T_{18}C	467	2.4	Dual specificity phosphatase, CL100	99.8/438	X68277,S46269
CC19	T_{11}CC	GATCATAGCC, T_{18}C	395	2.6	Dual specificity phosphatase, B23/hVH-3	100/294	U15932, U16996
AA2	T_{11}AA	TGGATTGGTC, T_{18}A	474	8.5 & 6.8	Gravin	99.5/445	M96322
GG11	T_{11}GG	TACCTAAGCG, T_{18}G	320	8.5 & 6.8	Rat mitogenic regulatory gene (gravin homolog)	78.9/289	U23146
CC1	T_{11}CC	TACAACGAGG, T_{18}C	205	1.6	Activating transcription factor-4 (ATF4)	98.5/176	M86842
AT30	T_{11}AT	ATTACCTGCC, T_{18}A	221	Undetectable	Heparin-binding EGF-like growth factor (HB-EGF)	100/192	M60278
Thrombosis-related proteins							
GT31	T_{11}GT	CTAATCAGCC, T_{18}G	254	3.6 & 2.6	Plasminogen activator inhibitor-1 (PAI-1)	100/225	J03764, M55991
GG17	T_{11}GG	GATCTGACAC, T_{18}G	460	3.0	Tissue plasminogen activator (tPA)	99.1/431	X13097
CA28	T_{11}CA	CAACCATTCC, T_{18}C	413	3.8	Thrombomodulin	94.4/384	M16552
Others							
GT15	T_{11}GT	GATCCAGTAC, T_{18}G	361	4.0	Stanniocalcin	99.4/332	U25997
AC27	T_{11}AC	GCTTTTGAGG, T_{18}A	482	2.5	NAD-dependent methylenetetrahydrofolate dehydrogenase-cyclohydrolase (NMDMC)	98.7/453	X16396
CG22	T_{11}CG	GATCGCATTG, T_{18}C	362	2.4	BENE	99.7/333	U17077
GC4	T_{11}GC	TTTTGGCTCC, T_{18}G	340	3.3	Reducing agents and tunicamycin-responsive protein (RTP)	100/312	D87953

[a]The fragment size includes both primer sequences. [b]mRNA size (kb) was estimated by Northern blot analysis. [c]bp and % idnetity indicate the size of the DNA fragment not including the two primer sequences.

