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 predominate 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 was independently shown that IGF-II is stronger than insulin in regulating glucose uptake (7). In accordance with our findings, it was more potent than IGF-II in stimulating the Shc/ERK pathway. As a result, IGF-II was more potent than insulin in inducing apoptosis protection (13). Taken together, these studies indicate that insulin and IGF-II, by binding to 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) 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 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). Gene expression was studied at growth factor (IGF-I, IGF-II, or G418) levels, and we found that 2314 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 have recently demonstrated that IR-A is the predominant isoform in fetal tissues and malignant cells producing IGF-II may acquire via IR-A overexpression.

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† The abbreviations used are: IR, insulin receptor; IGF, insulin-like growth factor; TGF, tumor 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.
ligand and 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 RIR-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, DNase 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—RIR-A mouse fibroblasts (mouse 3T3-like cells derived from animals with a targeted disruption of the IGF-IR gene, expressing ~5×10^5 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. RIR-A cells grown in 35-mm plates until 60–70% confluent were co-transfected with 2µg of pNTK2 expression vector containing the cDNA encoding for the A (Ex11−) isoform of the human IR (18) and with the pPVD6+ plasmid encoding for the puromycin resistance gene. Cells were subsequently subjected to antibiotic selection in medium supplemented with 2.4 µg/ml puromycin 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).

### Table I

| GenBank accession No. | Description | Insulin | IGF-II | Time |
|-----------------------|-------------|---------|--------|------|
| U73478                | Acidic nuclear phosphoprotein 32 | 2.4     | 3.8    | 0.5/8|
| AF064447              | Sex-determination protein homolog Fem1a | 2.7     | 3.1    | 3    |
| AB013839              | TIA     | 2.2     | 1.9    | 8    |
| U93583                | RAD51-associated protein 1 | 1.7     | 1.7    | 8    |

**Cell cycle**

- CDC47: 1.8  1.9  8
- Flap structure specific endonuclease 1: 1.8  1.9  8
- CDC45-related protein: 1.8  1.9  8

**Differential Transcriptional Effects of Insulin and IGF-II**

**Genes similarly up-regulated by both insulin and IGF-II**

| GenBank accession No. | Description | Insulin | IGF-II | Time |
|-----------------------|-------------|---------|--------|------|
| M28845                | Early growth response 1 | 3.5     | 3.4    | 0.5  |
| G41552                | Amphiuregulin | 2.8     | 2.7    | 3    |
| M59821                | Growth factor-inducible protein (pip92) | 2.7     | 2.6    | 0.5  |
| M14223                | Ribonucleotide reductase subunit 2 | 2.1     | 2.5    | 8    |
| AJ232087              | 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    |
| X67444                | Gly96 | 2.0     | 2.2    | 0.5  |
| M243777               | Early growth response 2 | 2.1     | 2.0    | 0.5  |
| M339960               | Mouse plasminogen activator inhibitor (PAI-1) | 2.1     | 2.0    | 3    |
| D86725                | MCM2 | 1.9     | 2.0    | 8    |
| M177280               | Nerve growth factor β | 2.4     | 1.9    | 3    |
| K02927                | Ribonucleotide reductase subunit 1 | 1.8     | 1.8    | 8    |
| D70264                | Heparin binding EGF-like<sup>a</sup> growth factor | 1.9     | 1.7    | 3    |
| M70642                | Fibroblast-inducible secreted protein | 1.7     | 1.6    | 3    |

### Other Functional Categories

- **Apoptosis**
  - RAD51: 1.7  1.9  8
- **Metabolism**
  - PDK1: 1.9  2.0  8
- **Cytoskeletal functions**
  - rhoB gene: 1.8  1.7  0.5
  - DNA mismatch repair: 1.6  1.7  8
- **Protein synthesis**
  - VEGF, epidermal growth factor: 2.8  2.7  3
- **Cell cycle**
  - CDC47: 1.8  1.9  8
  - Flap structure specific endonuclease 1: 1.8  1.9  8

