Gene Expression, Vol. 13, pp. 155–165
Printed in the USA.

1052-2166/06 $90.00 + .00
E-ISSN 1555-3884
www.cognizantcommunication.com

Growth Arrest-Inducing Genes Are Activated in Dbl-Transformed Mouse Fibroblasts

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The Dbl oncogene is a guanine nucleotide exchange factor for Rho GTPases and its activity has been linked to the regulation of gene transcription. Dbl oncogene expression in NIH3T3 cells leads to changes in morphological and proliferative properties of these cells, inducing a highly transformed phenotype. To gain insights into Dbl oncogene-induced transformation we compared gene expression profiles between Dbl oncogene-transformed and parental NIH3T3 cells by cDNA microarray. We found that Dbl oncogene expression is associated with gene expression modulation involving upregulation of 51 genes and downregulation of 49 genes. Five of the over-expressed genes identified are known to exert antiproliferative functions. Our observations suggest that the expression of Dbl oncogene in NIH3T3 may lead to the induction of genes associated with cell cycle arrest, possibly through the activation of stress-induced kinases.

Key words: Microarray; Dbl oncogene; Gene expression

INTRODUCTION

Guanine nucleotide exchange factors (GEFs) for Rho GTPases constitute a large family of proteins all characterized by a region of sequence similarity consisting of the catalytic Dbl homology (DH) domain and a Pleckstrin homology (PH) domain (39). Proto-Dbl is the prototype member of this family of proteins and functions as a GEF for RhoA and Cdc42 GTPases. Truncation of the amino-terminal 497 residues in proto-Dbl results in constitutive activation of the carboxyl-terminal sequences (onco-Dbl), whose expression in NIH3T3 fibroblast confers upon them a highly transformed phenotype (28).

Rho family proteins are tightly regulated molecular switches that cycle between a biologically active GTP-bound and an inactive GDP-bound form, controlling multiple important aspects of cellular functions. Evidence has emerged linking the Rho family proteins, and by extension the exchange factors that activate them, to the regulation of gene expression. Constitutively activated Rho GTPases Rac1 and Cdc42 are activators of Jun N-terminal kinase (JNK) and p38 MAPK (3,21). Rho, Rac, and Cdc42 have been shown to activate serum response factor (SRF), which cooperates with ternary complex factors (Elk-1 and SAP1) and the DNA elements found in certain promoters such as the c-fos promoter (13). Finally a link between RhoA, Rac, and Cdc42 and cell cycle progression has been made and this may be mediated by regulating the expression of cyclin D1 (23,35).

Many cellular activities induced by Dbl family members are associated with the activation of signaling pathways mediated by active Rho GTPases or by their effector targets. Thus, the common assumption is that any protein that contains the DH/PH domain tandem is a GEF for a Rho GTPase and that the high transformation capability exhibited by many members of the Dbl family, including Dbl itself, is the direct result of the activation of a Rho-related protein and of its downstream signaling pathway. This activation would ultimately lead to transcriptional induc-

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tion of genes involved in a diverse number of cellular processes.

To gain insights into the genes induced by onco-Dbl in mouse fibroblasts we compared gene expression profile of parental and Dbl-transformed NIH3T3 cells. Our study identified a cluster of genes that are linked to inhibition of cell proliferation.

MATERIALS AND METHODS

Plasmids and Antibodies

Construction of the pZIPNeo-ΔNDbl and proto-Dbl was previously described (29). IMAGE cDNA clones Gadd45a, Myd116, and Pdcd2 were purchased from the Resource Centre-Primary Database of Berlin. Chop10 and Btg1 clones were a kind gift of Dr. William D. Rees and Dr. Laura Corbo, respectively. Anti-Dbl polyclonal antibodies were generated as described (38). Antibodies anti-Chop10, Myd116, Gadd45, and p38 were from Santa Cruz Biotechnology. Anti-phospho-p38 (P-p38) was purchased from Cell Signalling.

Cell Culture and RNA Extraction

NIH3T3 onco-Dbl transfectants, cell lines 151-11-44 and 152-6-11, and proto-Dbl transfectant, cell line 533-13-11, have been previously described (4). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum to 80% confluency and detached by trypsinization. Total RNA was purified using the Trizol RNA reagent (Invitrogen-Life Technology) according to the manufacturer's instructions.

