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Microarray and Large-Scale *In Silico*–Based Identification of Genes Functionally Related to Haptoglobin and/or Hemopexin

SHARMILA FAGOONEE,[1] FERDINANDO DI CUNTO,[1,2] DIEGO VOZZI,[3] STEFANO VOLINIA,[4] MAURIZIO PELLEGRINO,[5] PAOLO GASPARINI,[3] LORENZO SILENGO,[1,2] FIORELLA ALTRUDA,[1,2] and EMANUELA TOLOSANO[1,2]

ABSTRACT

Haptoglobin and Hemopexin are plasma acute phase proteins that bind with high-affinity hemoglobin and heme, respectively. They play a key role in the protection against oxidative stress and inflammation. To dissect in more detail the mechanism of action of Haptoglobin and Hemopexin, it is important to identify their downstream effectors as well as genes functionally related to them. To this end, we performed a cDNA microarray analysis to compare gene expression profiles of the liver of Haptoglobin and Hemopexin single and double null mice to that of wild-type controls. Then, to extract the best candidates considered to be functionally related to Haptoglobin and/or Hemopexin from microarray-derived gene lists, we used a bioinformatic approach consisting in the screening of published microarray data for genes showing coexpression with Haptoglobin or Hemopexin. This strategy allowed us to identify a group of genes coexpressed with Haptoglobin or Hemopexin and transcriptionally modulated by their lack. These genes present a high probability to be functionally related to Haptoglobin and Hemopexin. Based on literature data, we picked up from this group of genes the ras suppressor Rsu1, the member of the G-protein signal transduction family Gnai2, and the cytokine Mdk as the best candidates mediating the anti-inflammatory action of Haptoglobin and Hemopexin.

INTRODUCTION

Haptoglobin (Hp) and Hemopexin (Hx) are the plasma acute phase proteins with the highest binding affinity for hemoglobin ($K_d \sim 1$ pM) and heme ($K_d < 1$ pM), respectively. They act as soluble scavengers by delivering free plasma hemoglobin and heme to the liver where they are catabolyzed. Their scavenging function is thought to become important under various pathologic conditions characterized by the release, into plasma, of high amounts of hemoglobin and heme, such as hemorrhage, hematoma, hemoglobinopathies, excessive blood transfusion, and muscle injury (Hoffman *et al.*, 1995). This role has been confirmed by the analysis of Hp- and Hx-null mice. Indeed, both Hp- and Hx-null mice develop severe renal injury after phenylhydrazine-induced hemolysis (Lim *et al.*, 1998, 2000; Tolosano *et al.*, 1999). Moreover, Hp has been shown to be important in modulating renal iron loading, as its deficiency leads to increased iron deposition in proximal tubules of the kidney (Fagoonee *et al.*, 2005). Studies on knockout models suggested that Hp and Hx cooperate in the resolution of oxidative damage, as plasma Hp and Hx levels increased in Hx-null and Hp-null mice, respectively, both under basal and pathologic conditions. Moreover, Hp and Hx double knockout mice showed a stronger phenotype after hemolytic stress than single null mice (Tolosano *et al.*, 2002). These data suggested that a group of genes common to Hp and Hx function could exist. To search for these genes, we performed cDNA microarray analysis and compared gene expression profiles of the liver of Hp- and/or Hx-null mice to that of wild-type controls. The liver is the main site for Hp and Hx synthesis, as well as for catabo-

[1]Department of Genetics, Biology, and Biochemistry, University of Torino, Torino, Italy.
[2]Experimental Medicine Research Center, San Giovanni Battista Hospital, Torino, Italy.
[3]Department of Reproductive and Developmental Sciences, University of Trieste, IRCCS-Burlo-Garofalo, Trieste, Italy.
[4]Telethon DAMA Facility, Department of Morphology and Embriology, University of Ferrara, Ferrara, Italy.
[5]Laboratory of Neurogenetics and Behavior, The Rockefeller University, New York, New York.

lism of hemoglobin–Hp and heme–Hx complexes. Hence, our choice of tissue.