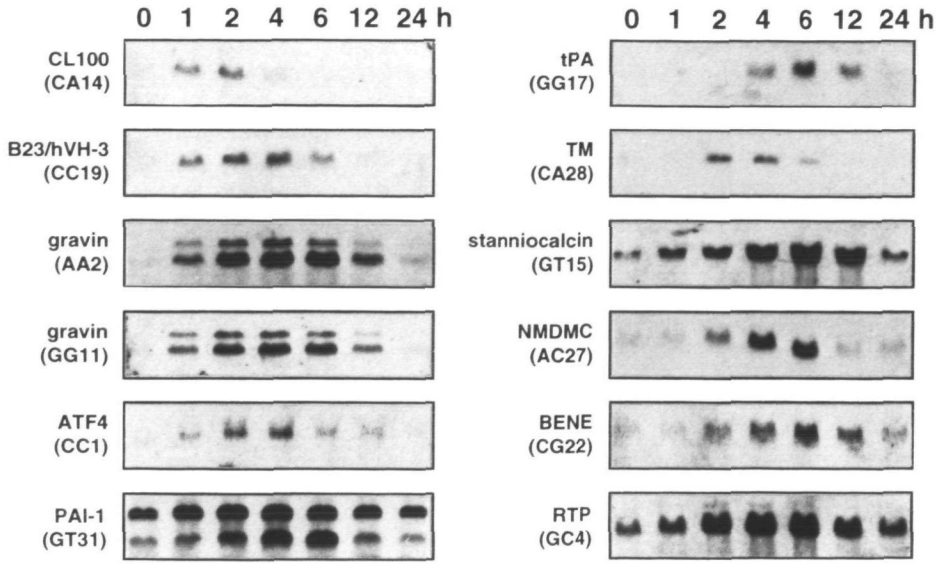

Fig. 2. **Time-dependent expression of genes in lysoPC-treated HUVEC.** At the indicated times after addition of 50 μM lysoPC, total RNA was isolated from the confluent HUVEC and subjected to Northern blot analysis using fluorescein-labeled probes and chemiluminescence detection procedures. Each lane contains 5 μg of total RNA. Equal loading of RNA was confirmed by staining of 18 S and 28 S ribosomal RNA by SYBR Green II (data not shown). The results shown are representative of two separate experiments. TM, thrombomodulin. Gravin and PAI-1 showed two distinct bands generated by alternative splicing.

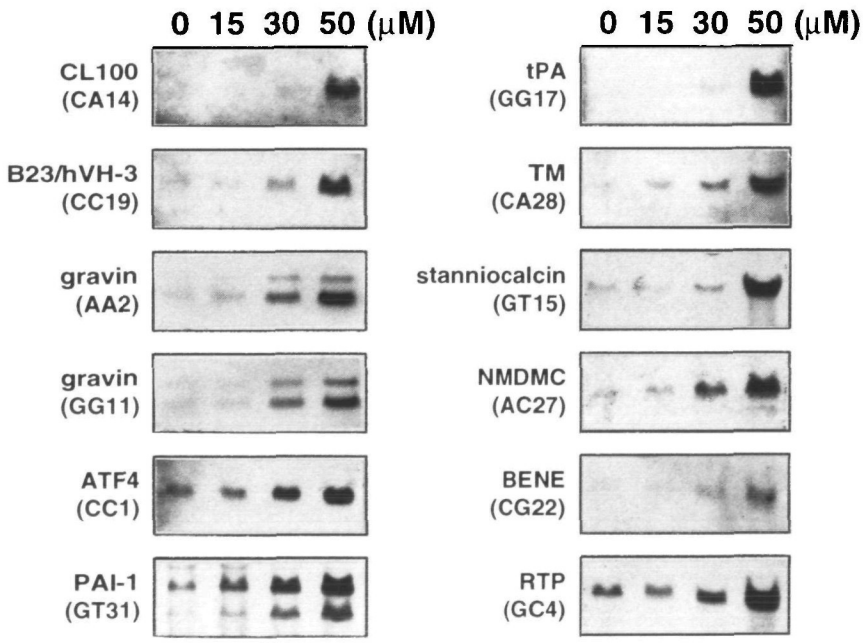

Fig. 3. **Dose-dependent expression of genes in lysoPC-treated HUVEC.** Confluent monolayers of HUVEC were treated with the indicated concentrations of lysoPC. Incubation periods were adjusted to attain maximum expression for each mRNA: 2 h for CA14 and CC19; 4 h for AA2, GG11, CC1, GT31, and CA28; and 6 h for GG17, GT15, AC27, CG22, and GC4. Total RNA was isolated and subjected to Northern blot analysis using fluorescein-labeled probes and chemiluminescence detection procedures. Each lane contains 5 μg of total RNA. Equal loading of RNA was confirmed by staining of 18 S and 28 S ribosomal RNA by SYBR Green II (data not shown). The results shown are representative of two separate experiments. TM, thrombomodulin. Gravin and PAI-1 showed two distinct bands generated by alternative splicing.

UTR of the following genes: stanniocalcin (*34, 35*), NAD-dependent methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase (NMDMC) (*36*), BENE (*37*), and reducing agents and tunicamycin-responsive protein (RTP) (*17*). RTP is a novel gene product, recently identified by our group, whose mRNA is up-regulated by homocysteine treatment in HUVEC. Thus, we identified 12 distinct up-regulated genes by lysoPC treatment in HUVEC.