cDNA Arrays, Probes, and Hybridization

Microarray experiments were performed on 2,700 features mouse oncochips (National Cancer Institute, Bethesda, MD, USA) version Mm-OC-6.1p 5-062200. The gene list is available at the web site (http://nciarray.nci.nih.gov) and cDNA set spanned 2,048 named genes, 398 expressed sequence tags (ESTs), and 202 unknown sequences. cDNA clones were selected from the Image Consortium and identified by their IMAGE ID. cDNA fluorescent-labeled probes were synthesized from 30–60 μg total RNA by oligo-dT primer polymerization using SuperScript II RT reverse transcriptase (Invitrogen-Life Technologies) in 40 μl of a reaction mix containing: 1 mM Cyanine dye-conjugated (Cy3 or Cy5)-dUTP (Amersham Pharmacia Biotech Inc.), 4.0 μg Oligo (dT) (Life Technologies), 10.0 mM dithiothreitol (DTT), 0.5 mM each dATP-dGTP and dCTP, 0.2 mM dTTP (Amersham Pharmacia Biotech Inc.), and 20 U RNAsin (Promega). Reactions were carried for 1 h at 42°C and labeled probes were purified and concentrated using Microcone YM-30 columns (Millipore, Bedford, MA). The appropriate Cy3 and Cy5 probes were combined and denaturated in a blocking solution containing: 10 μg of mouse COT-1 DNA (Boehringer Mannheim), 8–10 μg Poly(A) (Amersham Pharmacia Biotech Inc.), 4 μg yeast tRNA (Sigma-Aldrich). The probes were adjusted to 18 μl with 3.5× SSC, 0.3% SDS and applied to the slide. Hybridization was carried out at 65°C for 16 h under a cover slip in a humidified chamber submerged in a water bath. Slides were washed twice for 2 min each in 1× SSC/0.1% SDS, 1× SSC, 0.2× SSC, 0.05× SSC, and spun dried by centrifugation ($50 \times g$ for 3 min.). To control for gene-specific dye bias, in some experiments cyanine dyes were switched between control and test RNA.

Scanning Arrays and Analysis

Slides were analyzed with fluorescent confocal scanner (GenePix 4000, Axon Instruments, CA, USA) using two wavelengths of 532 and 635 nm for the green and the red, respectively, laser power between 0.93 and 1.03, and a photo multiplier tube gain (PMT) setting ranging from 790 to 760 volts. Image analysis and calculation of average signal for each channel was performed with GENEPIX Pro 3.0 Software. Image and signal intensity data were stored in a database supported by the Center for Information Technology of the National Institute of Health (NIH). Statistical Analysis was performed using the software mAdb-Tool Suite of the NCI/CCR mArray Centre (http://nciarray.nci.nih.gov). The signal intensity value was derived by subtracting the local background from the median fluorescent signal intensity of each spot and the Cy5/Cy3 intensity ratio was calculated. Acceptance of the spot for analysis was based on the following criteria: spots size greater than 50 μm, ratio of spot/background signal intensity greater than 2, and signal intensity in each channel greater than 500 relative fluorescent units. Data were normalized using the "50th percentile method," dividing the raw data by the median of all measurements (25). An "MA-plot" was used to represent the data, where $M = \log_2 \text{red/green}$ and $A = \log_2\sqrt{(\text{red} \times \text{green})}$ and genes were considered modulated in the test RNA when ratios were greater than 1 or smaller than −1, corresponding to a twofold increase or decrease, respectively (7,27). To specify differentially expressed genes between the two conditions a one-way analysis of variance (ANOVA) was performed. Genes were considered differentially expressed based on the results of a Student's two-sample *t*-test at a confidence

level of 95% ($p < 0.05$). Each modulated gene was associated with a Gene Ontology (GO) Consortium attribute (32).

Northern Blot Analysis

cDNA inserts were obtained by appropriate restriction enzyme digestion of recombinant plasmid clones, purified by gel extraction (Perfectprep, Eppendorf), and labeled with [α-^{32}P]dCTP (RadPrime DNA labeling system, Invitrogen, Life Technologies). A total of 20 µg of RNA from each sample was electrophoresed under denaturating conditions in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred onto Nytran membranes (Schleicher & Schuell, Keene, NH). Membranes were hybridized overnight at 65°C in Hybrisol solution (Oncor, Gaithersburg, MD, USA) with 10^6 cpm/ml of ^{32}P-labeled probe. Membranes were washed three times for 10 min each in 2× SSC/0.1% SDS at 42°C, two times for 15 min in 0.2× SSC/0.1% SDS at 65°C, and exposed to Kodak film.

Western Blot Analysis

Exponentially growing cells were lysed in lysis buffer containing 10 mM sodium phosphate, pH 7.4, 0.1 M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate (DOC), 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (ABSF), 1 mM leupeptin and aprotinin. Lysates were clarified, subjected to 10% SDS polyacrilamide gel electrophoresis (PAGE). and immunoblotted with specific antibodies. Immunocomplexes were visualized by West Dura extended chemiluminescence detection (Pierce) using protein G horseradish peroxidase—conjugated (Pierce).

Assay for Activation of p38 MAPK

To assess the levels of activated p38 MAPK, 80% confluent NIH3T3 transfectants were grown for 20 h in DMEM containing 0.5% fetal calf serum and lysed in a buffer containing 20 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton-X, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 0.5 mM NaF, 1 mM AEBSF, 10 µg/ml each of aprotinin and leupeptin. Whole cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with phospho-specific antibodies to detect P-p38 and anti-p38 to detect kinase expression levels.