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<sup>a</sup> EGF, epidermal growth factor.
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.
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 TRIzol reagent, and mRNA was purified from total RNA using 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 TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TRIzol reagent, and mRNA was purified from total RNA using TR

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 all experimental point with any other. Stimulation points were compared with the controls or between each other through pairwise log₂ ratio calculation and averaging. Standard deviations (S.D.s) of these average log₂ 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 log₂ ratios. In fact, although the S.D. of a single duplicate comparison can easily be abnormally 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 log₂ ratio for each experimental condition with respect to the control or to another experimental condition of choice; 3) S.D. for each average log₂ 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 log₂ 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. 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 a 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. 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 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/~medico/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 pre-developed TaqMan assay reagents (Applied Biosystems). Quantitative real-time PCR was performed on ABI Prism 7700 (PE Applied Biosystems) with the use of TaqMan 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

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co-amplifying in the same tube endogenous and target genes. To check reaction sensitivity, in preliminary experiments, serial dilutions of each cDNA (1: 1; 1: 2; 1: 4; 1: 8) were amplified for endogenous and target genes. The reaction efficiency resulted similar in simplex and duplex reactions (i.e. slope = -3.6 ≤ x ≥ 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

| GenBank accession No. | Description                     | Insulin | IGF-II | Time |
|-----------------------|---------------------------------|---------|--------|------|
| Y13087                | Apoptosis                       |         |        |      |
| L39822                | Caspase-8                       |         |        |      |
| M31418                | Max interacting protein 1       |         |        |      |
| Cell cycle            |                                 |         |        |      |
| U60453                | Ech1                            | −2.2    | −2.0   | 3    |
| U90937                | GADD45                          | −3.4    | −3.6   | 3    |
| 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    |
| X91617                | 5'-3' exonuclease                | −2.5    | −2.5   | 3/8  |
| Metabolism            |                                 |         |        |      |
| AF062071              | Zinc finger protein 216         | −1.5    | −1.4   | 3    |
| D53637                | 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 N1-acetyl transferase | −2.0    | −1.9   | 8    |
| D16195                | Granulin                        | −1.5    | −1.7   | 8    |
| AB012616              | 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                |         |        |      |
| AF037205              | RING zinc finger protein (Raf)   | −1.6    | −2.0   | 8    |
| M36116                | Zinc finger protein 35          | −1.7    | −1.4   | 3/8  |
| U60993                | Ndr 1                           | −1.5    | −1.6   | 3    |
| Y07609                | Max binding protein            | −1.7    | −1.5   | 3    |
| U52073                | TP53                            | −1.7    | −1.8   | 3/8  |
| AB014485              | RAF1                            | −1.7    | −1.5   | 8    |
| U08594                | Thyroid hormone receptor α      | −1.6    | −1.7   | 3    |
| X89749                | TGFβ                            | −1.5    | −1.6   | 8    |
| Cytokines             |                                 |         |        |      |
| M64849                | Platelet derived growth factor B| −2.1    | −1.6   | 8    |
| L07803                | Thrombopoietin 2                | −2.1    | −1.8   | 8    |
| X54542                | Interleukin 6                   | −3.5    | −1.6   | 8    |
| Signal transduction   |                                 |         |        |      |
| U90435                | Flotillin                       | −1.5    | −1.7   | 3    |
| Transcription factors |                                 |         |        |      |
| Y14296                | BTEB-1 transcription factor     | −1.5    | −1.9   | 8    |
| Miscellaneous         |                                 |         |        |      |
| M13945                | Proval integration site 1       | −1.4    | −1.6   | 8    |
| AF020308              | HRS                             | −1.5    | −1.5   | 3    |
| AF110520              | Major histocompatibility complex class II | −1.8   | −1.8 | 0.5/3 |
| Z60112                | Lcr-1 gene                      | −5.9    | −3.4   | 3/8  |
| Y8445                 | Cyc1-h-1                       | −1.9    | −2.0   | 3    |
| X95761                | New-Rhobin                     | −1.4    | −1.9   | 3    |

![Fig. 2. Number of transcripts similarly regulated by the two ligands at the different time points.](http://www.jbc.org/Downloaded from)
levels, ranged from hormones. Variations of gene expression, as compared with basal genes (132 known genes and 127 ESTs) regulated by one or both "with a similar pattern by both insulin and IGF-II (Fig. 1A were similarly down-regulated. Three genes, Il-6, JunB, and Mrp/plf, 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 A1606257), 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. 1A).

