Microarray Analysis of Insulin and Insulin-like Growth Factor-1 (IGF-1) Receptor Signaling Reveals the Selective Up-regulation of the Mitogen Heparin-binding EGF-like Growth Factor by IGF-1*

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Insulin and insulin-like growth factor-1 (IGF-1) act through highly homologous receptors that engage similar intracellular signaling pathways, yet these hormones serve largely distinct physiological roles in the control of metabolism and growth, respectively. In an attempt to uncover the molecular mechanisms underlying their divergent functions, we compared insulin receptor (IR) and IGF-1 receptor (IGF-1R) regulation of gene expression by microarray analysis, using 3T3-L1 cells expressing either TrkC/IR or TrkC/IGF-1R chimeric receptors to ensure the highly selective activation of each receptor tyrosine kinase. Following stimulation of the chimeric receptors for 4 h, we detected 11 genes to be differentially regulated, of which 10 were up-regulated to a greater extent by the IGF-1R. These included genes involved in adhesion, transcription, transport, and proliferation. The expression of mRNA encoding heparin-binding epidermal growth factor-like growth factor (HB-EGF), a potent mitogen, was markedly increased by IGF-1R but not IR activation. This effect was dependent on MAPK, but not phosphatidylinositol 3-kinase, and did not require an autocrine loop through the epidermal growth factor receptor. HB-EGF mitogenic activity was detectable in the medium of 3T3-L1 preadipocytes expressing activated IGF-1R but not IR, indicating that the transcriptional response is accompanied by a parallel increase in mature HB-EGF protein. The differential abilities of the IR and IGF-1R tyrosine kinases to stimulate the synthesis and release of a growth factor may provide, at least in part, an explanation for the greater role of the IGF-1R in the control of cellular proliferation.

The insulin receptor (IR) and the IGF-1 receptor (IGF-1R) are highly homologous, both being class II receptor tyrosine kinases with a disulfide-linked tetrameric structure. Both use similar signaling mechanisms, but in vivo the IGF-1R is associated with a more “mitogenic” response, whereas the IR is associated with a more “metabolic” response. This is graphically illustrated by the fact that mice in which the IR has been deleted transgenically are born of normal size but develop severe diabetes and fatal ketoacidosis shortly after birth (1, 2), whereas IGF-1R knockout mice have marked intrauterine growth retardation (3).

Whereas tissue-specific expression could contribute to the differential biological functions of these receptors, both receptors actually show quite widespread expression, and thus it appears likely that at least some of the in vivo specificity relates to differential signaling properties of the receptors. Most previous studies examining this issue have compared cells overexpressing the IR with those expressing the IGF-1R or have used IR/IGF-1R chimeras. The majority of these studies indicate that activation of the IGF-1R is more mitogenic than the IR (4–6), although others (7) have failed to demonstrate such differences. In addition, glycogen synthesis has been shown to be coupled more strongly to the IR than the IGF-1R (6, 8). However, there are several problems associated with the use of these systems, including the formation of hybrid IR/IGF-1Rs and cross-reactivity (ligand binding to non-cognate endogenous receptors), which is likely to occur at the concentrations usually used in ex vivo experiments), all of which may obscure any signaling specificity.

In an attempt to overcome these problems, we have generated TrkC receptor chimeras, consisting of the extracellular domain of the TrkC receptor fused to the intracellular domain of the IR or the IGF-1R (9). Expression of these receptors in cells without endogenous TrkC receptors allows specific stimulation of the receptors by neurotrophin-3 (NT-3) without any background signaling, hybrid formation, or non-cognate ligand binding. As the extracellular domains of the two chimeras are identical, any signaling differences observed are purely due to differences in the intracellular domains of the IR versus IGF-1R. In 3T3-L1 preadipocytes stably expressing these chimeras, we have reported previously (9) that the intracellular domain of the IR couples more effectively to glycogen synthesis than the intracellular domain of the IGF-1R, whereas both appear to couple equally to DNA synthesis. The two receptors also appear to be equally efficient in protecting fibroblasts and adipocytes against apoptosis (10). In 3T3-L1 adipocytes, the IR chimera (TIR) stimulated GLUT4 translocation and glucose uptake to a greater extent than the IGF-1R chimera (TIGR). Differences were also seen in signaling immediately downstream of the
receptors in adipocytes, with the IRS-1 signaling pathway being more effectively stimulated by the TIR chimera, and the Shc/MAPK signaling pathway being more stimulated by the TIGR chimera (11). Thus, it seems that intrinsic signaling differences between the two receptors do exist and are at least in part attributable to sequence differences in the intracellular domains of the IR and IGF-1R.