RESULTS

Identification of Genes Modulated in Onco-Dbl-Transformed Cells

To investigate onco-Dbl-induced gene expression patterns in NIH3T3 cells we performed cDNA microarray analysis. The clonal cell line 151-11-44 was selected among several onco-Dbl-transformed cells because of previous extensive biological and biochemical characterizations. These cells express onco-Dbl transcript and product (Fig. 1), and display all the features associated with onco-Dbl transforming activity (5). As control, parental NIH3T3 cells were used (Fig. 1).

Total RNA was used to synthesize fluorescently Cy3- or Cy5-labeled cDNA microarray probes. Microarray analysis was carried out using the 2.7K features murine oncochip previously used to study gene expression in experimental tumors (6). The relative abundance of each RNA was measured by directly comparing the signals from competitive hybridizations, resulting in a red/green expression ratio of each gene. The scatter plot (Fig. 2) displays the signal intensity versus the ratio of the signal from Dbl-transformed over control cells for each probe. One hundred cDNAs were found to be differentially expressed

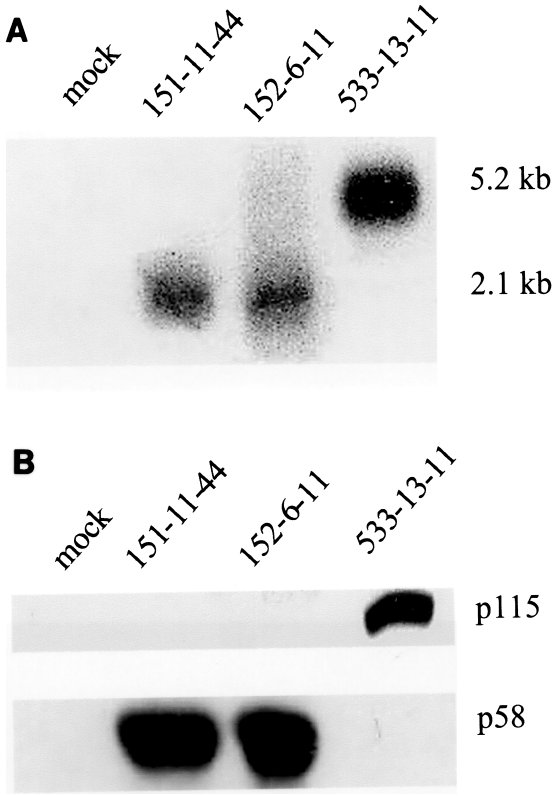

Figure 1. Expression of onco-Dbl and proto-Dbl in NIH3T3 transfectants. (A) Total RNA was harvested from mock transfected NIH3T3 cells, ΔNpDbl transfectant 151-11-44, ΔNpDbl transfectant 152-6-11, and proto-Dbl transfectant 533-13-11 and analyzed by Northern Blot hybridization. ^{32}P-Labeled onco-Dbl cDNA was used as probe. (B) Immunodetection of onco-Dbl and proto-Dbl products in NIH3T3 transfectants. Proteins were separated by SDS-PAGE and immunoblotted with anti-Dbl-specific antibody. Onco-Dbl p58 and proto-Dbl p115 products are indicated.