Microarray analysis identified a cluster of genes differentially modulated among the three genotypes (i.e., Hp-, Hx-, and HpHx-null versus wild type), as well as genes showing differential expression between each genotype and the control (i.e., Hp-null versus wild type; Hx-null versus wild type, and HpHx-null versus wild type). To extract the best candidates considered to be functionally related to Hp and/or Hx from these gene lists, we used a bioinformatic approach consisting of the screening of published microarray data for genes showing significant coexpression with Hp or Hx. The rationale for this is the observation that genes sharing common functions are often characterized by a high level of coregulation at the transcriptional level (Pellegrino *et al.*, 2004). Crossmatching of gene lists obtained by *in silico* analysis and by our microarray experiments allowed us to identify a group of genes coexpressed with Hp or Hx and transcriptionally modulated by their lack. These genes present a high probability to be functionally related to Hp and Hx.

Based on literature data, we picked up from this group, the ras suppressor Rsu1, the member of the G-protein signal transduction family Gnai2, and the cytokine Mdk as the best candidates capable of mediating the anti-inflammatory action of Hp and Hx.

MATERIALS AND METHODS

Animals

Hp-null mice were generated as previously described (Lim *et al.*, 1998) and kindly provided by F. Berger (University of South Carolina, Columbia, SC) and H. Baumann (Roswell Park Cancer Institute, Buffalo, NY). Hx-null and HpHx-null mice were generated in our laboratory as reported elsewhere (Tolosano *et al.*, 1999, 2002). The mice used in the following experiments, that is, wild-type ($Hp^{+/}Hx^{+/}$), Hp-null ($Hp^{-/-}Hx^{+/}$), Hx-null ($Hp^{+/}Hx^{-/-}$), and HpHx-null ($Hp^{-/-}Hx^{-/-}$), were littermates derived by breeding F1 double heterozygous $Hp^{+/-}Hx^{+/-}$ mice in the mixed genetic background C57/BLJ6 × 129Sv.

RNA extraction

Total RNA was extracted using the RNeasy MIDI kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The RNA concentration and purity were determined spectrophotometrically (DU530, Life Science UV/Vis Spectrophotometer, Beckman Coulter, Fullerton, CA), while integrity was tested on a 1% agarose gel. For microarray analysis, at least five adult mice per genotype were perfused via aorta with PBS, sacrificed and their liver pooled.

Microarray analysis

The mouse 15K cDNA microarray used in our study comprised 15,264 mouse ESTs derived from PCR amplification of synthetic EST clones (Mouse M15k3, Microarray center at the Clinical Genomics Centre, University Health Network, Toronto ON, Canada). Details of the clone identities are available at http://www.microarrays.ca/support/download/html. All ESTs were represented by two spots to ensure reproducibility. The chip included 480 spots derived from PCR amplification of *Arabidopsis thaliana* DNA clone as an internal negative control. The material spotted was in double-stranded form, and was coupled to a slide matrix covalently through an amino bonding.

Thirty micrograms of total RNA were subjected to direct labeling reaction by incorporation of cyanin 3 (Cy3) or cyanin 5 (Cy5) fluorescent dyes (Amersham Pharmacia Biotech, Frieburg, Germany) into the cDNA by priming with oligo(dT). Four replicates were set up for each experimental point. In order to exclude artifacts resulting from different dye usage, we employed the dye-switch approach, that is, incorporated Cy3-fluorescent dyes into the cDNA synthesized from wild-type mice RNA (control) and Cy5-fluorescent dyes into cDNA made from the experimental mice in two replicates and inversed the labeling for the other two. Cy3- and Cy5-labeled samples were purified using QIAquick™ (Qiagen, Hilden, Germany) and probes quantified. The purified Cy3- and Cy5-labeled cDNAs were then mixed and hybridized on chip at 42°C for 18 h.

TIFF images capture and signal quantification of hybridized cDNA arrays were done with the 428TM ArrayScanner (MWG, Florence, Italy) and ImaGene 4.1 software (BioDiscovery, Los Angeles, CA), respectively. The raw data were normalized using MIDAS software (Microarray Data Analysis System) (TIGR—The Institute for Genomic Research) and the Lowess normalization method was applied. The identification of genes with altered expression pattern between three conditions (Hp-null, Hx-null, and HpHx-null) with respect to wild-type genotype, was performed by a statistical ANOVA test. A cluster analysis was performed on these genes, using TIGR MeV software (The Institute for Genomic Research MultiExperiment Viewer), that outputs a hierarchical tree in which similar genes and experiments are connected by a series of "branches."

