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Differential Gene Expression Induced by Insulin and Insulin-like Growth Factor-II through the Insulin Receptor Isoform A*

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The human insulin receptor (IR) exists in two isoforms (IR-A and IR-B). IR-A is a short isoform, generated by the skipping of exon 11, a small exon encoding for 12 amino acid residues at the carboxyl terminus of the IR α-subunit. Recently, we found that IR-A is the predominant isoform in fetal tissues and malignant cells and binds with a high affinity not only insulin but also insulin-like growth factor-II (IGF-II). To investigate whether the activation of IR-A by the two ligands differentially activate post-receptor molecular mechanisms, we studied gene expression in response to IR-A activation by either insulin or IGF-II, using microarray technology. To avoid the interfering effect of the IGF-IR, IGF-II binding to the IR-A was studied in IGF-IR-deficient murine fibroblasts (R^- cells) transfected with the human IR-A cDNA (R^-/IR-A cells). Gene expression was studied at 0.5, 3, and 8 h. We found that 214 transcripts were similarly regulated by insulin and IGF-II, whereas 45 genes were differentially transcribed. Eighteen of these differentially regulated genes were responsive to only one of the two ligands (12 to insulin and 6 to IGF-II). Twenty-seven transcripts were regulated by both insulin and IGF-II, but a significant difference between the two ligands was present at least in one time point. Interestingly, IGF-II was a more potent and/or persistent regulator than insulin for these genes. Results were validated by measuring the expression of 12 genes by quantitative real-time reverse transcriptase-PCR. In conclusion, we show that insulin and IGF-II, acting via the same receptor, may differentially affect gene expression in cells. These studies provide a molecular basis for understanding some of the biological differences between the two ligands and may help to clarify the biological role of IR-A in embryonic/fetal growth and the selective biological advantage that malignant cells producing IGF-II may acquire via IR-A overexpression.

The human insulin receptor (IR)[1] exists in two isoforms (IR-A and IR-B). IR-A is a short isoform, generated by the skipping of exon 11, a small exon encoding for 12 amino acid residues at the carboxyl terminus of the IR α-subunit. The relative abundance of the two IR isoforms is regulated by tissue-specific factors, stage of development, and cell differentiation (1–3). Genetic studies carried out in transgenic mice have shown that fetal growth in response to IGF-II is partially mediated by the IR (4–6), and we have recently demonstrated that IR-A is the predominant isoform in fetal tissues and binds IGF-II with high affinity (7).

We also demonstrated that malignant transformation is associated with both IR overexpression and an increased relative abundance of IR-A, both in epithelial and in mesenchymal tumors (8–13), and that IR-A relative abundance may further increase with cells dedifferentiation, as observed in thyroid cancer (12, 14). Accumulating evidence also indicates that IR-A overexpression may play a significant role in growth promotion and apoptosis protection of malignant cells when tumors produce IGF-II (13, 15). In contrast, IR-B is the predominant IR isoform in normal adult tissues that are major targets for the metabolic effects of insulin (adipose tissue, liver, and muscle) (1, 2, 16).

The binding characteristics of insulin and IGF-II to IR-A and the biological effects of IR-A stimulation by IGF-II have been studied previously in a variety of models (7). In particular, we studied IGF-II binding to the IR-A in IGF-IR-deficient murine fibroblasts (R^- cells) transfected with the human IR-A cDNA (R^-/IR-A cells). This study revealed that IGF-II displaces labeled insulin from IR-A with a lower affinity than insulin (ED_{50} = 2.5 *versus* 0.9, respectively). However, unexpectedly, IGF-II was a more efficacious mitogen than insulin in these cells. In contrast, insulin was more potent than IGF-II in stimulating glucose uptake (7). In accordance with our findings, it was independently shown that IGF-II is stronger than insulin in inducing growth in IR-transfected R^- cells (17). These findings were confirmed and extended in SKUT-1 human rabdomyosarcoma cells, which lack functional IGF-IR and express almost only IR-A. In SKUT-1 cells, IGF-II was significantly more potent than insulin in stimulating the Shc/ERK pathway, whereas insulin was more potent than IGF-II in stimulating IR autophosphorylation and the IRS-1/phosphatidylinositol 3-kinase/Akt pathway. As a result, IGF-II was more potent than insulin in inducing cell chemoinvasion, whereas insulin was slightly more effective in apoptosis protection (13). Taken together, these studies indicate that insulin and IGF-II, by binding to the same receptor, may induce the preferential activation of different intracellular pathways. These differences may result in significant differences in the biological effects between the two ligands.

To gain further insights on the molecular mechanisms differentially activated by either IGF-II or insulin in R^-/IR-A cells, we investigated gene expression in response to either

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[1] The abbreviations used are: IR, insulin receptor; IGF, insulin-like growth factor; TGF, tumor necrosis growth factor; EST, expressed sequence tag; ERK, extracellular signal-regulated kinase; r.m.s.s.d., root mean square standard deviation; dChip, DNA chip analyzer.

TABLE I
Genes similarly up-regulated by both insulin and IGF-II

The 40 most strongly regulated genes are listed.

GenBank accession No.	Description	Insulin	IGF-II	Time
				h
	Apoptosis			
U73478	Acidic nuclear phosphoprotein 32	2.4	3.8	0.5/8
AF064447	Sex-determination protein homolog Fem1a	2.7	3.1	3
AB013819	TIAP	2.2	1.9	8
U93583	*RAD51*-associated protein 1	1.7	1.7	8
	Cell cycle			
D26091	CDC47	1.8	1.9	8
L26320	Flap structure specific endonuclease 1	1.8	1.9	8
AF098068	*CDC45*-related protein	1.8	1.9	8
D26089	Mini chromosome maintenance deficient 4	1.7	1.9	8
D13803	*Rad51* homolog (*Saccharomyces cerevisiae*)	1.6	1.7	8
M38724	Cell division cycle control protein 2a	1.8	1.6	8
J04620	Primase p49 subunit (priA)	1.8	1.6	8
D26090	CDC46	1.6	1.5	8
	Cytoskeletal functions			
X99963	rhoB gene	1.8	1.7	0.5
	DNA mismatch repair			
U28724	Postmeiotic segregation increased 2	1.6	1.7	8
	Metabolism			
U17132	Zinc transporter 1	1.7	2.0	8
AB000777	Cryptochrome 1 (photolyase-like)	1.6	1.7	3
X13752	δ-aminolevulinate dehydratase	1.8	1.7	8
AF043249	Mitochondrial outer membrane protein (Tom40)	1.6	1.6	8
	Proliferation			
M28845	Early growth response 1	3.5	3.4	0.5
L41352	Amphiregulin	2.8	2.7	3
M59821	Growth factor-inducible protein (pip92)	2.7	2.6	0.5
M14223	Ribonucleotide reductase M2 subunit	2.1	2.5	8
AJ223087	Cdc6-related protein	2.3	2.4	8
D87908	Nuclear protein np95	2.2	2.3	8
X60980	Thymidine kinase	2.6	2.3	8
X67644	Gly96	2.0	2.2	0.5
M24377	Early growth response 2	2.1	2.0	0.5
M33960	Mouse plasminogen activator inhibitor (PAI-1)	2.1	2.0	3
D86725	MCM2	1.9	2.0	8
M17298	Nerve growth factor β	2.4	1.9	3
K02927	Ribonucleotide reductase M1	1.8	1.8	8
L07264	Heparin binding EGF-like[a] growth factor	1.9	1.7	3
M70642	Fibroblast-inducible secreted protein	1.7	1.6	3
	Proliferation/apoptosis			
U77844	TRIP	1.7	1.5	8
	Proliferation/cell transformation			
U20735	Transcription factor *junB*	2.1	1.8	0.5
	Proliferation/differentiation			
U51000	Distal-less homeobox 1	2.4	2.6	8
D30782	Epiregulin	2.6	2.5	3
U03421	Interleukin 11	2.2	1.7	3
	Signal transduction			
U88328	Suppressor of cytokine signalling-3	1.9	2.0	0.5
D16497	Natriuretic peptide precursor type B	1.9	1.6	3

[a] EGF, epidermal growth factor.

ligand using microarray technology. Microarray techniques have emerged as a new potent approach for the global analysis of gene transcription. We used Affymetrix MG-U74A GeneChips to measure changes in mRNA levels for ~6,000 functionally characterized murine genes and ~6,000 expressed sequence tags (ESTs). We found that 45 genes are differentially transcribed in response to either insulin or IGF-II in R^-/IR-A cells. We also validated these results by evaluating the expression profile of 12 genes by quantitative real-time reverse transcriptase-PCR. These findings provide a molecular basis for understanding the biological differences between insulin and IGF-II after binding to the same receptor.