### Table III

| ID     | Description                        | Insulin (-fold change) | IGF-II (-fold change) | Δ^a | Time | Function                      |
|--------|------------------------------------|------------------------|-----------------------|-----|-----|-------------------------------|
| A1843384 | BLAST: BC019982 TK2                | -1.6                   | 1.2                   | 2.1 | 8   | Angiogenesis regulator          |
| A1853714 | BLAST: NM_007798 Cathepsin B       | -1.8                   | 1.2                   | 1.8 | 0.5 | Miscellaneous                 |
| A1514706 | Unknown                            | -1.4                   | 1.3                   | 1.7 | 8   |                                |
| X07439  | Hox-3.1                            | -1.2                   | 1.2                   | 1.6 | 0.5 | Angiogenesis regulator          |
| D74446  | TAK1 (TGF-β-activated kinase)      | -1.5                   | 1.1                   | 1.6 | 0.5 | Signal transduction            |
| A1853375 | BLAST: BC050902 Mdm2               | -1.7                   | -1.1                  | 1.6 | 8   | Cell cycle                    |
| A1851595 | 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                   | 1.1                   | 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          |

### Table IV

| ID     | Description                        | Insulin (-fold change) | IGF-II (-fold change) | Δ^a | Time | Function                      |
|--------|------------------------------------|------------------------|-----------------------|-----|-----|-------------------------------|
| U73478 | Acidic nuclear phosphoprotein 32   | 1.4                    | 3.8                   | 2.6 | 8   | Development                   |
| L09737 | GTP cyclohydrolase 1               | 1.4                    | -17                   | 23  | 8   | Angiogenesis regulator         |
| D18912 | Blot-14                            | -1.5                   | 1.5                   | 2.3 | 3   | Miscellaneous                 |
| AF021345 | Selenoprotein F (SELF)             | 1.0                    | 2.2                   | 2.2 | 3   | Metabolism                     |
| A184032 | BLAST: AK084414 corin 2B           | 1.5                    | -1.4                  | 2.2 | 0.5 | Miscellaneous                 |
| U03421 | Interleukin 11                     | -1.3                   | 1.6                   | 2.1 | 8   | Cytokine                      |
| X79003 | Integrin a 5                       | -1.5                   | 1.3                   | 2.0 | 8   | Adhesion                      |
| A1552528 | Unknown                           | -1.4                   | 1.4                   | 1.9 | 3   | Miscellaneous                 |
| A179018 | 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           |
| D63344 | Topoisomerase-inhibitor suppressed | -1.5                   | -2.5                  | 1.7 | 8   | Apoptosis regulator            |
| A185903 | Unknown                           | -1.3                   | -2.3                  | 1.7 | 8 | Miscellaneus                   |
| A1849191 | BLAST: AK003714 ZNDR1             | -1.3                   | 1.3                   | 1.6 | 3   | Transcription                 |
| U25691 | Helicase, lymphoid-specific        | -1.0                   | 1.6                   | 1.6 | 3 | DNA synthesis/repair           |
| AP057368 | 7-dehydrocholesterol reductase    | -1.2                   | 1.4                   | 1.6 | 8 | Metabolism                     |
| U66835 | Unknown protein                    | -1.7                   | -1.1                  | 1.6 | 3 | Miscellaneous                 |
| A1738776 | BLAST: BC026772 PSPC1             | -1.1                   | 1.5                   | 1.6 | 3 | Miscellaneous                 |
| Y16894 | Hsp11-like protein                 | 1.0                    | -1.6                  | 1.6 | 0.5 |                                |
| A1836322 | 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 | Metabolism                     |
| M73696 | Murine G1                          | -1.5                   | -1.0                  | 1.5 | 8 | Metabolism                     |
| X70472 | Myeloblastosis oncogene-like 2     | -1.1                   | -1.7                  | 1.5 | 0.5 | Oncogene                      |
| A1846118 | Unknown                           | 1.0                    | -1.5                  | 1.5 | 8 | Miscellaneous                 |
| U44088 | TDA51                             | -1.1                   | 1.3                   | 1.4 | 8 | Apoptosis regulator            |
| X65128 | Growth arrest-specific 1           | 1.4                    | -1.1                  | 1.4 | 8 | Cell cycle regulator           |