Time-Dependent and Dose-Dependent Gene Expression in LysoPC-Treated HUVEC—We evaluated time-dependent changes of individual gene expression by using Northern blot analysis (Fig. 2). The expression of heparin-binding epidermal growth factor-like growth factor mRNA was not detected, probably due to the lower specificity of the probe resulting from its short fragment size and/or to a low mRNA content. Since the fragment GT31 showed sequence identity with both PAI-1 (100%) and α-actinin (95%), a specific probe for PAI-1 was generated and used for the Northern blot analysis. HUVEC were incubated with 50 μmol/liter lysoPC for 0, 1, 2, 4, 6, 12, and 24 h, then total RNA was isolated. CL100 mRNA (CA14) was induced as early as 1 h and declined to the basal level by 4

Fig. 4. **Nucleotide and deduced amino acid sequence of human gravin.** The predicted amino acid sequence is shown by the single letter code under the nucleotide sequence of cDNA. The amino acid positions relative to initiating Met are shown on the right in italic numerals. The underlined nucleotide sequence was obtained from the 5′-RACE analysis. The shaded sequences indicate two peptides that show sequence similarities to the phosphorylation peptides in rat PKCBP (*27*). Five potential nuclear localization signals with the motif K(R/K)X(R/K) are black-boxed. The asterisk indicates a stop codon, and the polyadenylation signal sequence is boxed.