An analysis on a single genotype (Hp-null, Hx-null, and HpHx-null) was performed in comparison with the wild-type genotype. This kind of approach allows the identification of genes that show modulation of expression between each genotype versus the wild-type one. A permutation test was employed to this end, using the SAM (Significance of Analysis of Microarrays) software (Tusher *et al.,* 2001). Three gene lists of modulated genes with a low FDR (False Discovery Rate) (<0.05) were generated for Hp-null, Hx-null, and HpHx-null mice, respectively.

This microarray study followed MIAME (Minimum Information About a Microarray Experiment) guidelines issued by the Microarray Gene Expression Data group (Brazma *et al.*, 2001). The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) (Edgar *et al.*, 2002; Barrett *et al.*, 2005), and are accessible through GEO Series accession number GSE3656.

To functionally characterize the gene groups identified by the cluster analysis, their Gene Ontology (GO) annotation was analyzed as below, to reveal the presence of overrepresented keywords.

Quantitative real-time PCR (qRT-PCR)

Three micrograms of total RNA were reversed transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random hexamers (Ambion, Austin, TX) in

a final volume of 60 μl. Two microliters of cDNA were controlled for the presence of the actin transcript before proceeding to qRT-PCR.

For qRT-PCR, primers and probes were brought ready to use as Assays-on-Demand supplied by Applied Biosystems (Foster City, CA). The 18S rRNA TaqMan Predeveloped Assay Reagents (PDAR; Applied Biosystems) was used as an endogenous probe. An equal volume of endogenous and target gene probes and the required cDNA volume were added to TaqMan Master mix according to the manufacturer's instructions. The reactions were performed in triplicate using the standard protocol (95°C/2 min; 95°C/30 sec, 60°C/30 sec, 72°C/30 sec for 35 cycles) and monitored by the 7300 ABIPrism software (Applied Biosystems). We used the relative quantification study option to calculate the relative gene expression levels between two samples (comparative or ddCt method). The expression level of each gene under study was normalized to the 18S rRNA level to generate a ratio of the RNA of interest to 18S rRNA for each RNA sample. This ratio was further normalized to a control sample ("calibration"; Applied Biosystems). Thus, a relative ratio (relative quantity or RQ) between two samples was determined. The significance of difference in RQ values was determined by applying a Student's *t*-test.

Coexpression analysis

Single species and CLOE analysis were performed exactly as described (Pellegrino *et al.*, 2004) on all the human and mouse Hp and Hx probes found in the reference database. This corresponds to the January 2004 release of the Stanford Microarray Database. The correspondence between human genes and their mouse orthologs used for comparing human and mouse coexpression lists, as well as our mouse microarray data with human coexpression lists, were established on the basis of the INPARANOID tables. The *P*-values for keywords enrichment were calculated with respect to a null hypothesis derived on the basis of the appropriate hypergeometric distribution. In this case, *P*-values of 5 E^{-4} or lower were considered as significant, based on a Bonferroni correction, applied to account for multiple testing.

RESULTS

Identification of genes differentially expressed in Hp and/or Hx knockout mice

We used doubly spotted array containing 15,264 sequence-verified mouse ESTs to analyze the liver gene expression profiles of Hp-, Hx-, and HpHx-null mice under basal conditions with respect to wild-type mice. Upon data acquisition and normalization, we designated as differentially expressed the genes for which the SAM method (Tusher *et al.*, 2001) revealed at least twofold significant change at an FDR <0.05.

Hierarchical clustering of the data identified a group of 25 genes showing significant modulation among the three genotypes. Interestingly, genes belonging to this cluster were strongly upregulated in Hx-null mice, upregulated, but to a lesser extent, in HpHx-null mice, and not modulated in Hp-null mice. This group of genes included transcripts annotated to the ubiquitin–proteasome cycle (Pmse1, RFPL4, Smt3h1), ras protein signaling (Rsu1, Ulk1), carbohydrate metabolism (Gltp1), lipid catabolism (Plcg1), and regulation of transcription (Rcor1, Zdhhc8), besides to other not yet characterized ESTs and RIKEN cDNAs (Fig. 1). To assess whether the genes in this cluster share functional properties, we analyzed their GO annotation for overrepresented keywords, according to the appropriate hypergeometric distribution. The only significantly overrepresented annotation was "Ras protein signal transduction," contributed by the genes Rsu1 and Ulk1 ($P = 2.55$ e-04).