In order to elucidate further the reasons for the different effects of insulin and IGF-1, we chose to compare the regulation of gene transcription by the two chimeric receptors in 3T3-L1 preadipocytes, using microarray technology to gain a global perspective on transcriptional regulation. Overall, 3T3-L1 TIGR cells showed a greater transcriptional response than TIR cells. Of the differentially regulated mRNAs, the potent mitogen heparin-binding epidermal growth factor-like growth factor (HB-EGF) was shown to be highly selectively up-regulated by TIGR chimeras. The basis for this selective effect on HB-EGF mRNA was explored further, because it was thought to represent an excellent candidate for the preferential mitogenic effects of the IGF-1R.

**EXPERIMENTAL PROCEDURES**

*MATERIALS—Insulin (Actrapid) was supplied by Novo-Nordisk, and IGF-1 was supplied by GroPep. NT-3 was a generous gift from Regenon Pharmaceuticals, and HB-EGF was supplied by Sigma. The inhibitors PD153035, PD98059, and Wortmannin were all from Calbiochem. Cycloheximide and actinomycin-D were from Sigma. Anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology, Inc., and anti-active MAPK antibody (specific for dual-phosphorylated MAPK) and anti-Erk 1/2 antibody (which recognizes non-phosphorylated and phosphorylated forms) were purchased from Promega. Unless indicated otherwise, all cell culture and general purpose laboratory reagents were supplied by Sigma.

**Cell Culture—3T3-L1 TIR/TIGR preadipocytes were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) newborn calf serum, 50 units/ml penicillin/streptomycin, and 2 mm glutamine. NIH-3T3 IR/IGF-1R cells and HaCat cells (12) were maintained in DMEM supplemented with 10% fetal calf serum, 50 units/ml penicillin/streptomycin, and 2 mm glutamine.

**Microarray Analysis—**Confluent 3T3-L1 TIR/TIGR cells were serum-starved overnight and then stimulated with 4 nM NT-3 for 4 h. Total RNA from stimulated and unstimulated cells was extracted using the RNeasy mini kit (Qiagen). This RNA was then processed according to the protocol recommended by Affymetrix, using the Superscript Choice kit (Invitrogen) for double-stranded cDNA synthesis and the Enzo Bioarray kit for in vitro transcription and labeling of cRNA. 15-μg samples of each labeled cRNA were hybridized, in turn, to two cDNA-based arrays by probe synthesis using TIGR cDNA libraries (Affymetrix). Analysis of the scanned chips was carried out using Affymetrix Microarray Suite version 4.0, using default settings and a target intensity of 100 for each chip as a whole. Further analysis (sorting into groups) was carried out using a relational database program based on Filemaker Pro 5, developed for microarray analysis by G. Denyer. The program allowed the formation of clusters based on both data from the chips (mRNA intensity, fold change, etc.) and on known functionality (e.g. pathways, etc.). The clusters were combined in multiple comparison statements (AND/OR/NOT), and genes of interest were followed up by break out to web databases (e.g. SwissProt, BLAST, etc.) for the collection of sequence and functional information.