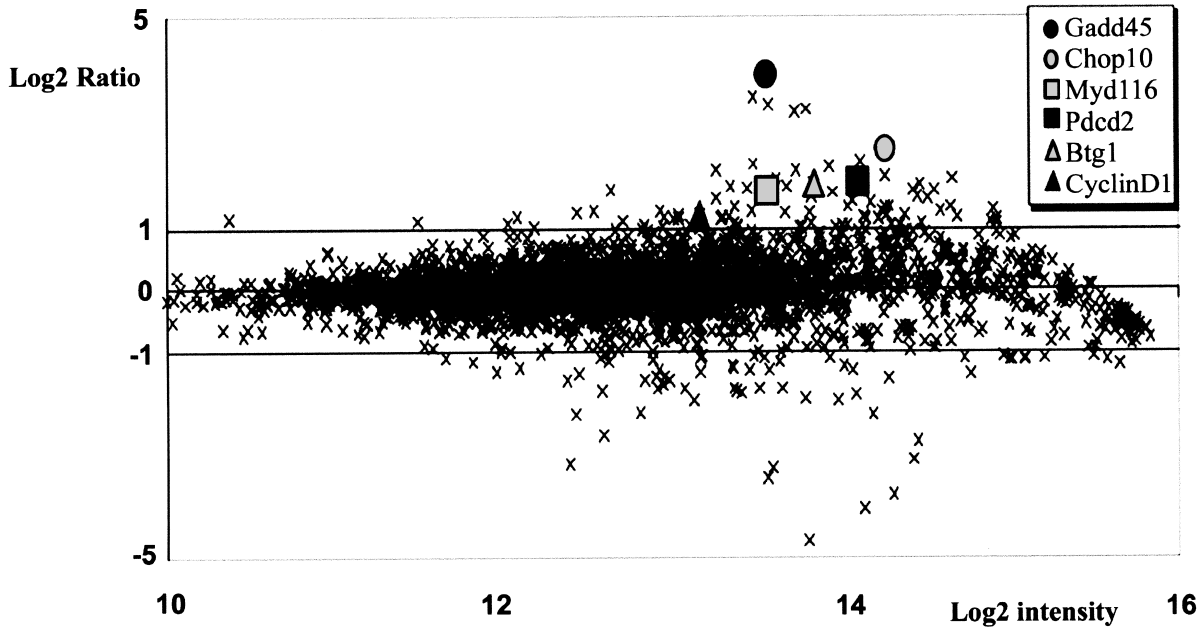

Figure 2. Scatter plot of differential gene expression. Results from the three chips were averaged. Graphical presentation of arrays was obtained by plotting M [$\log_2$(Cy5/Cy3)] on the y axis versus A [$\log_2\sqrt{(Cy5 \times Cy3)}$] on the x axis. The two parallel lines at $y = -1$ and $y = 1$ represent the fold change threshold. Values external to such lines are considered indicative of modulation of gene expression. The symbols shown in the graph indicate the intensity ratio of cyclin D1 and the five genes with antiproliferative functions induced in Dbl transfectants (inset).

in replicated arrays. As shown in Table 1, 51 genes were upregulated and 49 genes downregulated in onco-Dbl transformants relative to control NIH3T3. Of the outlier genes, 6 cDNAs were EST, 6 were Riken cDNAs, and 9 were uncharacterized sequences. To better understand the potential importance of each outlier gene, the GO annotation was assigned when possible (32). We ordered the genes regulated in Dbl transformants by the Biological Process annotation, which refers to a biological objective to which the gene product contributes. We observed that in Dbl transformants genes encoding proteins involved in cell cycle, cell organization, and transport were upregulated while genes encoding proteins involved in cell adhesion and signal transduction were downregulated. Conversely, a similar number of genes, whose function is associated with cell differentiation and metabolism, was stimulated as well as inhibited in Dbl transfectants. Among the genes assigned to the cell cycle control category, cyclin D1 was of particular significance, because its upregulation has been previously described as a hallmark of Dbl and Dbl-like gene-induced transformation (36).

We observed that five genes, which were assigned to different categories by the gene ontology annotation, could be better grouped in a new functional cluster. These genes, which include "apoptosis programmed cell death 2" (Pdcd2), "growth arrest and DNA-damage-inducible 45 alpha" (Gadd45a), "B-cell translocation gene" (Btg1), "DNA-damage inducible transcript 3" (Ddit3/Chop10/Gadd153), and "myeloid differentiation primary response gene 116" (Myd116/Gadd34), are all known to possess antiproliferative activity. Thus, the diversity of the genes found to be modulated in Dbl-transformed cells suggests that Dbl is involved in various biological processes, consistent with the data accumulated in the literature.

Northern Blot Validation of Selected Genes Found Differentially Expressed by Microarray Analysis

The finding that a group of five genes possessing antiproliferative function was overexpressed in onco-Dbl-transformed cells was contrasting with our expectations based on the knowledge that onco-Dbl is a potent oncogene. Thus, we concentrated our attention on these genes and utilized Northern blot hybridization to confirm microarray results. We used the same RNA employed for the microarray experiments as well as total RNA extracted from the independently generated onco-Dbl transformant 152-6-11 (Fig. 1). We also extended our analysis to the cell line overexpressing proto-Dbl protein 153-13-11 (Fig. 1).