To better characterize intergenotype differential gene expression, a single genotype (knockout versus wild type) analysis was performed, and a permutation statistical test was employed to identify genes characterized by a modulation in their expression. Data revealed two upregulated and nine downregulated transcripts in Hp-null mice; 21 overexpressed and one

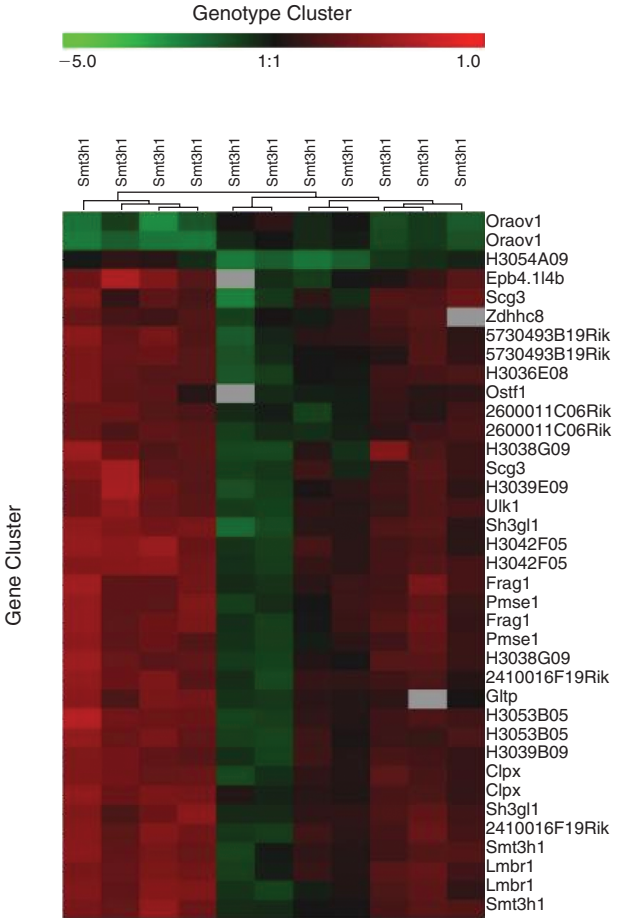

FIG. 1. Hierarchical clustering of significantly modulated genes in Hp-, Hx-, and HpHx-null mice versus wild-type controls. Experiments were run in quadruplicates, except for HpHx-null mice (triplicate). Each column represents separate liver samples and rows show individual genes. At some points, two rows represent the same gene. The first four columns represent clustered genes from Hx-null mice, the four middle columns from Hp-null mice, and the last three columns from HpHx-null mice versus a common wild-type RNA pool. The normalized expression index for each gene is indicated by a color code. Green cells show downregulated genes, red cells show upregulated genes, and black cells show nonmodulated genes.

underexpressed genes in Hx-null mice, and 69 overexpressed and 3 underexpressed genes in HpHx-null mice. The lists from the three genotypes included genes involved in cell proliferation (Mdk, Pin1, Anapc4, Ppp1r8, Nfkbia, Men1), in signal transduction (Rsu1, Ulk1, Ednrb, Ptger4, Gnai2, Wnt4), in transcriptional regulation (E4f1, Rcor1, Eya1, Ndn, Nr1h2, Rai17), and in the proteasome and ubiquitin cycle (Pmse1, Smth3h1, Anapc4, Trim37). As for the cluster, the only significantly overrepresented GO annotation was "Ras protein signal transduction" in the Hx-deficient liver gene list.