In order to find transcripts expressed at a similar level in two chips, we first eliminated all transcripts that were classified by the Affymetrix software as increasing to or decreasing from a detectable signal. We also eliminated all those sequences that were more than 1.5-fold different between the two chips, unless they were undetectable in both. To find transcripts that were different between two chips, we selected for mRNAs that were classified as increasing to or decreasing from a detectable signal, with a difference of more than 2-fold. Transcripts were only included in the final lists if they fulfilled these selection criteria in two independent microarray experiments.

**Northern Blotting—**Confluent cells were serum-starved and treated as indicated in the text. Total RNA was extracted using the RNeasy mini-kit from Qiagen and quantified by GeneQuant (Amerham Biosciences). Equal amounts of RNA were resolved by formaldehyde gel electrophoresis in MOPS buffer before transfer and cross-linking to Nytran N membrane (Schleicher & Schuell). Membranes were hybridized to HB-EGF probe in Quickhyb solution (Stratagene) for 3–4 h at 65 °C. Membranes were then washed in 2× SSC, 0.1% SDS (2 times for 15 min), followed by a final wash in 0.1× SSC, 0.1% SDS for 30 min (all at 65 °C), and subsequent visualization using a PhosphorImager (Fuji). HB-EGF probe was made by nick translation of the TIGR cDNA sequence. A radiolabeled probe was used for a single hybridization. The probe was sequenced to verify its identity.

**Western Blotting—**Confluent cells were serum-starved overnight, before being treated as indicated in the text. They were then rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM NaVO₄, 30 mM NaF, 10 mM NaF₂PO₄, 2.5 mM benzamidine, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100). Cleared lysates were assayed for total protein concentration (Bio-Rad Dc kit), and equal amounts of total protein were resolved by SDS-PAGE (using 8% minigels) and transferred by electroblotting to polyvinylidene difluoride membrane (Millipore). The membrane was then placed in blocking buffer (PBS, 0.05% Tween 20, 1% bovine serum albumin) for 1 h at room temperature, followed by incubation with primary antibody overnight at 4 °C. After extensive washing in PBS, 0.05% Tween 20, detection was carried out with horseradish peroxidase-conjugated anti-mouse/rabbit antibody (Dako) and ECL Western blotting detection reagents (Amerham Biosciences).

**Thymidine Incorporation Assay—**HaCat cells were seeded into 12-well plates at equal density and allowed to grow to ~80% confluency. They were then serum-starved for 24 h, followed by treatment for 16 h as described in the text. Culture medium was then replaced with serum-free medium containing 1 μCi/well [3H]thymidine for 4 h (37 °C), followed by two washes in ice-cold PBS and precipitation at 4 °C for 30 min in 10% trichloroacetic acid. Cells were then washed once in 5% trichloroacetic acid and solubilized first in 0.2 M NaOH and then in 0.4 M HCl. Lysates were combined for counting.

**RESULTS**

**TIGR Chimera Signaling Stimulates Transcription to a Greater Extent Than TIR Signaling in 3T3-L1 Preadipocytes—**We first compared transcript levels in NT-3-stimulated TIR/TIGR cells with levels of the same transcripts in untreated cells. Only genes/ESTs that were similar at base line in untreated TIGR and TIR cells according to our selection criteria (see “Experimental Procedures”) were considered further. From this set of transcripts with similar base-line levels, we selected those that were increased or decreased from base line in either cell line after chimeric receptor stimulation. This resulted in a list of 185 transcripts that were up-regulated and 38 that were down-regulated in either cell line. Fig. 1 shows the fold changes from base line for each of these genes/ESTs, represented as a scatter plot with the fold change from base line after TIR stimulation along the horizontal axis, and that for TIR stimulation along the vertical axis. Simple inspection of this plot demonstrates clearly the greater effect of TIGR compared with TIR in terms of numbers of genes whose expression level increased and the extent of that change. Down-regulated genes, on the other hand, did not show such a bias. The bias toward TIGR-mediated stimulation was confirmed by linear regression analysis of the data that yielded a gradient of 0.66.