TABLE 1
GENES DIFFERENTIALLY EXPRESSED BETWEEN NORMAL AND ONCO-Dbl-TRANSFORMED NIH3T3

Category	IMAGE ID	Fold Regulation	Gene Description
Upregulated genes*			
Cell adhesion	476416	1.4	CD151 antigen
	373219	2.1	Thrombospondin 4
Cell death	441810	2.0	Apoptosis programmed cell death 2
Cell cycle	697296	4.2	Growth arrest and DNA damage-inducible 45 alpha
	483527	2.6	DNA damage-inducible transcript 3
	374778	1.2	Cycle D1
	388688	1.2	Forkhead box G1
Cell differentiation and proliferation	598433	2.6	Myeloid differentiation primary response gene 116
	607129	2.8	General BTG1 protein
	581111	2.9	N-myc downstream regulated 1
	596827	2.7	Lung carcinoma myc related oncogene 1
Cell organization and biogenesis	616054	2.6	High movility group AT-hook 1
	579958	1.4	Peroxisome membrane protein PEX2
	391736	1.7	Ribosomal protein L44
Metabolism	579744	1.3	Thieredoxin reductase 2
	551565	2.4	Ornithine decarboxylase, structural
	387240	1.1	Lysophospholipase 3
	571468	2.6	Hypothetical protein E430004F23
	599018	1.6	G1 to phase transition 1
	582144	1.4	Hypothetical protein LOC236710
	558111	1.4	General Muller.inhib. weakly similar to KIAA0116
	352436	1.8	Asparaginyl-tRNA synthetase
	699094	2.0	Hormorn/GF NGF-B moderately similar to Gu protein
	676330	3.4	High mobility group protein 14
	584391	2.0	High mobility group protein I isoform C
Transport	477981	1.3	Solute carrier family 6 (neurotransmitter transporter)
	475661	2.1	Lipoprotein lipase
	619051	1.1	Effector ADP ribosyl. for ARF4
Response to external stimulus	557512	3.6	Stress diamine
	574849	1.6	Heat shock 70 kDA protein 4
Signal transduction	1263639	1.4	Fyn proto-oncogene
Unknown genes or without GO annotation	478392	1.7	Hsp27 ERE-TATA-binding protein
	418576	1.4	Nucleoporin 210
	634664	1.2	Guanine nucleotide binding progein (G protein), gamma 5
	577545	1.6	26S proteasome-associated pad1 homolog
	474139	2.3	RIKEN cDNA 1500041N16 gene
	537328	2.0	RIKEN cDNA 2600016B03 gene
	634942	1.7	RIKEN cDNA 3930401B19 gene
	578508	1.5	RIKEN cDNA 4432411E13 gene
	443986	1.4	RIKEN cDNA 5830443C21 gene
	515877	1.3	ESTs
	556047	1.8	ESTs
	374937	1.8	ESTs
	535320	1.1	ESTs
	467499	1.4	ESTs
	402938	1.8	ESTs
	586330	1.8	Unknown
	653249	3.3	Unknown
	464735	1.9	Unknown
	409194	1.5	Unknown
	336024	1.4	Unknown

TABLE 1
CONTINUED

Category	IMAGE ID	Fold Regulation	Gene Description
Downregulated genes†			
Cell adhesion	439174	−1.9	Cytoskeletal tenascin C
	577893	−1.8	Catenin beta
	574605	−1.9	Procollagen, type VI, alpha 3
	599085	−2.0	Procollagen, type IV, alpha 1
	478422	−1.6	Chondroitin sulfate proteoglycan 2
	368182	−2.7	Thrombospondin 1
Cell cycle	635180	−1.3	Cyclin D3
	851086	−1.3	Cyclin-dependent kinase inhibitor 2C
Cell differentiation and proliferation	440922	−1.4	Tissue inhibitor of metalloproteinase 1
	482009	−1.8	Insulin-like growth factor 2
	641632	−2.1	Colony stimulating factor macrophage
Cell organization and biogenesis	469046	−3.1	General CENP-B
Metabolism	466306	−1.8	Bone morphogenetic protein 1
	567544	−1.9	Ex matrix epican
	672409	−1.9	Enhancer trap locus 1
	350912	−1.5	Sine oculis-related homeobox 1 homolog
	574265	−1.6	Cytochrome b-5
	478504	−1.9	Calpain 6
	444392	−1.3	Transcription factor 3
	615406	−1.7	26S protease regulatory subunit 7
	535475	−2.4	Retinol binding protein 1, cellular
	608153	−2.6	Early growth response 1
Response to external stimulus	464496	−1.6	Major intrinsic protein of eye lens fiber
	634949	−2.0	CD24a antigen
	579629	−1.4	Fc receptor, IgG, alpha chain transporter
	481649	−1.9	Fibroblast growth factor receptor 1
	438455	−1.6	TF ZnFing.Mch
	386757	−1.2	Signal transducer and activator of transcription 3
Unknown genes or without GO annotation	465722	−1.0	Cytoskeletal hevin matrix protein 2
	551196	−3.3	G-protein-lie LRG-47
	479131	−1.6	Granulin
	438329	−2.3	Perlecan (Haparan sulfate proteoglycan 2)
	467863	−4.6	Delta-liek 1 homolog (Drosophila)
	598609	−3.3	Zinc finger protein 36, C3H type-like 1
	618018	−2.2	Latexin
	333540	−1.8	Secretory leukocyte protease inhibitor
	463711	−2.0	DNA segmnt, Chr7, ERATO Doi 671
	440285	−2.7	General vav
	335536	−1.1	C46011 cysteine proteinase inhibitor stefin 3
	303868	−1.3	Vang (van gogh)-like 2 (Drosophila); loop tail
	577899	−1.5	L 18.5 KD Pprotein C12G12.05C in Chr I
	523390	−4.0	General ski
	426726	−1.4	Hormone/GF RA bind.
	MP:6E7	−2.1	Modification tastin
	582160	−1.4	RIKEN cDNA 1110004C05 gene
	459072	−1.4	Unknown
	537201	−1.9	Unknown
	493030	−2.2	Unknown
	444699	−1.1	Unknown

The category was assigned according to the Gene Ontology for the description of the biological process of gene products. Only those genes with statistically significant ($p < 0.05$) modulation were included. Identification of genes was done by their IMAGE ID. Fold regulations are expressed as $\log_2$.