Experimental validation of microarray data

Experimental validation of microarray data was performed by quantitative real-time PCR (qRT-PCR) on total liver RNA extracted from wild-type, Hp-deficient, and Hx-deficient mice. We chose for validation the genes Smt3h1, Rsu1, and Ulk1, found by cluster analysis, and Mdk, overexpressed both in Hx-null and HpHx-null mice.

qRT-PCR analysis showed that Smt3h1, Mdk, and Rsu1 were significantly more expressed ($P < 0.01$ for Smt3h1; $P < 0.05$ for Mdk and Rsu1) in Hx-deficient mice than in wild-type and Hp-deficient mice, whereas their expression levels were similar in Hp-null and wild-type mice (Fig. 2A–C). Fold changes in expression between Hx-deficient and wild-type mice were about 1.5–2 for all transcripts.

In the case of Ulk1, the difference in expression level among the three genotypes did not reach statistical significance. However, for Ulk1 also, changes in expression levels were directionally similar to those determined by microarray experiments, that is, higher expression in Hx-deficient liver than in wild-type and Hp-null ones (Fig. 2D). Even in this case, expression level was comparable between Hp-null and wild-type mice (Fig. 2D).

Identification of genes coexpressed with Hp and Hx

Since genes sharing common functions are often characterized by a high level of coregulation at the transcriptional level, databases of published microarray data represent a very useful resource for functional discoveries, as they allow to systematically identify genes displaying related expression profiles. We searched the Stanford Microarray Database for human and mouse Hp or Hx probes. For each probe, we calculated a normalized Pearson correlation coefficient with every other probe of the same species, ranked them on the basis of this score, and extracted the first 1% rank, that contains most of the biologically relevant coexpressed genes (Pellegrino *et al.*, 2004). In the case of Hp, only two human probes were identified (GeneBank acc. AI870548 and AI985788), whereas two informative human (GeneBank acc. AI204645 and AI283497) and two mouse (GeneBank acc. AI415648 and AV104547) Hx probes were found.

Genes obtained by this procedure were analyzed for their GO annotation as in the case of microarray experiments. According to the expectations, we found that both the human Hp and mouse Hx lists showed a significant overrepresentation of acute phase genes, and that mouse Hx lists contained a strong overrepresentation of genes related to hemoglobin metabolism. Interestingly, human Hp and mouse Hx lists shared other annotation terms, related to lipid metabolism and serine proteases inhibition. On the other hand, apart from the keyword "extracellular," that was common to the three lists, human Hx lists did not share terms with human Hp and, more surprisingly, with mouse Hx (Table 1). Nevertheless, a systematic search of orthologous sequences (CLOE analysis) revealed 12 genes showing conserved coexpression with Hx in human and mouse (Table 2).