**Similar and Differential Regulation of a Transcript by TIR and TIGR Chimeras—**As would be expected from the similarity of their receptors, insulin and IGF-1 do have overlapping effects, so we searched for transcripts that were similarly regulated by the two chimeric receptors after stimulation with NT-3, using stringent selection criteria (see “Experimental Procedures”). From a list of transcripts expressed at similar levels in TIR versus TIGR comparisons at base line and after stimulation of the chimeric receptors, we then selected only those genes that were increased or decreased from base line in TIR
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Fig. 1. Changes in gene expression from basal after a 4-h stimulation of TIR and TIGR chimeras. 3T3-L1 TIR/TIGR cells were serum-starved overnight and then treated with 4 nM NT-3 for 4 h before RNA extraction. Untreated samples were extracted at the start of the stimulation period to provide a base line, and RNA expression was studied by microarray analysis. The scatter plot shows all genes that were up-regulated or down-regulated from similar base lines by either receptor (see "Experimental Procedures" for selection criteria). The dotted line indicates a 1:1 ratio, and data from a representative of two independent experiments are shown.

...cells. This approach revealed 23 transcripts that were up-regulated similarly by the TIR and TIGR (Table I, part A). Some of these molecules have known roles in various processes including proliferation, gene expression from transcription to translation, protein modification and degradation, and cellular adhesion. In addition, two secreted products (protein related to DAN and cerberus, small inducible cytokine A2) were up-regulated by both TIR and TIGR stimulation. These could be involved in signaling to neighboring cells, thus adding another layer of complexity to the downstream effects of TIR/TIGR signaling. Fewer mRNAs (13) were equally down-regulated by the two receptors, in agreement with the general expression patterns observed. These transcripts encoded proteins involved in metabolism, apoptosis, signaling, and vesicular transport (Table I, part B).

We defined differentially regulated transcripts as those that were different in a comparison of the two stimulated cell lines but expressed at similar levels in unstimulated cells, again using stringent selection criteria. Fewer mRNAs (11) were differentially regulated compared with those similarly regulated between the two cell lines, consistent with the broadly overlapping effects of insulin and IGF-1. Some of these molecules are involved in signaling to neighboring cells, thus adding another layer of complexity to the downstream effects of TIR/TIGR signaling. Fewer mRNAs (13) were equally down-regulated by the two receptors, in agreement with the general expression patterns observed. These transcripts encoded proteins involved in metabolism, apoptosis, signaling, and vesicular transport (Table I, part B).

Only a fraction of the 223 genes identified as being affected by TIR/TIGR activation were found to be equally or differentially regulated. The remainder fell outside the boundaries of our selection criteria, for example by being between 1.5- and 2-fold different in stimulated cells. The numbers of genes observed as being similarly or differentially regulated were small because we designed our selection criteria with high stringency to reduce the risk of false positive results. Representative genes from each of the three groupings (equally up-regulated, equally down-regulated, and differentially regulated) were examined by Northern blotting, and in each case those results confirmed the microarray result, showing a stimulation of HB-EGF gene transcription in TIGR but not TIR cells. The differences seen were not significant until the 4-h time point and continued up to 24 h, the last time point studied.

We proceeded to determine whether the differences in HB-EGF expression observed in the cells expressing chimeric receptors would also be seen when insulin and IGF-1/holoreceptors were activated. NIH-3T3 cells overexpressing IR or IGF-1R were serum-starved for at least 4 h, followed by 4 h of stimulation with varying concentrations of insulin or IGF-1 (for NIH-3T3 IR or IGFR cells respectively). Equal amounts of RNA extracts were then Northern-blotted for HB-EGF. Three independent experiments showed that although insulin did cause an increase in HB-EGF transcription in this system, IGF-1 clearly stimulated HB-EGF transcription to a greater extent than insulin, at both 5 and 10 nM concentrations (Fig. 2B). Therefore, the selective regulation of HB-EGF by chimeric receptors is also a property of whole IR/IGF-1Rs.