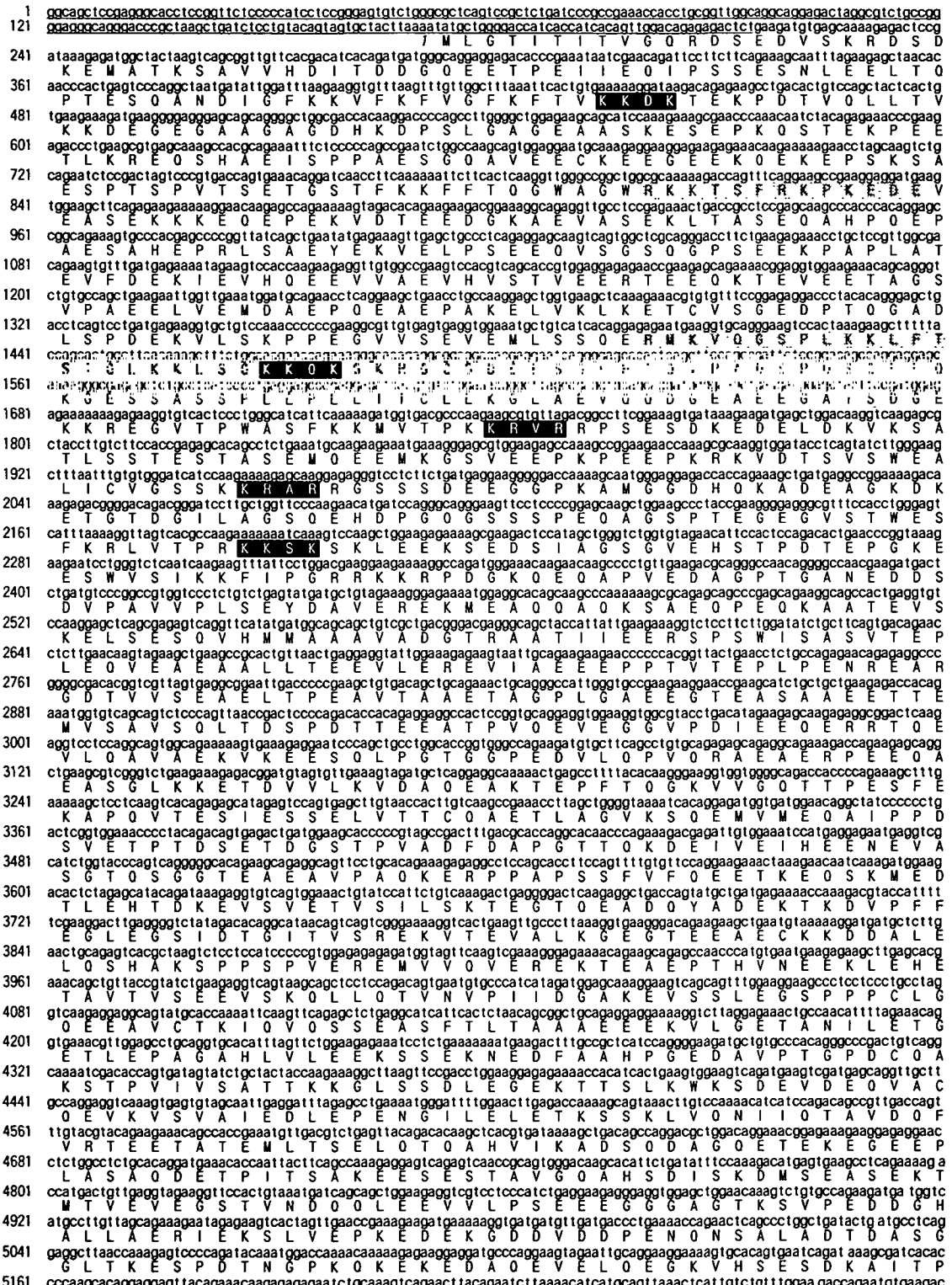

Fig. 4

h. The transcripts of B23/hVH-3 (CC19), gravin (AA2, GG11), PAI-1 (GT31), and RTP (GC4) exhibited a slight increase at 1 h, reached a peak at 4 h, and gradually decreased to the basal level by 24 h. The induction of ATF4 (CC1), thrombomodulin (CA28), and NMDMC (AC27) was observed within a relatively short period (by 2 to 6 h). For tPA (GG17), stanniocalcin (GT15), and BENE (CG22), the maximum response was observed after 6 h of treatment. The elevation of each transcript level was transient and did not persist at the maximum level.

We then evaluated the dose-dependent expression of each gene. HUVEC were incubated with 0, 15, 30, or 50 μM lysoPC, then total RNA was isolated for Northern blot analysis (Fig. 3). Concentrations of 75 μM or higher were not appropriate for the analysis because lysoPC was cytotoxic to HUVEC. At concentrations of 10 μM or below, lysoPC did not affect the expression levels of the genes. The induction of PAI-1 and thrombomodulin was observed even at 15 μM concentration of lysoPC. Expression of most of the genes was observed at 30 μM concentration, and the maximum response was observed at 50 μM.

cDNA Cloning and Nucleotide Sequence of Human Gravin—The lysoPC up-regulated genes other than gravin and BENE were well-characterized. Human gravin mRNA is estimated to be 6.8 or 8.5 kb in size (*24*). Therefore, we attempted to identify a full-length cDNA of human gravin. We isolated eight clones encoding gravin from screening of the HUVEC cDNA library. The longest human gravin clone, pGRA-b, was 6,072 bp in length. Homologies with this nucleotide sequence were searched for with the GenBank and the EMBL databases. The search demonstrated that this sequence is homologous with rat mitogenic regulatory protein gene (*25*) or rat PKCBP gene (*26*), but encodes a partial cDNA. To obtain the 5′-nucleotide sequence of human gravin, we performed the 5′-RACE assay using poly(A)$^+$ RNA prepared from lysoPC-treated HUVEC and isolated a 374-bp fragment. Figure 4 shows a contiguous cDNA sequence of 6,287 bases and the deduced amino acid sequence of human gravin. It shows an open reading frame of 1,684 amino acids with a calculated molecular mass of 181,671 Da. Human gravin is a highly acidic protein with 18% Glu residues and its calculated isoelectric point is 4.25. It has at least five potential nuclear localization signals with the motif K(R/K)X(R/K): KKDK (amino acids 88 to 91), KKQK (amino acids 433 to 436), KRVK (amino acids 523 to 526), KRAK (amino acids 592 to 595), and KKSK (amino acids 672 to 675). Fragments AA2 and GG11 obtained from the differential display analysis were positioned, respectively, in 3′-UTR (nucleotide number: 5830–6287) and in the coding sequence (nucleotide number: 1881–2200).

The previously isolated partial cDNA of human gravin (1,981 bp in length) (*24*) corresponds to approximately one-third of the full-length cDNA we isolated and shows 99.5% identity with the 3′-portion of our sequence, with one inframe deletion and two missense mutations in the coding sequence, and three additional mutations in the 3′-UTR. The human gravin showed extensive sequence homologies to rat mitogenic regulatory protein (*25*) and rat PKCBP (*26*) with 58.2 and 54.7% amino acid identity, respectively. PKCBP can be phosphorylated *in vitro* and two phosphorylation peptides were identified (*26*). The potential phosphorylation sites, Ser and Thr residues, in these two phosphorylation peptides are highly homologous with the corresponding region of human gravin (shaded sequences in Fig. 4).