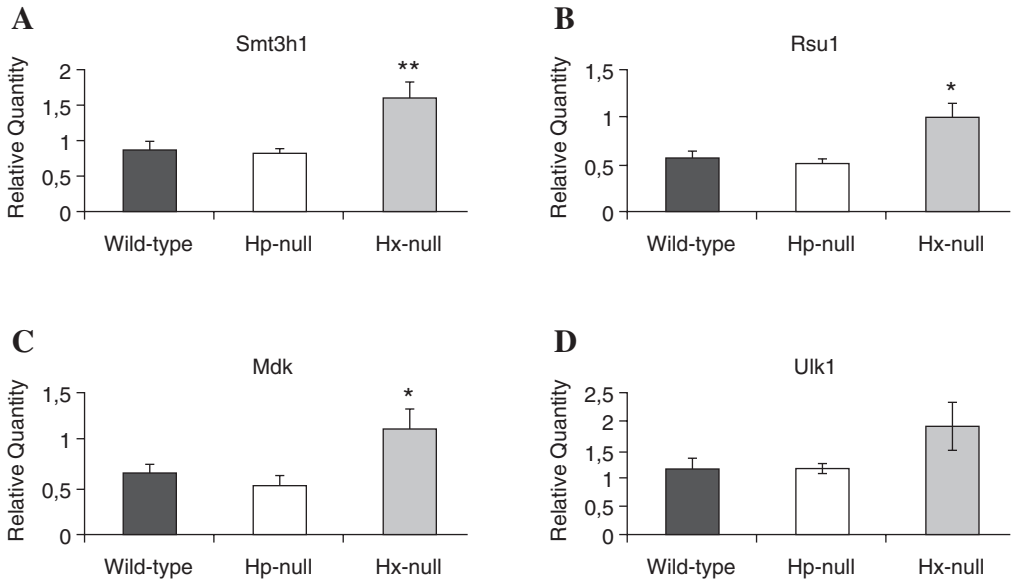

FIG. 2. Validation of microarray results by qRT-PCR. Primers and probes for Smt3h1 (**A**), Rsu1 (**B**), Mdk (**C**), and Ulk1 (**D**) were brought ready to use as described in Materials and Methods. Total RNA from 11 mice for each genotype was used, and mean Relative Quantity ± SE is shown. The differences in the level of expression between Hx-null and wild-type mice were significant for Smt3h1, Rsu1, and Mdk. The levels of expression were comparable between Hp-null and wild-type mice. *$P < 0.05$; ** $P < 0.01$.

Table 1. Common Overrepresented GO Annotations Obtained by Analyzing Human and Mouse Hp and Hx Probes from Stanford Microarray Database

GO annotation	Homo sapiens *Hp*	Homo sapiens *Hx*	Mus musculus *Hx*
Acute-phase response	*		*
Extracellular	*	*	*
Lipid transporter	*		*
Serine protease inhibitor	*		*

Several terms are common between human Hp and mouse Hx lists. The term "extracellular" is shared by all three gene lists.

Identification of genes coexpressed with Hp and Hx and modulated in their absence

We then set out to identify the genes that are normally coexpressed with Hp and Hx, and transcriptionally modulated in their absence. To this aim, we crossmatched the single species coexpression lists with the results of wild-type versus single or double knockout microarray experiments (Table 3).

Crossmatch of bioinformatics data with our microarray data evidentiated the following genes: (1) Rsu1 and Ostf1 that were present in the cluster as differentially modulated genes in the three genotypes with respect to wild-type mice, were upregulated in Hx-null and HpHx-null mice and were coexpressed with human Hx; (2) Gnai2, that was upregulated in Hp-null and HpHx-null mice (microarray experiments) and was coexpressed with mouse and human Hx (CLOE analysis); (3) Mdk that was upregulated in Hx-null and HpHx-null mice and was coexpressed with Hp. In addition, some other transcripts were evidentiated by crossmatching bioinformatics data with microarray results on HpHx double null mice.

DISCUSSION

Microarray analysis

We performed microarray analysis on the liver of Hp- and/or Hx-null mice versus wild-type controls. Clustering of microarray data identified 25 differentially modulated genes. Most of them were upregulated in Hx- and HpHx-null mice and not modulated in Hp-deficient mice. A higher expression in the liver of Hx-null mice was confirmed for three (Rsu1, Mdk, and Smt3h1) out of four genes selected for experimental validation of microarray data. Only for Ulk1, even if the level of expression was higher in Hx-deficient liver than in wild-type control, the difference did not reach statistical significance. On the other hand, the expression levels of the four selected genes, assayed by qRT-PCR, were similar in Hp-deficient and wild-type mice as in microarrays.

None of the 25 selected genes was involved in hemoglobin/heme catabolism. A possible explanation might be that the function of Hp and Hx as scavengers of hemoglobin and heme, respectively, is mainly mediated by Kupffer cells that represent a minor component of the liver. Changes in gene expression in Kupffer cells could be masked by the more abundant hepatocyte RNA. Thus, our results may reflect how Hp and Hx gene inactivation affects hepatocyte transcriptome. The fact that the most relevant differences in microarrays were seen in Hx-null mice supports this conclusion as Hx exerts its action through the LRP receptor, found on hepatocytes (Schmoelzl *et al.*, 1998; Hvidberg *et al.*, 2005) while Hp acts through CD163, the expression of which in the liver is confined to Kupffer cells (Kristiansen *et al.*, 2001).