In order to determine whether TIGR signaling was acting on transcription of HB-EGF or on the stability of its mRNA, we stimulated 3T3-L1 TIGR cells with NT-3 for 4 h and then incubated with actinomycin D (to block further RNA synthesis) with or without NT-3. HB-EGF mRNA levels at various time points after actinomycin D treatment were studied by Northern blotting to observe any effect of TIGR signaling on mRNA degradation. TIGR signaling did not have any effect on mRNA stability, with both NT-3-treated and -untreated samples degrading to ~35% of their initial levels by 4 h (Fig. 2C). Thus it appears likely that TIGR signaling stimulates transcription of the HB-EGF gene.

Finally, we determined whether the up-regulation of HB-EGF was a primary transcriptional response or a secondary effect. 3T3-L1 TIGR cells were stimulated with NT-3 for 4 h in the presence or absence of cycloheximide. Cycloheximide did not inhibit HB-EGF mRNA induction, and in fact caused a slight superinduction relative to NT-3 alone (Fig. 2D). Therefore, TIGR-mediated up-regulation of HB-EGF is a primary transcriptional response that does not require de novo protein synthesis.

The Activation of the IGF-1R but Not the IR Also Increases the Release of Bioactive EGF Receptor Ligands from 3T3-L1 Preadipocytes—To determine whether the selective increase in HB-EGF mRNA was accompanied by a similarly selective release of HB-EGF bioactivity, we examined conditioned medium from cells expressing activated TIGR or TIR. Briefly, 3T3-L1 TIR or TIGR cells were stimulated with NT-3 for 24 h. Conditioned medium from these cells was used to treat HaCat cells (12), a human keratinocyte cell line that expresses abundant EGF receptors (EGFR). TIGR-conditioned medium caused a significant increase in MAPK phosphorylation in HaCat cells, whereas TIR-conditioned medium had no significant effect (Fig. 3A and B). This pattern was mirrored almost exactly at the level of proliferation in HaCat cells, with TIGR-conditioned medium stimulating thymidine uptake to the same extent as seen for MAPK phosphorylation, whereas TIR-conditioned medium had no effect (Fig. 3C). In the case of both MAPK phosphorylation and thymidine incorporation, this stimulatory ef-
fect was completely abrogated by concomitant treatment with the EGFR kinase inhibitor PD153035, indicating that the mitogenic activity in the medium is due to an EGFR ligand. It is, of course, possible that other EGFR ligands are contributing to this effect. Of the other members of the EGF family, only epiregulin was detectable in the microarray experiments, with

### TABLE I

**Genes regulated in a similar fashion by TIR and TIGR after 4 h stimulation**

From a list of genes with similar base line values, genes that changed from base line in both TIR and TIGR cells were selected. Genes that were expressed at similar levels in a comparison of the two stimulated cell lines were selected from this second list as genes regulated in a similar fashion by TIR and TIGR signaling. See “Experimental Procedures” for details of selection criteria. This analysis was carried out on data from two independent experiments, and only genes that fulfilled the criteria in both experiments were included in the final lists. “BLAST” indicates ESTs with homology to a known gene. Representative fold changes from a single experiment are shown, with stimulated genes and suppressed genes listed.