DISCUSSION

In the present study, we applied differential display analysis to investigate the change of endothelial gene expression induced by lysoPC, and identified 12 up-regulated genes in lysoPC-treated HUVEC. These 12 genes can be categorized into three groups: growth-related genes, thrombosis-related genes, and functionally uncharacterized genes. One of the striking findings of our present study is the up-regulation by lysoPC of growth-related genes that have not been reported in endothelial cells: two dual specificity phosphatases, gravin and ATF4.

Two phosphatases, CL100 and B23/hVH-3, inactivate activated forms of mitogen-activated protein kinase through dephosphorylation (*23, 38, 39*). Growth stimuli such as growth factors, serum, and phorbol ester induce the gene expression of these two phosphatases (*21-23*). In addition, certain kinds of stress such as heat shock, ischemia, or oxidative stress also induce the gene expression of CL100 (*21*).

One of the growth-related genes up-regulated by lysoPC is gravin, a ~250-kDa cytoplasmic protein originally identified as a protein recognized with the serum from a patient with myasthenia gravis (*24*). We isolated human gravin cDNA and clarified its biochemical features: gravin is an acidic protein consisting of 1,684 amino acids with 18% Glu residues and containing multiple nuclear localization signals. More importantly, full sequencing of the cDNA enabled us to identify two rat homologues of human gravin and to identify this gene as growth-related. Rat mitogenic regulatory gene was identified as one of the genes transcriptionally suppressed in NIH3T3 cells transformed by *src, ras*, and *fos*. Overexpression of this gene product resulted in decreased cell proliferation rate (*25*). Rat PKCBP has been isolated as a binding protein of protein kinase C. PKCBP is also a substrate of protein kinase C (*26*). This growth-related nature of rat homologues or related proteins suggests that human gravin is a growth-related protein. Recently, the complete amino acid sequence of human gravin has been deduced from the nucleotide sequence of its full-length cDNA clone (*40*). The alignment showed that the amino-terminal 8 amino acids of our sequence has been replaced by 106 amino acids in their sequence, indicating the possible alternative splicing in this region.

Another growth-related gene up-regulated by lysoPC is ATF4. ATF4, also known as cAMP response element binding protein-2 (*28*) or TAXREB67 (*27*), is a member of the ATF/cAMP response element binding protein subfamily of basic region/leucine zipper-containing transcription factors. Since overexpression of ATF4 resulted in a significant repression of cAMP response element-dependent transcription (*28*), ATF4 is likely to be involved in the regulation of cell growth (*41*). It is not clear whether the changes in these mRNA levels for growth-related genes reflect the mitogenic activity of lysoPC for HUVEC or endothelial responses to growth-regulatory effects of lysoPC. However, our preliminary observation that lysoPC induces tyrosine phosphorylation of MAPK (Sato *et al.*,

unpublished observation) favors the possibility that lysoPC stimulates proliferation of HUVEC, as reported with other cell types (*7, 8*).

We also identified 3 lysoPC-up-regulated endothelial genes involved in thrombosis and fibrinolysis (tPA, PAI-1, and thrombomodulin). Up-regulation of PAI-1 by lysoPC would account for the increased PAI-1 antigen release from HUVEC elicited by lysoPC (*9*). The increased mRNA expression produced by lysoPC of both PAI-1 and tPA makes it difficult to interpret the effect of lysoPC on the net fibrinolytic activity of endothelial cells. However, unlike that of tPA, the mRNA expression of PAI-1 was augmented even at 15 μmol/liter lysoPC concentration, and the augmentation persisted for a longer period of time. Furthermore, it has been reported that lysoPC does not effect tPA antigen release (*9*). Therefore, enhanced expression of PAI-1 rather than tPA seems likely to have the more predominant up-regulatory effect. Thrombomodulin, another up-regulated gene, is an endothelial cell surface anticoagulant protein that promotes activation of the anticoagulant protein C and inhibits the procoagulant activities of thrombin. We have observed in other experiments that lysoPC suppresses the mRNA and antigen levels of coagulation factors Xa/VIIa inhibitor, tissue factor pathway inhibitor, in HUVEC, whereas no change of the mRNA level of procoagulant tissue factor was induced (*42*). Therefore, the net effects of lysoPC on endothelial cell surface anticoagulant activity seem to be very complex.