The expression profile of the HpHx-null liver was more similar to that of the Hx-deficient liver than to that of Hp-deficient one as shown by cluster analysis. This result further suggests

Table 2. CLOE Analysis of Hx Probes

Gene name	*EntrezGene ID*	*Gene description*
HPX	3263	Hemopexin
ACOX2	8309	Acyl-coenzyme A oxidase 2, perosixomal
AKR1C1	1645	Aldo-keto reductase family 1 member C1
COL3A1	1281	Collagen alpha 1(III) chain precursor
CYP11A1	1583	Cytochrome P450 11A1, mitochondrial precursor
CYP17A1	1586	Cytochrome P450, family 17, subfamily A, polypeptide 1
GNAI2	2771	Guanine nucleotide-binding protein G(i), alpha-2 subunit
GUCY1A3	2982	Guanylate cyclase 1, soluble, alpha 3
MAPK14	1432	Mitogen-activated protein kinase 14
PON1	5444	Serum paraoxonase/arylesterase 1
PRLR	5618	Prolactin receptor precursor (PRL-R)
SNCA	6622	Alpha-synuclein (Non-A beta component of AD amyloid)
TDO2	6999	Tryptophan 2,3-dioxygenase

Most significant orthologs obtained by comparing Hx human and mouse datasets from the Stanford Microarray Database are shown.

TABLE 3. CROSSMATCH OF SINGLE SPECIES COEXPRESSION LISTS (VERTICAL) WITH SINGLE EXPERIMENT MICROARRAY GENE LISTS (HORIZONTAL)

		Probes found in Stanford Microarray Database		
		Homo sapiens *Hp*	Homo sapiens *Hx*	Mus musculus *Hx*
cDNA microarrays	Cluster of 3 genotypes		Rsu1 (20163) Ostf1 (20409)	
	HpHx-null *vs.* wild-type: upregulated	Bag1 (12017) Mdk (17242)[a] Trim37 (68729)[a] 2810422O20Rik (69962) Rps21 (66481) Ednrb (13618)	Rsu1 (20163) BC019537 (228993) 4930548G07Rik (75339) Prkcn (75292) Krt2-7 (110310) Gnai2 (14678) Pin1 (23988) 2810422O20Rik (69962) Ostf1 (20409) Asrgl1 (66514) Banp (53325)	Pdzk1 (59020) 4930442L01Rik (67583) AW552393 (99922) Gnai2 (14678) C1qtnf3 (81799) Tmem32 (236792)
	Hp-null *vs.* wild-type: upregulated		Gnai2 (14678)	Gnai2 (14678)
	Hx-null *vs.* wild-type: upregulated	Mdk (17242)[a]	Rsu1 (20163)	

Common genes are represented by gene name and EntrezGene ID.
[a]Indicates genes found with both human Hp probes in the Stanford Microarray Database.

that in our experiments the effect of Hx gene inactivation on liver transcription was "dominant" with respect to the effect of Hp knockout. Moreover, a greater number of genes were modulated in HpHx-null mice compared to single knockout mice. The lack of two genes simultaneously probably imposes a major stress to the system, hence influencing the expression of other genes not strictly related to Hp or Hx.

GO analysis of the genes in the cluster and in single genotypes lists identified the functional category "Ras protein signal transduction" as overrepresented both in the cluster and in Hx-deficient mice. The gene Ras suppressor 1 (Rsu1), coding for an inhibitor of ras signaling, has been annotated to this category. Rsu1 is overexpressed in the liver of Hx-deficient mice, thus suggesting that the lack of Hx leads to a perturbation in ras signaling.

In silico *analysis*

GO annotation of gene lists obtained by *in silico* search for genes coexpressed with Hp or Hx has evidentiated as overrepresented, in both the human Hp and mouse Hx lists, the obvious categories "acute-phase response" and "extracellular" as well as the unexpected "lipid transporter" and "serine protease inhibitor."

Surprisingly, human and mouse Hx gene lists were quite different apart from the overrepresentation of the GO category "extracellular." This could be due to the fact that the analyzed data, contained in the Stanford Microarray Database, are representative of very different experimental situations. However, this difference might also reflect a species-specific transcriptional regulation of these two genes. This conclusion is supported by the fact that the well-characterized human and rat Hx promoters contain the same HxA motif, which is a cytokine-responsive element strongly activated by C/EBP-β and -δ in humans, but inactive in the rat (Immenschuh *et al.*, 1994).