EXPERIMENTAL PROCEDURES

Materials—The pNTK2 expression vector containing the cDNA for the A (Ex11−) isoform of the human IR was kindly provided by Dr. Axel Ullrich (Martinsried, Germany). Fetal calf serum, glutamine, LipofectAMINE, DNAase I were from Invitrogen; RPMI 1640 medium, Dulbecco's modified Eagle's medium, bovine serum albumin (BSA, radioimmunoassay grade), bacitracin, phenylmethylsulfonyl fluoride, puromycin, porcine insulin were from Sigma; IGF-II was obtained from Calbiochem Laboratories. TRIzol reagent and Superscript Choice system were purchased from Invitrogen; Oligotex mRNA kit and RNeasy Mini kit were obtained from Qiagen; BioArray HighYield RNA transcript labeling kit (ENZO Bioarray kit) was obtained from Affymetrix .

Cells—R^- mouse fibroblasts (mouse 3T3-like cells derived from animals with a targeted disruption of the *IGF-IR* gene, expressing ~5×10^3 native insulin receptors/cell) were kindly provided by Dr. R. Baserga (Philadelphia, PA) and were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. R^- cells grown in 35-mm plates until 60–70% confluent were co-transfected with 2 μg of pNTK2 expression vector containing the cDNA encoding for the A (Ex11−) isoform of the human IR (18) and with the pPDV6+ plasmid encoding for the puromicyn resistance gene. Cells were subsequently subjected to antibiotic selection in medium supplemented with 2.4 μg/ml puromicin for 3 weeks. Stably transfected cells were then cloned, and a cell clone with ~5×10^5 receptors/cell was obtained, as described previously (7). Receptor content was evaluated in selected cell clones by enzyme-linked immunosorbent assay (10).

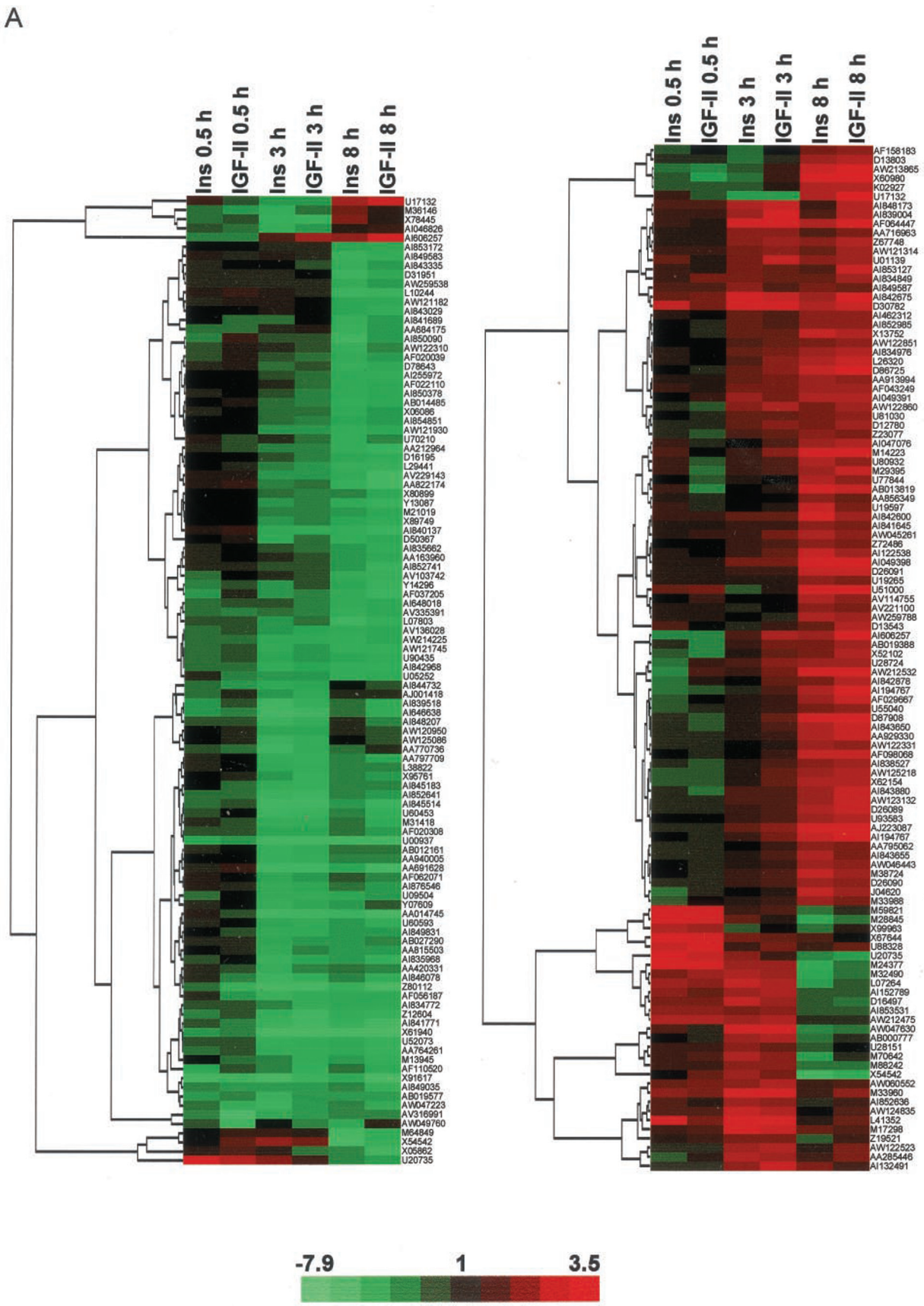


FIG. 1. **Cluster analysis of genes regulated by either insulin or IGF-II or both in R-/IR-A cells.** Two hundred and fifty nine genes and ESTs demonstrated themselves to be either up-regulated or down-regulated at least at one time point (0.5, 3, or 8 h) by one or both ligands on the basis of microarray hybridization technique using Affymetrix MG-U74A GeneChips. These genes were subjected to three different hierarchical cluster analysis and represented: *A*, genes and ESTs similarly down- or up-regulated by the two ligands; *B*, genes and ESTs differentially expressed in response to either insulin or IGF-II. Genes regulated only by insulin are indicated in *red*; genes regulated only by IGF-II are indicated in *blue*. The scale of gene expression, as -fold changes, is shown.