In addition to the genes involved in cell growth or thrombosis, four other genes were induced by lysoPC in HUVEC. Stanniocalcin regulates calcium homeostasis (*34, 35*). Since elevated intracellular Ca^{2+} concentration is known to induce stanniocalcin mRNA (*35*), the enhancement of stanniocalcin mRNA expression by lysoPC might be mediated by increased intracellular Ca^{2+} concentration. Indeed, it was reported that the intracellular Ca^{2+} concentration was increased through Ca^{2+} influx induced by lysoPC (*43*). NMDMC is a mitochondrial bifunctional enzyme which has the activities of both 5,10-methylenetetrahydrofolate dehydrogenase and 5,10-methenyltetrahydrofolate cyclohydrolase, and is involved in tetrahydrofolate metabolism (*36*). Partial cDNA of BENE has been isolated from PC-3 cells (*37*). The nucleotide sequence showed 58% identity to that of T-cell differentiation protein, MAL, although the precise function is unknown. RTP has been identified as one of the up-regulated genes in homocysteine-treated HUVEC by our group (*17*). This gene was also up-regulated by reducing agents such as mercaptoethanol and by glycosylation inhibitor tunicamycin (*17*), strongly suggesting that RTP is induced by the unfolded protein response pathway (*44*).

We have previously studied changes in endothelial gene expression induced by another atherogenic stimulus, homocysteine, and identified six up-regulated and one down-regulated genes by using differential display analysis (*17*). The interesting common feature of changes in gene expression induced by homocysteine and lysoPC is that both share the up-regulation of three genes, ATF4, NMDMC, and RTP, all of which have hitherto been regarded as atherosclerosis-unrelated. Although the mechanisms of action of homocysteine and lysoPC on endothelial gene expression seem to be quite different, they might be involved in atherogenesis through the expression of common genes. Here we identified a variety of genes induced by lysoPC using differential display analysis. Additional studies are necessary to determine whether increased expression of these proteins is crucial for atherogenesis.