However, despite the fact that the human and mouse Hx gene lists were so different, we identified 12 genes coexpressed with Hx in both species (CLOE analysis). Three of these genes were annotated in the categories "lipid metabolism and transport," suggesting a role for Hx in lipid homeostasis. To this regard, it has been previously shown that Hx is able to inhibit plasma LDL oxidation, thus suggesting an active role in preventing LDL-mediated vascular damage (Miller and Shaklai, 1999). Moreover, Hx may have a role in lipid homeostasis by virtue of its capability to bind LRP, thus interfering with its function in mediating the recovery of lipoproteins (Herz and Strickland, 2001).

Crossmatch between experimental and bioinformatics data

Crossmatch of gene lists derived by *in silico* analysis and by microarrays identified a group of genes coexpressed with Hp and/or Hx and modulated by their lack. These genes present a high probability to be functionally related to Hp and/or Hx. One of these genes is the ras suppressor Rsu1 that is overexpressed in the liver of Hx- and HpHx-deficient mice and is coexpressed with mouse Hx. Rsu1 was cloned on the basis of its ability to suppress transformation by v-Ras (Cutler *et al.*, 1992). Its expression renders cells resistant to Ras transformation in spite of activation of Erk-2. This is because Rsu1 expression, other than

activating Erk-2, inhibits Jun kinase activation (Masuelli and Cutler, 1996). On the other hand, Eskew and coworkers demonstrated that stimulation of hepatoma cells with the heme–Hx complex activates Jun kinase (Eskew *et al.*, 1999). Finally, Barnes and coworkers (2003) have shown that LRP can be phosphorylated by src and other kinases of the same family, and that, once phosphorylated, it can bind Shc, an adaptor protein involved in the activation of Ras. Taken together, these data suggest that ras signaling may be downstream of Hx action.

Another gene evidentiated from both experimental and bioinformatics data is Gnai2 (G-protein alpha inhibiting 2). It has been found by microarrays as overexpressed in the Hp-null and HpHx-null liver and by CLOE analysis, as coexpressed with Hx. The fact that it is upregulated in Hp-deficient mice and is coexpressed with Hx is intriguing, as we have previously shown that the plasma level of Hx is increased in Hp knockout mice, and that these mice are partially able to compensate for the lack of Hp (Tolosano *et al.*, 2002). Therefore, upregulation of Hx and Gnai2, could account for the capability of Hp-null mice to compensate for the lack of Hp.

Gnai2 encodes for a member of the G protein signal transduction family that is important to transduce inflammatory signals (Blatt *et al.*, 1988). Indeed, Gnai2-deficient mice show B- and T-cell defects. Moreover, Han and coworkers (2005) have recently demonstrated that Gnai2 plays a key role in regulating the entrance of B lymphocytes into lymph nodes and B-cell motility within lymph node follicle. Given that Gnai2 is involved in antiinflammatory reactions, it could be a target for the Hp- and Hx-mediated anti-inflammatory processes.

Finally, the cytokine Mdk (Midkine) has been found by microarray as upregulated in Hx-null and HpHx-null mice and by *in silico* analysis as coexpressed with Hp. Mdk, as Hx, is a ligand for LRP (Muramatsu *et al.*, 2000), and it is also involved in the response to inflammatory stimuli by promoting migration of neutrophils and macrophages (Kadomatsu and Muramatsu, 2004). Upregulation of Mdk in Hx-null mice would potentiate their ability to recruit inflammatory cells, thus allowing them to sustain a normal anti-inflammatory reaction as demonstrated elsewhere (Tolosano *et al.*, 1999).

CONCLUSIONS

The main novelty of this work has been the utilization of a bioinformatic approach to extract information from gene lists obtained by a standard microarray analysis on the liver of Hp- and/or Hx-null mice. This strategy allowed us to identify some potential functional targets of Hp and Hx actions. As discussed in the last section, the ras suppressor, Rsu1, and the member of the G-protein signal transduction family, Gnai2, are good candidates for mediating the anti-inflammatory action of Hp and Hx. Moreover, the cytokine, Mdk, could be a partner of Hp and Hx in the acute phase response. Further studies are needed to validate these conclusions. To this end, knockout models represent a unique and powerful tool.

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Address reprint requests to:
Emanuela Tolosano, Ph.D.
Department of Genetics, Biology, and Biochemistry
University of Torino
Via Santena 5bis
10126 Torino, Italy

E-mail: emanuela.tolosano@unito.it

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