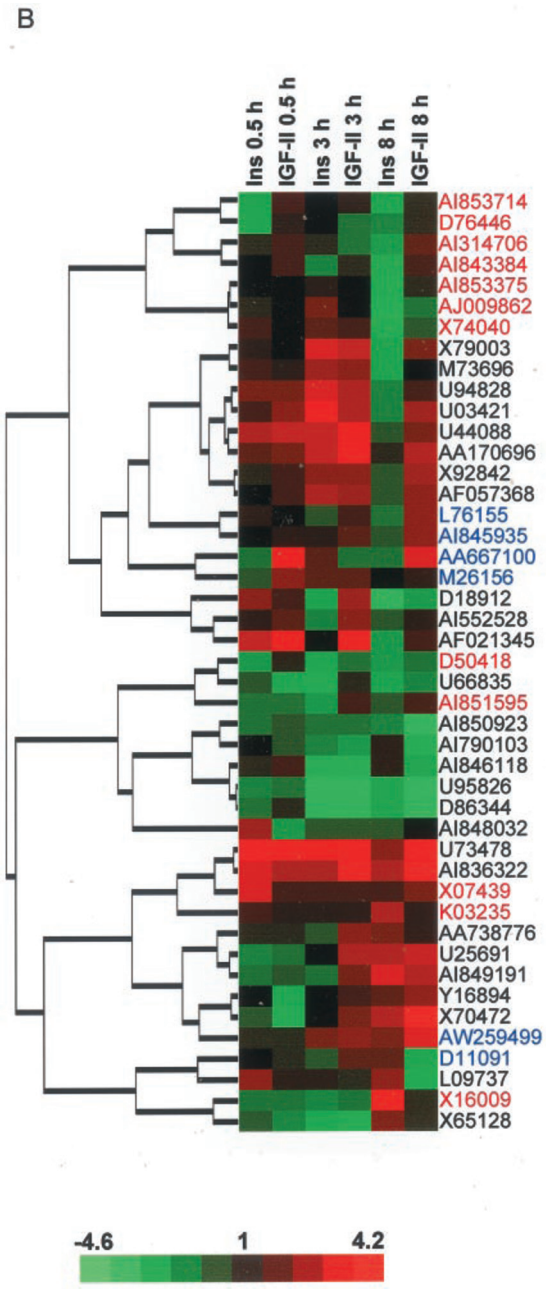


FIG. 1—*continued*

cRNA Preparation—R⁻/IR-A cells were grown until 80% confluent and serum-starved for 24 h. Cells were then stimulated with 10 nM of either insulin or IGF-II for 30 min or 3 or 8 h. Total RNA was isolated by TRIzol reagent, and mRNA was purified from total RNA using Oligotex mRNA kit, according to the protocol recommended by Affymetrix. mRNA (2 μg) was then used to synthesize double-stranded cDNA by Superscript Choice system with T7-(dT)24 as a primer. Biotin-labeled cRNAs were *in vitro* transcribed using the ENZO BioArray kit and fragmented to produce a distribution of RNA fragments with size ranging from ~35 to 200 bases. Samples of fragmented cRNA (15 μg) were hybridized for 16 h at 45 °C to MG-U74A mouse arrays (Affymetrix). Analysis of the scanned chips was carried out using Affymetrix Microarray Suite version 5.0 (MAS5).

Data Treatment—Raw data from GeneChip microarrays were converted with the MAS5 software into a single, tab-delimited text file reporting, for each probe set, the "signal" and "detection" values from all experimental points. This file was subsequently processed with Microsoft Excel as follows. For each probe set, average signal was calculated across all experimental points. The average signal column was used to sort rows by increasing signal and to normalize individual microarray columns using a moving average (window of 200 probe sets) of increasing signal. Such normalization corrected signal non-linearity and allowed comparison of any experimental point with any other. Stimulation points were compared with the controls or between each other through pairwise $\log_2$ ratio calculation and averaging. Standard deviations (S.D.s) of these average $\log_2$ ratios were also calculated. To obtain a more reliable estimate of variability, for each probe set, we also calculated the root mean square standard deviation (r.m.s.s.d.), encompassing all S.D.s of the average $\log_2$ ratios. In fact, although the S.D. of a single duplicate comparison can easily be aberrantly high or low by chance, the r.m.s.s.d. from many duplicate comparisons is a more stable and reliable parameter.

An additional test was performed on these data, based on the "detection" call (present, absent, marginal). In synthesis, if a gene is induced in a certain experimental point, it must be called expressed in that point (not necessarily in the control). Otherwise, if it is suppressed, it must be called expressed in the control. At the end of this process, the following data were obtained for each gene: 1) normalized expression levels for all individual control and stimulated points; 2) average $\log_2$ ratio for each experimental condition with respect to the control or to another experimental condition of choice; 3) S.D. for each average $\log_2$ ratio, and r.m.s.s.d.; 4) call compatibility for each comparison. The first filter was the call compatibility, after which the other parameters were included and "tuned" in a statistical test aimed at identifying significantly regulated genes. The test requires that after subtraction of m*S.D. or of m*r.m.s.s.d., the average $\log_2$ ratio is still higher than a threshold value of T. The tunable values in this test are m, the S.D./r.m.s.s.d. multiplier, and T, the threshold -fold change. To optimize test tuning, we systematically evaluated the false discovery rate, that is, the percentage of the sequences that could have passed the test by chance. False discovery can be estimated by generation, through data permutation, of mixed couples of microarray data that are not expected to display significant gene regulation. Existing microarray analysis tools such as significance analysis of microarrays (19) support data permutation. Differently from significance analysis of microarrays, our modified test weights overall variability of each probe set across all duplicates, which allows more reliable detection of tiny differences in gene expression.[2] We also implemented a permutation strategy and estimated the false discovery rate of our analysis based on 1,260 permutations. The test tuning parameters showing the best performance with the present data were $T = 0.4$ and $m = 1.5$, with which we could detect 259 regulated genes with an false discovery rate below 10%. Test tuning for identifying genes differentially regulated by insulin and IGF-II was slightly different, with $T = 0.4$ and $m = 1$. The false discovery rate above 10% indicated the necessity for real-time PCR validation of these data.

As a control of data robustness, we also used dChip (20) to normalize the data. We saw a lower coefficient of variation in dChip-normalized triplicates and could confirm >90% of the genes originally identified on MAS5-normalized data as regulated by insulin and/or IGF-II. Interestingly, dChip normalization rendered non-significant the regulation of a gene we had already validated by real-time PCR, which indicates that different normalization procedures may also yield non-overlapping false negatives. We therefore decided to make available for download the two spreadsheets containing, respectively, MAS5-normalized and dChip-normalized data.

The analysis spreadsheets and the raw CEL files can be downloaded (www.ircc.it/~emedico/FOG/data). The original data will also be submitted to the NCBI's Gene Expression Omnibus public data base (www.ncbi.nlm.nih.gov/geo) at a later date. Further information is available from the authors on request.

Hierarchical Clustering—Hierarchical clustering of the selected genes was performed using the computer program Cluster (21) and visualized using the program TreeView (available at rana.stanford.edu/software).

Real-time Polymerase Chain Reaction—Primer Express software (PE Applied Biosystems, Foster City, CA) was used to design appropriate primer pairs and fluorescent probes. Primer pairs and probes with 5′-FAM reporter dye and 3′-TAMRA quencher dye were synthesized by MWG-Biotech (Ebersberg, Germany). Probe and primers for endogenous control (glyceraldehyde-3-phosphate dehydrogenase) were from predeveloped TaqMan assay reagents (Applied Biosystems). Quantitative real-time PCR was performed on Abi Prism 7700 (PE Applied Biosystems) using Sybr Green PCR Master Mix and Taqman Universal PCR Master Mix (PE Applied Biosystems) following manufacturer's instructions. To normalize gene expression, a parallel amplification (six replicates) of endogenous and target genes was performed with Sybr Green reagents. For Taqman analysis, all reactions (six replicates) were performed by

[2] E. Medico, M. Riba, L. D'Alessandro, J. Aach, G. M. Church, and P. M. Comoglio, manuscript in preparation.

TABLE II
Genes similarly down-regulated by both insulin and IGF-II

The 40 most strongly regulated genes are listed.