*Among the 51 cDNA, 4 are characterized sequences without a gene ontology annotation and 16 are not completely characterized sequences, including 6 ESTs and 5 Riken cDNAs.

†As in the upregulated genes, 16 cDNA do not have an ontology annotation and 5 are still uncharacterized sequences.

We observed that Btg1, Chop10, Gadd45, Myd116, and Pdcd2 were reproducibly overexpressed in onco-Dbl-transformed cell lines (Fig. 3). The difference observed in the Northern blot in levels of expression among the two onco-Dbl expressing cell lines analyzed is likely due to the clonal variability compounded with the selection of cells needed to generate stable transfectants. This can give rise to similar but not identical cell lines. Nevertheless, the variability observed was within the technical/biological error and does not alter the conclusion that genes associated with cell cycle arrest are upregulated in onco-Dbl transfectants. In proto-Dbl transfectants, only Btg1 showed a high level of expression, while Chop10, Myd116, and Pdcd2 were only weakly upregulated and Gadd45 was not induced (Fig. 3). The low transforming activity of proto-Dbl may be responsible for the differences of gene modulation observed between onco-Dbl and proto-Dbl transfectants (28).

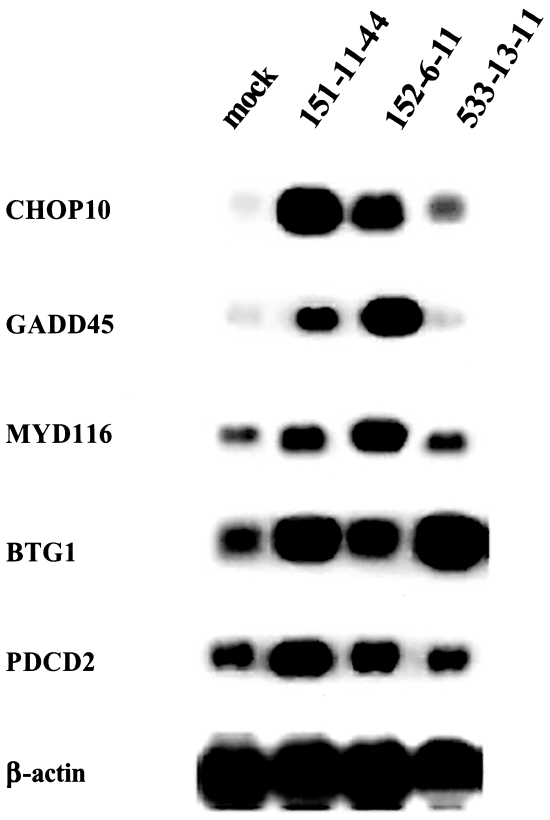

Figure 3. Verification of upregulated gene expression by Northern blot analysis. Total RNA was harvested from mock transfected NIH3T3 cells, ΔNpDbl transfectant 151-11-44, ΔNpDbl transfectant 152-6-11, and proto-Dbl transfectant 533-13-11 and analyzed by Northern Blot hybridization. ^{32}P-Labeled cDNAs of Btg1, Chop10, Gadd45, Myd116, and Pdcd2 were used as probes. Actin levels were determined to ensure that comparable amounts of RNA were loaded in each lane.

Gadd Proteins Are Overexpressed in Onco-Dbl-Transformants

To verify whether the induction of mRNA of Gadd45, Chop10, and Myd116 genes translated into changes in protein expression, cell lysates derived from the same two Dbl transfectants were subjected to immunoblotting analysis using antibody specific to each Gadd protein. As shown in Figure 4A, Gadd45 protein was induced in both onco-Dbl transformants. Similarly, when anti-Chop10 and anti-Myd116 antibody were used, a high level of the specific corresponding protein was detected in the onco-Dbl transfectants analyzed in comparison with control NIH3T3 cells (Fig. 4B, C). These results indicate that Gadd gene products are overexpressed in onco-Dbl-transformed mouse fibroblasts.

Expression of Onco-Dbl in NIH3T3 Cells Induces Activation of p38 Kinase

Rho family GTPases exert their effect at least in part through the activation of serine–threonine kinase signaling pathways. Some of these kinases, namely JNK and p38, are associated with stress-induced cell growth arrest. To explore whether the stimulation of expression of the genes possessing antiproliferative function was associated with onco-Dbl-induced activation of p38 kinase, NIH3T3 transfectants were harvested 20 h after incubation in serum-free medium and lysed. The activation of p38 kinase was evaluated by Western blot analysis with anti-P-p38 antibodies.