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REFERENCES

1. Ross, R. (1993) The pathogenesis of atherosclerosis; a perspective for the 1990's. *Nature* **362**, 801-809
2. Steinberg, D.S., Parthasarathy, T.E., Carew, J., Khoo, C., and Witztum, J.L. (1989) Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320**, 915-924
3. Witztum, J.L. and Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88**, 1785-1792
4. Quinn, M.T., Parthasarathy, S., and Steinberg, D. (1988) Lysophosphatidylcholine: A chemotactic factor for human monocytes and its potential role in atherogenesis. *Proc. Natl. Acad. Sci. USA* **85**, 2805-2809
5. McMurray, H.F., Parthasarathy, S., and Steinberg, D. (1993) Oxidatively modified low density lipoprotein is a chemoattractant for human T lymphocytes. *J. Clin. Invest.* **92**, 1004-1008
6. Kugiyama, K., Kerns, S.A., Morrisett, J.D., Roberts, R., and Henry, P.D. (1990) Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* **344**, 160-162
7. Sakai, M., Miyazaki, A., Hakamata, H., Sakaki, T., Yui, S., Yamazaki, M., Shichiri, M., and Horiuchi, S. (1994) Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. *J. Biol. Chem.* **269**, 31430-31435
8. Locher, R., Weisser, B., Mengden, T., Brunner, C., and Vetter, W. (1992) Lysolecithin actions on vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **183**, 156-162
9. Kugiyama, K., Sakamoto, T., Misumi, I., Sugiyama, S., Ohgushi, M., Ogawa, H., Horiguchi, M., and Yasue, H. (1993) Transferable lipids in oxidized low-density lipoprotein stimulate plasminogen activator inhibitor-1 and inhibit tissue-type plasminogen activator release from endothelial cells. *Circ. Res.* **73**, 335-343
10. Kume, N., Cybulsky, M.I., and Gimbrone, M.A. Jr. (1992) Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J. Clin. Invest.* **90**, 1138-1144
11. Kume, N. and Gimbrone, M.A. Jr. (1994) Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. *J. Clin. Invest.* **93**, 907-911
12. Nakano, T., Raines, E.W., Abraham, J.A., Klagsbrun, M., and Ross, R. (1994) Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. *Proc. Natl. Acad. Sci. USA* **91**, 1069-1073
13. Hirata, K., Miki, N., Kuroda, Y., Sakoda, T., Kawashima, S., and Yokoyama, M. (1995) Low concentration of oxidized low-density lipoprotein and lysophosphatidylcholine upregulate constitutive nitric oxide synthase mRNA expression in bovine aortic endothelial cells. *Circ. Res.* **76**, 958-962
14. Zembowicz, A., Tang, J., and Wu, K.K. (1995) Transcriptional induction of endothelial nitric oxide synthase type III by lysophosphatidylcholine. *J. Biol. Chem.* **270**, 17006-17010
15. Zembowicz, A., Jones, S.L., and Wu, K.K. (1995) Induction of cyclooxygenase-2 in human umbilical vein endothelial cells by lysophosphatidylcholine. *J. Clin. Invest.* **96**, 1688-1692
16. Liang, P. and Pardee, A.B. (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**, 967-971