GenBank accession No.	Description	Insulin	IGF-II	Time
				h
	Apoptosis			
Y13087	Caspase-6	−1.6	−1.9	8
L38822	Max interacting protein 1	−1.6	−1.7	3
M31418	Interferon activated gene 202	−1.7	−1.7	3
	Cell cycle			
U60453	*Ezh1*	−2.2	−2.0	3
U00937	*GADD45*	−3.4	−3.6	3
	Cell-to-matrix interaction			
AF022110	Integrin β-5	−1.6	−1.5	8
X06086	Cathepsin L	−1.6	−1.4	8
Z12604	Matrix metalloproteinase 11	−1.8	−2.2	3
D31951	Osteoglycin	−2.0	−1.5	8
	Cytoskeletal functions			
U05252	*SATB1*	−1.8	−1.7	8
	DNA repair			
X91617	5-3 exonuclease	−2.5	−2.5	3/8
	Metabolism			
AF062071	Zinc finger protein 216	−1.5	−1.4	3
D50367	*KAP3B*	−1.8	−1.5	3/8
AF020039	NADP-dependent isocitrate dehydrogenase (*Idh*)	−1.6	−1.5	8
U17132	Zinc transporter 1	−1.4	−1.4	3
	Proliferation/differentiation			
L10244	Spermidine/spermine *N*1-acetyl transferase	−2.0	−1.9	8
D16195	Granulin	−1.5	−1.7	8
AB012161	*KF-1*	−1.8	−1.7	3
X61940	Growth factor-inducible immediate early gene	−2.9	−4.0	3/8
AB019577	UNC-51-like kinase (*ULK*) 2	−1.7	−1.5	3
D78643	Seizure-related	−1.7	−1.5	8
AF037205	RING zinc finger protein (*Rzf*)	−1.6	−2.0	8
M36146	Zinc finger protein 35	−1.7	−1.4	3/0.5
U60593	*Ndr1*	−1.5	−1.6	3
Y07609	Max binding protein	−1.7	−1.5	3
U52073	*TDD5*	−1.7	−1.8	3/8
AB014485	*RA70*	−1.7	−1.5	8
U09504	Thyroid hormone receptor α	−1.6	−1.7	3
X89749	*TGIF*	−1.5	−1.6	8
	Cytokines			
M64849	Platelet derived growth factor B	−2.1	−1.6	8
L07803	Thrombospondin 2	−2.1	−1.8	8
X54542	Interleukin 6	−3.5	−1.6	8
	Signal transduction			
U90435	Flotillin	−1.5	−1.7	8
	Transcription factors			
Y14296	*BTEB-1* transcription factor	−1.5	−1.9	8
	Miscellaneous			
M13945	Proviral integration site 1	−1.4	−1.6	8
AF020308	*HRS*	−1.5	−1.5	3
AF110520	Major histocompatibility complex class II	−1.5	−1.8	0.5/3
Z80112	*Lcr-1* gene	−5.9	−3.4	3/8
X78445	*Cyp1-b-1*	−1.9	−2.0	3
X95761	New-Rhobin	−1.4	−1.9	3

co-amplifying in the same tube endogenous and target genes. To check reaction sensitivity, in preliminary experiments, serial dilutions of each cDNA (1, 1:10, 1:100, 1:1,000) were amplified for endogenous and target genes. The reaction efficiency resulted similar in simplex and duplex reactions (*i.e.* slope = $3.6 \leq x \geq 3$; correlation coefficient ≥0.99). Relative quantitative evaluation (PE Applied Biosystems user bulletin number 2) of target gene levels was performed by comparing ΔCt, as described previously (22).

RESULTS

Genes Regulated by Insulin and/or IGF-II in R⁻/IR-A Cells

To analyze gene expression profiles following IR-A activation by either insulin or IGF-II, R⁻/IR-A cells were stimulated with either ligand (10 nM) for various time intervals (0.5, 3, and 8 h). Biotinylated cRNA probes were generated from the RNA extracted from control and ligand-stimulated cells and hybridized to microarray membranes containing the entire mouse genes, according to Affymetrix procedure. Using the analysis strategy

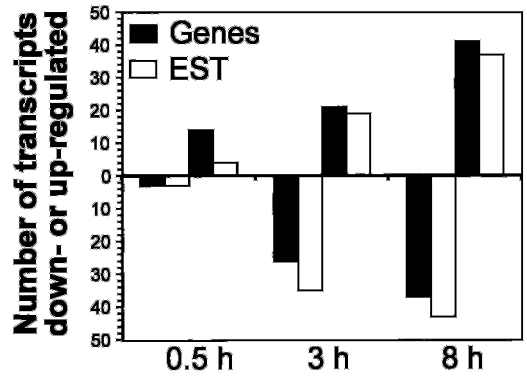


FIG. 2. **Number of transcripts similarly regulated by the two ligands at the different time points.** The figure represents the total number of genes and ESTs down- or up-regulated at a given time point. In some genes, regulation was transient and only detected at one time point, whereas it was more prolonged in other cases.

TABLE III
Genes regulated by only one ligand

ID	Description	Insulin (-fold change)	IGF-II (-fold change)	Δ[a]	Time	Function
					h	
A. Regulated only by insulin						
X16009	*Mrp/plf*	2.0	−1.0	2.1	8	Angiogenesis regulator
AI843384	BLAST: BC019982 *TK2*	−1.6	1.2	1.9	8	DNA synthesis/repair
AI853714	BLAST: NM_007798 Cathepsin B	−1.8	1.2	1.8	0.5	Miscellaneous
AI314706	Unknown	−1.4	1.3	1.7	8	
X07439	*Hox-3.1*	1.9	1.2	1.6	0.5	Angiogenesis regulator
D76446	*TAK1* (TGF-β-activated kinase)	−1.5	1.1	1.6	0.5	Signal transduction
AI853375	BLAST: BC050902 *Mdm2*	−1.7	−1.1	1.6	8	Cell cycle
AI851595	Unknown	−1.4	1.2	1.6	3	Miscellaneous
X74040	Mesenchyme fork head-1	−1.8	−1.2	1.5	8	Signal transduction
D50418	Mouse mRNA for *AREC3*	−1.6	−1.2	1.4	3	Metabolism
AJ009862	Transforming growth factor-β 1	−1.8	−1.3	1.4	8	Cytokine
K03235	Proliferin	1.6	1.1	1.1	8	Angiogenesis regulator
B. Regulated only by IGF-II						
AA667100	BLAST: XM_128828 *GATA-6*	−1.2	2.1	2.6	0.5	Transcription
D11091	Protein kinase C θ	1.3	−1.5	1.9	8	Signal transduction
L76155	*Bat-4*	−1.2	1.4	1.8	8	Metabolism
AI845935	BLAST: AB042855 *GNB-1*	−1.1	1.5	1.7	8	Signal transduction
M26156	Histocompatibility 2	−1.2	1.4	1.6	0.5	Miscellaneous
AW259499	BLAST: XM_194355 similar to hypothetical protein	1.2	1.9	1.6	8	

[a] Δ indicates the ratio of IGF-II stimulation/insulin stimulation.

TABLE IV
Genes and EST regulated by both insulin and IGF-II but with a significant difference between the two ligands in at least one time point

ID	Description	Insulin (-fold change)	IGF-II (-fold change)	Δ[a]	Time	Function
					h	
U73478	Acidic nuclear phosphoprotein 32	1.4	3.8	2.6	8	Development
L09737	GTP cyclohydrolase 1	1.4	−17	2.3	8	Angiogenesis regulator
D18912	BLAST: BC029234 ADP-ribosylation-like 4	−1.5	1.5	2.3	3	Miscellaneous
AF021345	Selenoprotein P (*SELP*)	1.0	2.2	2.2	3	Metabolism
AI848032	BLAST: AK087414 coronin 2B	1.5	−1.4	2.2	0.5	Miscellaneous
U03421	Interleukin 11	−1.3	1.6	2.1	8	Cytokine
X79003	Integrin α 5	−1.5	1.3	2.0	8	Adhesion
AI552528	Unknown	−1.4	1.4	1.9	3	Miscellaneous
AI790103	BLAST: BC002102 *Ifitm3l*	1.1	−1.7	1.9	8	
X92842	Surfeit gene 6	−1.2	1.5	1.8	8	Nucleolar matrix
AA170696	BLAST: BC016198 *ICAM*	−1.0	1.6	1.7	8	Adhesion
U95826	Cyclin G2	−1.5	−2.5	1.7	8	Cell cycle regulator
D86344	Topoisomerase-inhibitor suppressed	−1.5	−2.5	1.7	8	Apoptosis regulator
AI850923	Unknown	−1.3	−2.3	1.7	8	Miscellaneous
AI849191	BLAST: AK003714 *ZNRD1*	−1.3	1.3	1.6	3	Transcription
U25691	Helicase, lymphoid-specific	−1.0	1.6	1.6	3	DNA sinthesis/repair
AF057368	7-dehydrocholesterol reductase	−1.2	1.4	1.6	8	Metabolism
U66835	Unknown protein	−1.7	−1.1	1.6	3	Miscellaneous
AA738776	BLAST: BC026772 *PSPC1*	−1.1	1.5	1.6	3	
Y16894	Hus1+-like protein	1.0	−1.6	1.6	0.5	
AI836322	BLAST: AB055070 *RhoGDI-1*	1.6	2.5	1.5	8	Signal transduction
U94828	Regulator of G-protein signaling	−1.3	1.2	1.5	8	
M73696	Murine *Glvr-1*	−1.5	−1.0	1.5	8	Metabolism
X70472	Myeloblastosis oncogene-like 2	−1.1	−1.7	1.5	0.5	Oncogene
AI846118	Unknown	1.0	−1.5	1.5	8	Miscellaneous
U44088	*TDAG51*	−1.1	1.3	1.4	8	Apoptosis regulator
X65128	Growth arrest-specific 1	1.4	−1.1	1.4	8	Cell cycle regulator