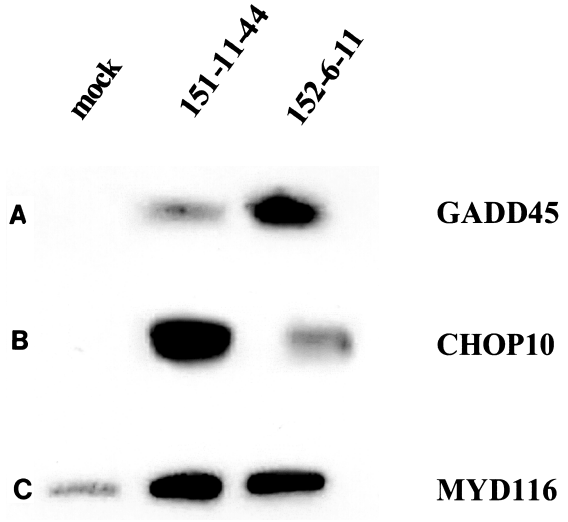

Figure 4. Detection of Gadd family proteins in onco-Dbl transformants. Mock transfected NIH3T3, stable onco-Dbl transfectants, clone 151-11-44, and clone 152-6-11 were lysed and analyzed by Western blot for Gadd family protein expression using anti-Gadd45 (A), anti-Chop10 (B), and anti-Myd116 (C) specific antibody.

As shown in Figure 5 expression of proto-Dbl and onco-Dbl induces activation of p38 by ~5- and 10-fold, respectively, compared with mock transfected cells. These results indicate that onco-Dbl and proto-Dbl stimulate the p38 MAP kinase signaling pathway.

DISCUSSION

The Dbl family GEFs exhibit a broad range of signaling activities consistent with their activation of Rho family proteins in vivo. Their activity has been linked to the regulation of gene expression through Rho GTPases substrates. Onco-Dbl expression in cells stimulates the activation of JNK, efficiently activates transcription from NF-κB responsive elements, and induces SRF transcriptional activation. Moreover expression of transforming onco-Dbl protein correlates with p38 kinase activation and stimulation of cyclin D1 expression (13,20,36,37). Thus, the link between onco-Dbl protein activation of nuclear signaling pathways has been established but a correlation between a signaling event and Dbl protein biological activities is largely unknown.

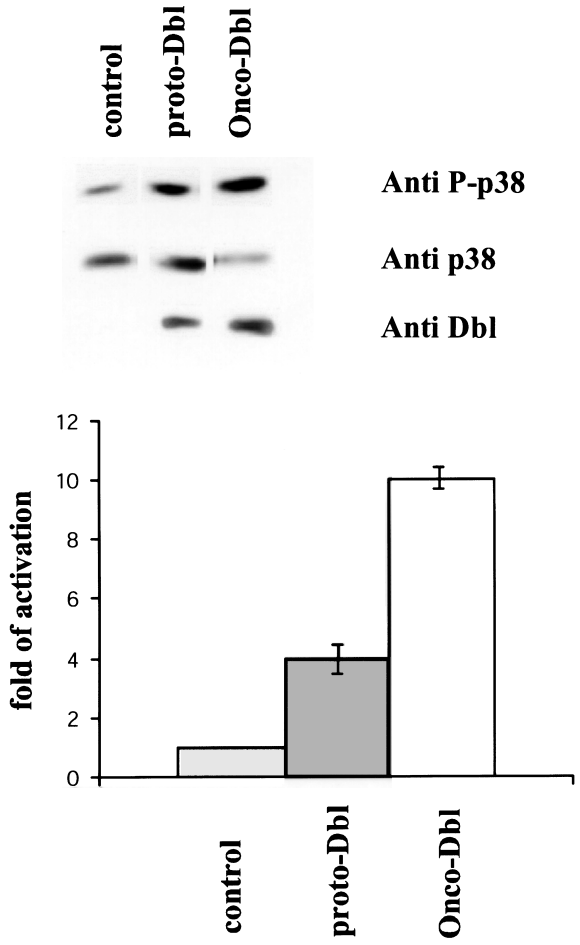

Figure 5. Expression of onco-Dbl in NIH3T3 cells induces activation of p38 kinase. Mock transfected NIH3T3 (control), stable onco-Dbl transfectants (Onco-Dbl), and proto-Dbl transfectant (proto-Dbl) were lysed and analyzed by Western blot for activated p38 kinase using anti-P-p38-specific antibody. The amount of p38 kinase was determined by Western blotting with anti-p38-specific antibodies. The amount of phosphorylated p38 for each sample was quantified. Bars represent the fold of activation over control determined by densitometric analysis from three independent experiments, one of which is shown as a representative.

We used a cDNA-based microarray analysis to obtain a comprehensive view of genes activated by Dbl oncogene in mouse fibroblasts. In the cDNA microarray experiments presented herein we identified 100 genes that were differentially expressed between onco-Dbl-transformed and control NIH3T3. Most of the genes identified have not been described previously as associated with Rho GTPase- induced signaling pathways. Nevertheless, some of these genes have been linked to cancer, control of cell proliferation, and adhesion. The classification of the genes according to the GO system allowed us to assign about 50% of the genes to a basic function classification group. The upregulated genes could be grouped into several categories, among which were cell cycle control, metabolism, transport, and transcription regulation. One gene in Dbl-transformed cells could be categorized into cell death, 1 in signal transduction, 2 genes categorized into cell adhesion and response to external stimulus, 3 into cell organization and transport, 4 in cell cycle and into cell differentiation and proliferation, 11 into metabolism, and 20 into uncharacterized gene-related functions or without GO annotation.