Δ^a 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. 1A). 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 other cases, as indicated by cluster analysis (Fig. 1A). The
The 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/
scripts encoding for TGF regulation. Genes down-regulated by insulin include the homeobox family that is involved in development—proliferin is a promoter of angiogenesis (23). Mrp/plf is an inhibitor of angiogenesis, whereas sis, and migration. However, these two factors have opposite remodeling by affecting endothelial cell proliferation, apoptosis, and formation and maintenance of actin in the cytoskeleton (35).

Time Pattern
Twenty-seven transcripts (17 genes and 10 ESTs) was responsive only to IGF-II and down-regulated, protein family of the major histocompatibility complex. Only one gene from inhibition (33, 34).

Transcripts that are responsive and up-regulated only by IGF-II include GNB-1, a negative growth regulator, and Bat-4-like EST and the gene. 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). Mesenchymal 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-α5) 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 (Glur-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
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.

**DISCUSSION**

Both IR isoforms (IR-A and IR-B) are expressed by most human tissues (1, 2). The relative abundance of these two...
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).
the various time points after insulin or IGF-II stimulation were plotted-

fold changes of transcripts measured by quantitative real-time PCR at-

logically relevant (47).

that a gene classified as regulated could be a false positive. In-

transcript relative abundance with a very small probability-

allowed us to define as significant a 1.3-fold difference in the-

three time points, and used stringent selection criteria that-

obtained data from two separate experiments, each containing-

addressed in a careful way the problem of data variability, a-

genes may vary with the time elapsed after stimulation. We-

of the two hormones and carried out a three-point time course-

by homologous recombination, which ex-

ated the IR-A isoform. Many genes stimulated by insulin in our-

studies addressed the differences in gene expression between-

metabolism of cholesterol but is also an important factor in-

strongly involved in survival.

In 12 of them (validation group), the relative-

nal transduction. In 12 of them (validation group), the relative-

were responsive only to IGF-II. The remaining 27 differentially-

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, $\alpha_3\beta_3$ 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. $\alpha_3\beta_3$ integrin has been recently-

described to be an important determinant of the IGF-IR activa-

tion (49) and to play a role in DNA synthesis, cell proliferation,

and migration. Previously, we have shown that IR-A preferen-
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tion (49) and to play a role in DNA synthesis, cell proliferation,

and migration. Previously, we have shown that IR-A preferen-
tial expression, in contrast to IR-B predominant expression,

plays a role in the activation of the IGF-1 system by multiple-

mechanisms, including direct binding of IGF-II and formation-

of IR/IGF-IR hybrid receptor (hybrid-R$^H$), that bind both IGFs-

with high affinity and insulin with low affinity (45). Up-regu-
lation of $\alpha_3\beta_3$ integrin by IR-A phosphorylation (more potently-

stimulated by IGF-II) may be an additional mechanism con-

tributing to the activation of the IGF system (50). TDAG51 is-

a positive regulator of apoptosis in T-cells (44), and transcription-

al up-regulation of this molecule by two antiapoptotic fac-

tors 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-

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).
motions, 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/pif 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 (59), and the regulator of Mrp, a selenium supply protein involved in the prevention of oxidative stress (57–59), and the regulator of 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.

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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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