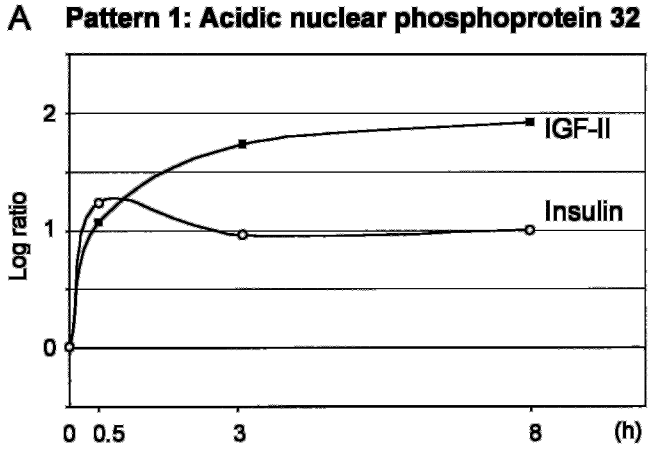
[a] Δ indicates the ratio IGF-II stimulation/insulin stimulation.

described under "Experimental Procedures," we identified 259 genes (132 known genes and 127 ESTs) regulated by one or both hormones. Variations of gene expression, as compared with basal levels, ranged from +1.3 to +4.2 and from −1.3- to −7.9-fold changes.

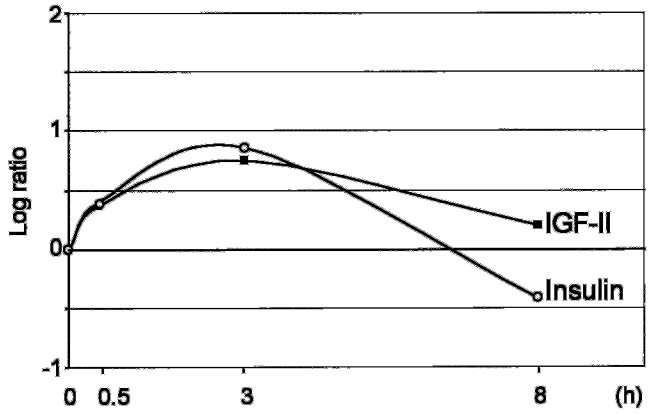
Genes Similarly Regulated by Both Insulin and IGF-II

Two hundred and fourteen genes and ESTs were regulated with a similar pattern by both insulin and IGF-II (Fig. 1*A*). Sixty genes (Table I) and 52 ESTs were similarly up-regulated by the two hormones, whereas 48 genes (Table II) and 58 ESTs were similarly down-regulated. Three genes, *JunB*, *IL-6*, and zinc transporter 1, and one EST (GenBank™ accession number AI606257), are present in both tables because they are up-regulated and down-regulated at different time points: *JunB* and *IL-6* were up-regulated at 30 min and 3 h, respectively, and then down-regulated at 8 h; zinc transporter 1 was down-regulated at 3 h and subsequently up-regulated at 8 h. Most of the genes regulated are considered regulators of apoptosis, cell cycle, proliferation, signal transduction, metabolism, and differentiation (Tables I and II).

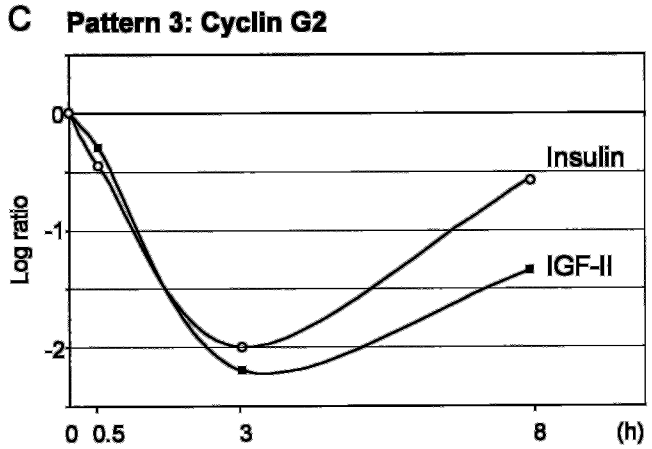
In some genes, up- or down-regulation was transient and only detected at one time point, whereas it was persistent in other cases, as indicated by cluster analysis (Fig. 1*A*). The



ID	Gene
U73478	Acidic nuclear phosphoprotein 32
AI836322	RhoGDI-1
X92842	Surfeit gene 6
AI849191	Trascription-associated zinc ribbon
U25691	Helicase, lymphoid specific



ID	Gene
X79003	Integrin α 5
AA170696	ICAM
U44088	TDAG51
U94828	Regulator of G-protein signaling
AF057368	7-dehydrocholesterol reductase
M73696	Murine Glvr-1
AF021345	Selenoprotein P
U03421	Interleukin 11



ID	Gene
U95826	Cyclin G2
D86344	Topoisomerase-inhibitor suppressed
X65128	Growth arrest specific 1

FIG. 3. **Expression patterns in genes differentially regulated by insulin and IGF-II.** The expression profile of 16 differentially regulated genes fell into one of the three patterns shown. In *A*, genes were persistently up-regulated by both ligands with IGF-II being more potent, especially at 8 h. In *B*, genes were up-regulated by both ligands; gene expression, however, remained above basal levels after IGF-II, whereas it decreased significantly after insulin. In *C*, genes were persistently down-regulated by both ligands with IGF-II being more potent than insulin at 8 h.

number of genes and ESTs similarly up-regulated or down-regulated by the two ligands at the different time points is indicated in Fig. 2.

Genes Differentially Regulated by Insulin and IGF-II

Comparative analysis of the ~6,000 genes and ~6,000 ESTs on the cDNA microarrays revealed that 45 transcripts (27 genes and 18 ESTs) were differentially regulated by insulin and IGF-II.

Transcripts Regulated by Only One Ligand—Eighteen of these differentially regulated genes (10 genes and 8 ESTs) were responsive to only one of the two ligands. Twelve transcripts (7 genes and 5 ESTs) responded only to insulin (3 up-regulated and 9 down-regulated, Table III), whereas 6 transcripts (3 genes and 3 ESTs) responded only to IGF-II (5 up-regulated and 1 down-regulated; see Table III).