17. Kokame, K., Kato, H., and Miyata, T. (1996) Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis: GRP78/BiP and novel genes. *J. Biol. Chem.* **271**, 29659-29665
18. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403-410
19. Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**, 2444-2448
20. Ny, T., Sawdey, M., Lawrence, D., Millan, J.L., and Loskutoff, D.J. (1986) Cloning and sequence of cDNA coding for human β-migrating endothelial-cell-type plasminogen activator inhibitor. *Proc. Natl. Acad. Sci. USA* **83**, 6776-6780
21. Keyse, S.M. and Emslie, E.A. (1992) Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. *Nature* **359**, 644-647
22. Ishibashi, T., Bottaro, D.P., Michieli, P., Kelley, C.A., and Aaronson, S.A. (1994) A novel dual specificity phosphatase induced by serum stimulation and heat shock. *J. Biol. Chem.* **269**, 29897-29902
23. Kwak, S.P. and Dixon, J.E. (1995) Multiple dual specificity protein tyrosine phosphatases are expressed and regulated differentially in liver cell lines. *J. Biol. Chem.* **270**, 1156-1160
24. Gordon, T., Grove, B., Loftus, J.C., O'Toole, T., McMillan, R., Lindstrom, J., and Ginsberg, M.H. (1992) Molecular cloning and preliminary characterization of a novel cytoplasmic antigen recognized by myasthenia gravis sera. *J. Clin. Invest.* **90**, 992-999
25. Lin, X., Nelson, P.J., Frankfort, B., Tombler, E., Johnson, R., and Gelman, I.H. (1995) Isolation and characterization of a novel mitogenic regulatory gene, 322, which is transcriptionally suppressed in cells transformed by *src* and *ras*. *Mol. Cell. Biol.* **15**, 2754-2762
26. Chapline, C., Mousseau, B., Ramsay, K., Duddy, S., Li, Y., Kiley, S.C., and Jaken, S. (1996) Identification of a major protein kinase C-binding protein and substrate in rat embryo fibroblasts. Decreased expression in transformed cells. *J. Biol. Chem.* **271**, 6417-6422
27. Tsujimoto, A., Nyunoya, H., Morita, T., Sato,T., and Shimotohno, K. (1991) Isolation of cDNAs for DNA-binding proteins which specifically bind to a *tax*-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I. *J. Virol.* **65**, 1420-1426
28. Karpinski, B.A., Morle, G.D., Huggenvik, J., Uhler, M.D., and Leiden, J.M. (1992) Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. *Proc. Natl. Acad. Sci. USA* **89**, 4820-4824
29. Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C., and Klagsbrun, M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936-939
30. Ginsburg, D., Zeheb, R., Yang, A.Y., Rafferty, U.M., Andreasen, P.A., Nielsen, L., Dano, K., Lebo, R.V., and Gelehrter, T.D. (1986) cDNA cloning of human plasminogen activator-inhibitor from endothelial cells. *J. Clin. Invest.* **78**, 1673-1680
31. Youssoufian, H., McAfee, M., and Kwiatkowski, D.J. (1990) Cloning and chromosomal localization of the human cytoskeletal a-actinin gene reveals linkage to the β-spectrin gene. *Am. J. Hum. Genet.* **47**, 62-72
32. Harris, T.J.R., Patel, T., Marston, F.A.O., Little, S., Emtage, J.S., Opdenakker, G., Volckaert, G., Rombauts, W., Billiau, A., and de Somer, P. (1986) Cloning of cDNA coding for human tissue-type plasminogen activator and its expression in *Escherichia coli*. *Mol. Biol. Med.* **3**, 279-292
33. Suzuki, K., Kusumoto, H., Deyashiki, Y., Nishioka, J., Maruyama, I., Zushi, M., Kawahara, S., Honda, G., Yamamoto, S., and Horiguchi, S. (1987) Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. *EMBO J.* **6**, 1891-1897
34. Wagner, G.F., Guiraudon, C.C., Milliken, C., and Copp, D.H. (1995) Immunological and biological evidence for a stanniocalcin-like hormone in human kidney. *Proc. Natl. Acad. Sci. USA* **92**, 1871-1875
35. Chang, A.C.M., Janosi, J., Hulsbeek, M., de Jong, D., Jeffrey, K.J., Noble, J.R., and Reddel, R.R. (1995) A novel human cDNA highly homologous to the fish hormone stanniocalcin. *Mol. Cell. Endocrinol.* **112**, 241-247
36. Peri, K.G., Belanger, C., and Mackenzie, R.E. (1989) Nucleotide sequence of the human NAD-dependent methylene tetrahydrofolate dehydrogenase-cyclohydrolase. *Nucleic Acids Res.* **17**, 8853
37. Lautner-Rieske, A., Thiebe, R., and Zachau, H.G. (1995) Searching for non-Vk transcripts from the human immunoglobulin k locus. *Gene* **159**, 199-202
38. Sun, H., Charles, C.H., Lau, L.F., and Tonks, N.K. (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. *Cell* **75**, 487-493
39. Sun, H., Tonks, N.K., and Bar-Sagi, D. (1994) Inhibition of ras-induced DNA synthesis by expression of the phosphatase MKP-1. *Science* **266**, 285-288
40. Nauert, J.B., Klauck, T.M., Langeberg, L.K., and Scott, J.D. (1996) Gravin, an autoantigen recognized by serum from myasthenia gravis patients, is a kinase scaffold protein. *Curr. Biol.* **7**, 52-62
41. Mielnicki, L.M., Hughes, R.G., Chevray, P.M., and Pruitt, S.C. (1996) Mutated ATF4 suppresses c-Ha-ras oncogene transcript levels and cellular transcription in NIH3T3 fibroblasts. *Biochem. Biophys. Res. Commun.* **228**, 586-595
42. Sato, N., Kokame, K., Miyata, T., and Kato, H. (1998) Lysophosphatidylcholine decreases the synthesis of tissue factor pathway inhibitor in human umbilical vein endothelial cells. *Thromb. Haemost.* **79**, 217-221
43. Chen, Y., Morimoto, S., Kitano, S., Koh, E., Fukuo, K., Jiang, B., Chen, S., Yasuda, O., Hirotani, A., and Ogihara, T. (1995) Lysophosphatidylcholine causes Ca^{2+} influx, enhanced DNA synthesis and cytotoxicity in cultured vascular smooth muscle cells. *Atherosclerosis* **112**, 69-76
44. Cox, J.S. and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* **87**, 391-404