Among the upregulated genes, of interest is cyclin D1, previously shown to be induced in cells expressing Dbl and Dbl-like oncogenes (36). Fyn, a Src-related gene involved in the regulation of Ras-Erk signaling pathway, cell cycle progression, and cell adhesion-mediated signaling was upregulated as well. Interestingly, Fyn has been associated with Rho family GTPase functions because it activates the Dbl family GEF Vav by phosphorylation (14). Three high motility group (HMG) genes were also found to be upregulated in Dbl transformants: an increased expression of HMG-1 proteins may lead to neoplastic transformation (26). Thus, the upregulation of cyclin D1, Fyn proto-oncogene, and HMG genes may contribute to the capacity of onco-Dbl to induce cell proliferation and transformation.

Adhesion to extracellular proteins is necessary for several cell functions such as actin cytosckeleton organization, cell proliferation, and motility. The neoplastic transformation is determined in part by alterations of the cell adhesive properties. We found that several genes that are involved in cell–cell and cell–extracellular matrix interactions are downregulated in Dbl-transformed NIH3T3 cells. Among these are ten-

ascin C (15), collagen IV (22), collagen VI (17), and catenin-beta (11). We have previously reported that Dbl expression alters actin cytoskeleton polymerization in response to adhesion to different extracellular proteins (4). Thus, the modulation of expression of several adhesion proteins may be related to the structural and morphological changes observed in Dbl-transformed cells.

Our analysis identified five genes that are upregulated in onco-Dbl-transformed cells and implicated in cell cycle arrest: Chop10 (Gadd153), Gadd45α, and Myd116 (Gadd34), which belong to the family of growth arrest and DNA damage (Gadd)-inducible genes (9,10), Pdcd2 (16,24), and Btg1 (19). These genes are stress response genes, inducible by UV radiation, chemical carcinogens, starvation, oxidative stress, and apoptosis-inducing agents. Overexpression of each Gadd gene causes growth inhibition and combined overexpression of the Gadd genes leads to synergistic or cooperative antiproliferative effects.

We confirmed microarray results by Northen blot analysis and, in the case of Gadd45, Chop10, and Myd116, by Western blot as well. Overexpression of these genes both at the RNA and at the protein level was evident in more than one onco-Dbl transfectant. On the contrary, only Btg1 seemed to be upregulated in NIH3T3 cells expressing proto-Dbl. The different ability of proto-Dbl to modulate gene expression in NIH3T3 cells is probably due to its low GEF activity on Rho GTPases, which is determined by the inhibitory function of the N-terminus sequences through an intramolecular interaction with the PH domain (2).

The findings that onco-Dbl-transformed cells overexpress genes that act as negative regulator of cell cycle are somehow contradictory with the expectations derived by the fact that onco-Dbl is a potent oncogene. Nevertheless, our results are compatible with some established data. Firstly, Dbl oncogene strongly activates kinases, namely JNK and p38, which are associated with stress-induced cell growth arrest. Activated p38 phosphorylates transcription factors important in the regulation of cell growth and apoptosis, promoting G_1 arrest as part of the response to genotoxic stress (1,34). Moreover, it has been shown that Cdc42 can inhibit cell cycle progression at G_1/S through a mechanism requiring activation of p38 (21) and, in *S. cerevisiae*, Cdc42p is an essential component of the mating pheromone response pathway that results in G_1 arrest (12,31). Secondly, cells transformed by onco-Dbl show a morphology, associated with roughly 8–10% of the cell population, that indicates a block in the mitotic process, probably due to the uncoupling of the cell cycle progression and nuclear division from cell division (30). Thirdly, onco-Dbl oncogene serves as a GEF for Cdc42, whose constitutive activation or deregulation is detrimental for cell proliferation (8,18,33).

In summary, constitutive activation of Cdc42 by onco-Dbl oncogene may cause a strong activation of JNK and p38 MAPK, inducing in turn activation of genes involved in cell cycle arrest. On the other hand, activation of RhoA may lead to activation of cell proliferation-promoting signaling, inducing cell transformation. Thus, our observations suggest that constitutive activation of Cdc42 by onco-Dbl interferes with the cell division process and reveals that proliferation-inducing genes and growth-arresting genes are concurrently upregulated in Dbl-transformed mouse fibroblasts.

ACKNOWLEDGMENTS

We thank of Dr. William D. Rees and Dr. Laura Corbo, for providing reagents. We also thank Dr. A. Rapisarda for support. This work was supported by grants from the Italian Association for Cancer Research (AIRC), from the Italian Health Ministry, and from Compagnia di S. Paolo, Torino.

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