Three genes selectively up-regulated by insulin are genes involved in angiogenesis regulation and differentiation: *mrp/*

TABLE V
Primer and probe sequences used for real-time PCRfs

Gene	ID	Primer and probe
Phosphoprotein 32	U73478	sense (28) GGAGATGGACAAACGGATTTATT antisense (82) TATCCAGGACCAGCTCTTTCACA probe (54) AGCTGCGGAACAGGACGCCCTCT
Proliferin	X16009	sense (145) GTTGCCTCATTTCCCATGTGT antisense (203)GCCGGCTAATTCAAATGTGTCT probe (167) CAATGAGGAATGGTCGTTGCTTTATGTCCTT
RGS-r	U94828	sense (754) GGAGTCGCCCGTCCCTAA antisense (828) TCCACTATCCTCTTGCACTTGCT probe (780) CCCTGTGTGGGAGGCAGATCC
TDAG51	U44088	sense (832) AGCGCAAGGGCAAGTACATG antisense (900) GGCACCGAAAGTCGATCTCTT probe (857) CACTGTGGTGATGACGGAGGGC
Bat-4	L76155	sense (102) TCACAGGCATGTCTAGGCCA antisense (159) TCACTGGAGTCAGCCGCTG
CyclinG2	U95826	sense (902) GCTGCAAGCCTGATCTGAAGA antisense (969) GCTGTGGAGGTTCTGCGC
7-Dehydrocholesterol reductase	AF057368	sense (43) GCTTCGAAATCCCAGCACA antisense (96) AGCCTTGCCGTTGGGAC
GNB-1	AI845935	Sense (740) TCTTTCCCAATGGCAATGC Antisense (805) GGAGGTCAAACAGCCTGCA
ICAM	AA170696	Sense (210) AACGCCGTGCGCCC Antisense (274) CCGTAAAGCTTCTAGCTCCATCTC
Integrin 5 α	X79003	Sense (1537) GCCGTACCCCAGACTTCTTTG Antisense (1616) AACGATTAGATCAGGGTATCCATTG
Thymidine kinase 2	AI843384	Sense (839) GATCTACAAGACAGCGCCAGG Antisense (904) CTTCCAAATGCTCCTAGCAGCT
Topoisomerase inibitor	D86344	Sense (723) GCCAGCCACCGGGAG Antisense (776) CACCGTCCCGCAAAGGT

plf, proliferin, and *Hox-3.1*. *Mrp/plf* and proliferin are highly homolog proteins that belong to the superfamily of prolactin/growth hormone and act as endogenous regulators of angiogenesis. These factors play a role in blood vessel formation and remodeling by affecting endothelial cell proliferation, apoptosis, and migration. However, these two factors have opposite functions as mrp/plf is an inhibitor of angiogenesis, whereas proliferin is a promoter of angiogenesis (23–26). *Hox-3.1* is a gene of the homeobox family that is involved in development regulation. Genes down-regulated by insulin include transcripts encoding for TGFβ1, a negative growth regulator, and for TAK1 (TGF-β-activated kinase), a caspase-independent antiapoptotic factor (27–30). Mesenchyme fork head-1, a transcription factor that affects adipocyte metabolism and that is increased by high fat diet, seems to counteract most of the symptoms associated with obesity, including hypertriglyceridemia and diet-induced insulin resistance, a protection against type 2 diabetes (31, 32).

Transcripts that are responsive and up-regulated only by IGF-II include *GNB-1*-like EST and the *Bat-4* gene. *GNB-1* encodes for a protein that releases cGMP-phosphodiesterase from inhibition (33, 34). *Bat-4* encodes for a protein of the family of the major histocompatibility complex. Only one gene was responsive only to IGF-II and down-regulated, protein kinase C θ, which plays a role in insulin receptor signaling, differentiation, and survival of T-cells and in multiple processes essential for angiogenesis, regulation of cell cycle progression, and formation and maintenance of actin in the cytoskeleton (35–37).

Transcripts Regulated by Both Ligands but with a Different Time Pattern—Twenty-seven transcripts (17 genes and 10 ESTs) were regulated by both insulin and IGF-II but showed, at least in one time point, a significant difference between the two ligands (Table IV). The expression profile of 16 of these transcripts followed three major patterns: pattern a transcription was persistently up-regulated by both ligands with IGF-II being more potent; pattern b transcription was transiently up-regulated by both ligands; it persisted, however, above basal levels after IGF-II, whereas levels after insulin were significantly lower; pattern c transcription was persistently down-regulated by both ligands with IGF-II being more potent than insulin.

Five genes fell in pattern a (Fig. 3). These genes encode for: acidic nuclear phosphoprotein 32, a protein that is expressed at high levels during embryogenesis and decreases as tissues terminally differentiate (38–40); RhoGDI-1, a GTP-binding protein; surfeit 6, a nucleolar matrix protein ubiquitously expressed with nucleic acid binding properties (41–43); transcription-associated zinc ribbon protein, a protein that binds to transcription factors and to DNA; helicase-lymphoid-specific, a protein involved in DNA repair.

Eight genes followed pattern b (Fig. 3). Up-regulation of these genes was more persistent following IGF-II than following insulin. Two of these genes play an important role either in cell-substrate adhesion (integrin-α_5) or in cell-to-cell interaction (*ICAM*). One gene is a regulator of G-protein signaling (retinally abundant regulator G-protein). *TDAG51* is a gene involved in apoptosis regulation of T-cells that is required for Fas expression and is also involved in apoptosis resistance and growth dysregulation in cancer, as shown in the melanoma model (44). Three genes in this group are involved in various aspects of the metabolism: cholesterol metabolism (7-dehydrocholesterol reductase), phosphate transport (*Glvr-1*), selenium supply, and prevention of oxidative stress (selenoprotein P).

Three genes followed pattern c: these included topoisomerase inhibitor-suppressed and two negative regulators of cell cycle, cyclin G2 and *Gas1*. These genes were more potently down-regulated by IGF-II with a significant difference at 8 h (Fig. 3).

Validation of Microarray Data

To validate the microarray transcription data, 12 genes belonging to different functional categories were selected for real-time PCR confirmation. To minimize data variation, the same cDNA samples used for microarray analysis were used also for real-time PCR. Genes selected and primer pairs used are reported in Table V. At least two independent experiments were

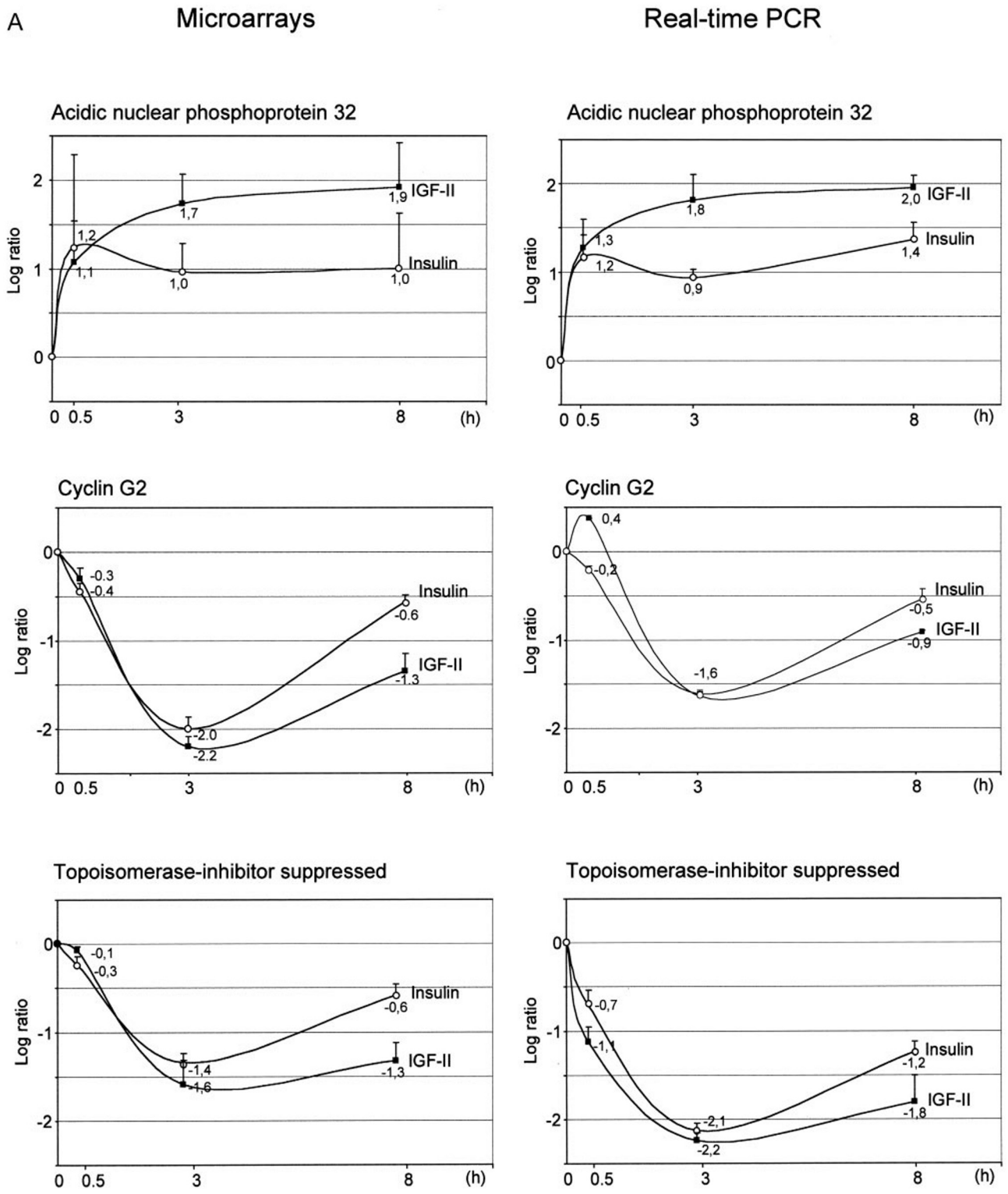


FIG. 4. **Validation of microarray data by quantitative real-time PCR.** Among the 45 genes differentially regulated by insulin and IGF-II, 12 genes were analyzed also by real-time PCR (*A* and *B*). The -fold induction was computed using a standard curve analysis and normalized to the level of glyceraldehyde-3-phosphate dehydrogenase as described under "Experimental Procedures." All PCR reactions were performed at least in duplicate. The expression profile after either insulin or IGF-II stimulation (-fold change, mean ± S.D.) is shown for 6 representative genes and compared with data obtained by microarray analysis.

carried out for each gene. In these 12 genes, the magnitude of the regulation obtained by real-time PCR was similar to that obtained by microarray analysis. The expression profile of 6 representative genes by the two techniques is shown in Fig. 4. Data obtained in the 12 genes of the validation group at the different time points by either microarrays or real-time PCR are highly correlated ($r^2 = 0.7096$, $p < 0.0001$, Spearman correlation) (Fig. 5).

DISCUSSION

Both IR isoforms (IR-A and IR-B) are expressed by most human tissues (1, 2). The relative abundance of these two

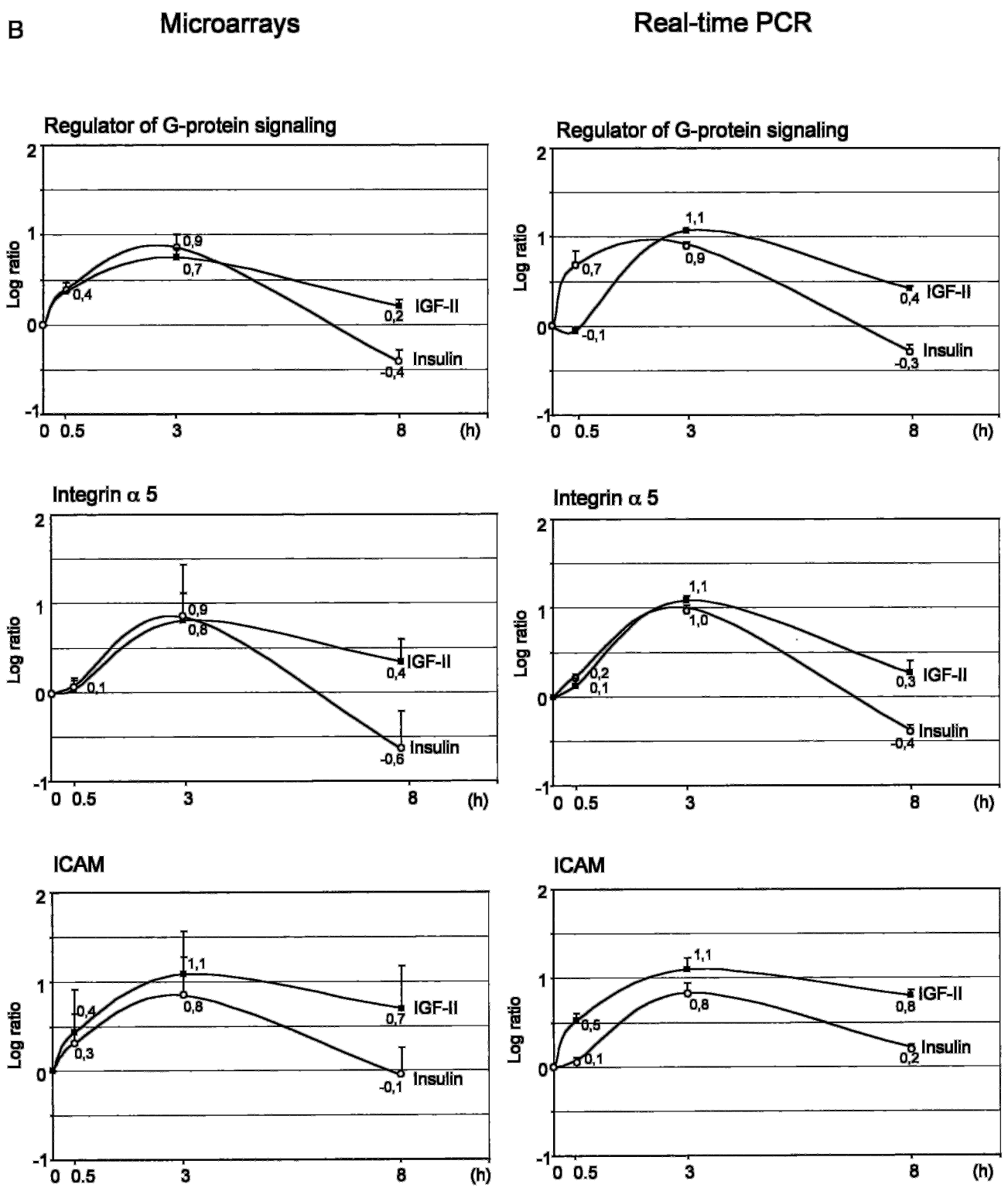


FIG. 4—*continued*

isoforms is strictly regulated by tissue-specific factors (1, 2). In general, IR-B is more expressed than IR-A by classical insulin target tissues (1, 2). Recently, we have tried to clarify the biological role of IR-A. We found that IR-A binds not only insulin but also IGF-II with high affinity and behaves as a second physiologic receptor for IGF-II in fetal cells (7). Moreover, IR-A was found overexpressed both in epithelial and mesenchymal malignancies, including cancer of the breast, colon, lung, thyroid, and myosarcomas (11–13). In these malignancies, IR-A overexpression activates an autocrine/paracrine loop involving IGF-II that appears to promote growth, protection from apoptosis, and cancer progression (12). Recently, an independent study has extended these findings to ovary cancer (15). Furthermore, we have shown that IR-A overexpression profoundly affects the binding and signaling specificities of IR/IGF-IR hybrids (hybrid-R), heterodimeric receptors containing an IR, and an IGF-I-R hemireceptor, which are abundant in all tissues that express both IR and IGF-IR (10, 45). Taken together, these data suggest that IR isoform switching is a major regulator of the IGF system (45).

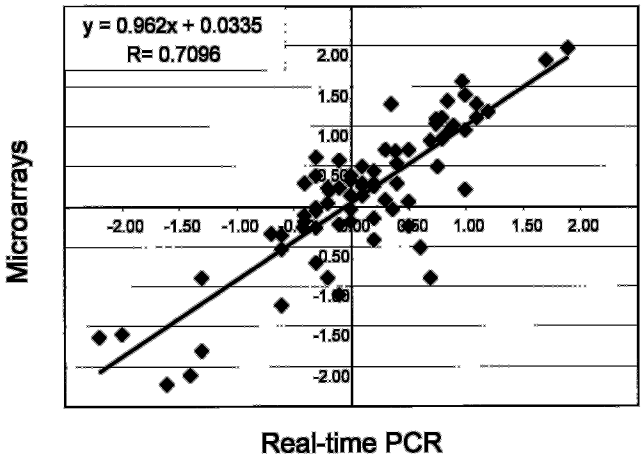


FIG. 5. **Correlation of microarray and real-time PCR data.** -Fold changes of transcripts measured by quantitative real-time PCR at the various time points after insulin or IGF-II stimulation were plotted against the corresponding values obtained by microarray analysis. Data obtained with the two techniques were highly correlated ($r^2 = 0.7096$, $p < 0.0001$, Spearman correlation).

A puzzling finding was the observation that, after IR-A binding, IGF-II is a stronger mitogen than insulin, whereas insulin is a stronger activator of glucose uptake (7). For these studies, to avoid the interference of IGF-IR that may also bind IGF-II with high affinity, we used R^- mouse embryo fibroblasts, cells with disrupted *igf-1r* by homologous recombination, which express low endogenous IR levels (17, 46). When transfected with human IR-A cDNA, these cells (R^-/IR-A cells) expressed the human IR-A at high level ($\sim 5 \times 10^5$/cell). Such a model avoids cross-reactivity of ligands with receptors other than IR-A.

By using the same model, we now show that upon binding to IR-A, insulin and IGF-II cause a differential gene expression. To analyze the physiological profile of gene expression by microarrays, we exposed these cells to a low dose (10 nM) of each of the two hormones and carried out a three-point time course (30 min and 3 and 8 h) as it is known that the expression of genes may vary with the time elapsed after stimulation. We addressed in a careful way the problem of data variability, a limitation of microarray analysis. To deal with variability, we obtained data from two separate experiments, each containing three time points, and used stringent selection criteria that allowed us to define as significant a 1.3-fold difference in the transcript relative abundance with a very small probability that a gene classified as regulated could be a false positive. In accordance with these selection criteria, it was recently reported that a <2-fold variation in gene expression can be biologically relevant (47).

Out of ~6,000 genes and ~6,000 ESTs, a total of 259 genes and ESTs were regulated by one or both ligands (insulin and IGF-II). The majority of these genes ($n = 214$) were similarly regulated by insulin and IGF-II. The magnitude of gene expression variation ranged from +1.3 to +4.2 and from −1.3 to −7.9, values similar to those reported by other authors (47, 48).

Two recent studies have reported microarray analysis of gene expression in IR-transfected cells (47, 48). However, these authors have used a different cell model and different stimulation and analysis modalities. Dupont *et al.* (48) used NIH-3T3 cells transfected with IR and stimulated cells for 90 min. Mulligan *et al.* (47) used 3T3-L1 cells transfected with a TrkC/IR chimeric receptor and stimulated cells for 4 h. In addition, both studies addressed the differences in gene expression between insulin and IGF-I on their cognate receptor, and none evaluated the IR-A isoform. Many genes stimulated by insulin in our model had not been reported previously (47, 48). Interestingly, 6 genes (integrin α_5, early growth response 1, *Jun* oncogene, IGF binding protein 10, T-cell death-associated gene 51, immediate early protein *Gly96*) were reported previously to be regulated only by IGF-I in cells overexpressing the IGF-IR but not in cells overexpressing the IR (48). The reason for this discrepancy is unclear. Possible explanations may include the different experimental system and methodology. Moreover, one intriguing possibility is that IR-B and IR-A isoforms have a different capability to regulate gene transcription.

Remarkably, 45 genes and ESTs were differentially expressed in response to either insulin or IGF-II. These genes were mainly involved in cell cycle and apoptosis regulation, angiogenesis, cell-to-matrix or cell-to-cell interaction, and signal transduction. In 12 of them (validation group), the relative abundance of differentially regulated genes was also confirmed by quantitative real-time PCR with expression profiles very similar to those obtained by the microarray analysis.

Eighteen of these transcripts (40%) were responsive to only one ligand; 12 of them were responsive only to insulin, and 6 were responsive only to IGF-II. The remaining 27 differentially regulated genes (60%) responded to both ligands but with a different magnitude or a different time course; in all of them, IGF-II was more potent than insulin either in inducing up-regulation or in inducing down-regulation.

The greatest difference in favor of IGF-II was observed for acidic nuclear phosphoprotein 32 (*ANP32*) gene (40). ANP32 is a protein involved in embryogenesis that is switched off in differentiated cells. Although *ANP32* is not an oncogene and can actually inhibit transformation, it increases resistance to apoptosis (38–40). In humans, several ANP32 isoforms have been described; one of these isoforms is increased in prostate cancer and has been suggested to play a role in this malignancy (38–40).

Two genes, $\alpha_5\beta_3$ integrin and *TDAG51*, reported previously to be responsive to IGF-I and not to insulin, in our system demonstrated themselves to be more strongly regulated by IGF-II than by insulin. $\alpha_5\beta_3$ integrin has been recently described to be an important determinant of the IGF-IR activation (49) and to play a role in DNA synthesis, cell proliferation, and migration. Previously, we have shown that IR-A preferential expression, in contrast to IR-B predominant expression, plays a role in the activation of the IGF-I system by multiple mechanisms, including direct binding of IGF-II and formation of IR/IGF-IR hybrid receptor (hybrid-R^A), that bind both IGFs with high affinity and insulin with low affinity (45). Up-regulation of $\alpha_5\beta_3$ integrin by IR-A phosphorylation (more potently stimulated by IGF-II) may be an additional mechanism contributing to the activation of the IGF system (50). TDAG51 is a positive regulator of apoptosis in T-cells (44), and transcriptional up-regulation of this molecule by two antiapoptotic factors such as insulin and IGF-II may be an unexpected finding. However, although considered proapoptotic, *TDAG51* is also up-regulated by IGF-I (48) and platelet-derived growth factor (51) through the activation of the Akt pathway (51), which is strongly involved in survival. *TDAG51* regulation by IR-A may reflect a mechanism common to different growth factors and may underscore the fact that apoptosis is also regulated by the IR-A isoform, by both proapoptotic and antiapoptotic factors, as shown previously for IGF-IR (44, 52, 53).

The gene encoding for 7-dehydrocholesterol reductase was also significantly more responsive to IGF-II than to insulin. The protein encoded by this gene is a key enzyme in the metabolism of cholesterol but is also an important factor in morphogenesis. Mutations of this gene lead to the Smith-Lemli-Opitz syndrome, characterized by multiple congenital anomalies such as microcephaly, cleft palate, visceral malfor-

mations, postnatal failure to thrive, and mental retardation (54–56). This observation is consistent with the important role of IGF-II during embryogenesis and fetal development (4, 5). Among genes responsive to IGF-II more than to insulin are also selenoprotein P, a selenium supply protein involved in the prevention of oxidative stress (57–59), and the regulator of G-protein signaling. The preferential stimulation of these genes by IGF-II may reflect the protective role of IGF-II on cell survival and G-protein receptor differential regulation by the two ligands. Finally, two genes involved in the negative control of cell cycle (cyclin G2- and growth arrest-specific 1) were more strongly down-regulated by IGF-II than by insulin.

In contrast, *Mrp/plf* and proliferin, both involved in angiogenesis regulation, were significantly up-regulated only by insulin and not by IGF-II. These results have not been reported before and may indicate a possible, previously unrecognized, role of IR-A in the control of angiogenesis.

In conclusion, we show for the first time that insulin and IGF-II may differentially affect gene expression in cells expressing the fetal IR isoform and lacking the IGF-IR. Although some genes appear to be regulated only by insulin, 6 genes are selectively regulated by IGF-II, and other genes show a more potent and/or persistent regulation after IGF-II than after insulin. These findings may appear surprising when considering that IR-A binds IGF-II with a lower affinity than insulin (7). However, these data are in agreement with our previous data showing that IGF-II is a more potent mitogen than insulin in R^- cells transfected with the IR-A (7). Similar data were also reported by Morrione *et al.* (17). Moreover, we also previously found that IGF-II is more potent than insulin in stimulating chemoinvasion in SKUT-1 leiomyosarcoma cells that overexpress IR-A and lack IGF-IR (13). In cells that express both IR and IGF-IR, the relative abundance of the two IR isoforms affects the relative potency of insulin and IGF-II signaling to hybrid-Rs, as hybrid-R^A binds IGF-II much better than insulin (45), and according to the present data, we may expect that the two ligands elicit partially different transcriptional effects also via hybrid-R^A. These findings may help to clarify the biological role of IR-A in embryonic/fetal growth and the biological advantage that malignant cells acquire by overexpressing IR-A.

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