| ID       | Description                                                                 | TIR4/TIR0 | Function     |
|----------|-----------------------------------------------------------------------------|-----------|--------------|
| U88064   | Basonuclin                                                                  | 3.2       | Proliferation |
| Z31432   | Tmx1 mRNA, neoplastic progression 3                                         | 2.7       | Proliferation |
| M35970   | Tumor metastasis process-associated protein (NM23)                          | 3.1       | Proliferation |
| A1836093 | BLAST, NM_019553 DEAD/H box polypeptide 21                                  | 3.1       | RNA processing|
| X98511   | PR264 gene                                                                  | 2.5       | RNA processing|
| A1838915 | BLAST, AF188591 cyclin ania-6b; AB049645 mitochondrial ribosomal protein L20 | 2.1       | RNA processing/translation |
| AW123694 | BLAST, BC06974 glycoprotein 110                                             | 2.2       | Adhesion     |
| D28841   | α-2,3-Sialyltransferase                                                     | 3.1       | Protein/lipid modification |
| D84096   | Ubiquitin carboxyl-terminal hydrolase (UBP)                                 | 2.4       | Protein degradation |
| AB011030 | Protein related to DAN and cerberus, PRDC                                  | 3.1       | Secreted     |
| M19681   | Small inducible cytokine A2                                                 | 2.1       | Secreted     |
| AW120925 | Unknown                                                                     | 7.9       | Miscellaneous |
| A1846023 | Unknown                                                                     | 3.6       | Miscellaneous |
| AW121447 | Unknown                                                                     | 3.6       | Miscellaneous |
| A1117157 | Unknown                                                                     | 3.4       | Miscellaneous |
| A1852608 | Unknown                                                                     | 3.2       | Miscellaneous |
| A1852985 | Unknown                                                                     | 2.9       | Miscellaneous |
| A1785289 | BLAST, BC018560 similar to putative nucleotide-binding protein, estradiol-induced | 2.9 | Miscellaneous |
| AW123801 | BLAST, BC019444 coronin, actin-binding protein 1C                           | 2.6       | Miscellaneous |
| A1853476 | Unknown                                                                     | 2.1       | Miscellaneous |
| A1843466 | Unknown                                                                     | 2.1       | Miscellaneous |
| A1845934 | Unknown                                                                     | 2.1       | Miscellaneous |
| AA615831 | Unknown                                                                     | 2.1       | Miscellaneous |
| A1848841 | Pyruvate dehydrogenase kinase 4                                            | −4.3      | Metabolism   |
| A1224738 | Death adaptor molecule Rald/Cradd                                           | −3.3      | Apoptosis    |
| AB016835 | SH3 domain-binding protein                                                  | −2.4      | Signaling cascade |
| AB027290 | SID 99 mRNA for small GTP-binding protein, Rab9                           | −2.2      | Vesicular transport |
| A1848841 | Unknown                                                                     | −3.4      | Miscellaneous |
| AA260005 | Unknown                                                                     | −2.7      | Miscellaneous |
| A1851172 | Unknown                                                                     | −2.6      | Miscellaneous |
| A1844151 | Unknown                                                                     | −2.6      | Miscellaneous |
| AW047728 | Unknown                                                                     | −2.5      | Miscellaneous |
| U13371   | Kidney cell line derived transcript 1                                       | −2.4      | Miscellaneous |
| AA688938 | Unknown                                                                     | −2.4      | Miscellaneous |
| M29280   | Histone 1–0 gene, 5' end                                                   | −2.4      | Miscellaneous |
| AA532174 | Unknown                                                                     | −2.1      | Miscellaneous |

### TABLE II

**Genes differentially regulated by TIR/TIGR signaling after 4 h stimulation**

Genes that were different at base line were first eliminated, and from the resulting list, genes were selected that were differentially regulated by TIR/TIGR signaling (see "Experimental Procedures" for details of selection criteria). Representative data are shown for genes that fulfilled these criteria in two independent experiments.

| ID       | Description                                                                 | TIGR4/TIR4 | TIGR4/TIGR0 | TIR4/TIR0 | Function     |
|----------|-----------------------------------------------------------------------------|------------|-------------|-----------|--------------|
| L07264   | Heparin-binding EGF-like growth factor                                       | 4.4        | 6.4         | 1.6       | Proliferation |
| X69902   | Integrin α2                                                                  | 3.4        | 3.9         | 2.3       | Adhesion     |
| M63801   | Connexin 43                                                                  | 2.4        | 1.7         | 1.0       | Adhesion     |
| U72680   | Ion channel homologue RIC                                                    | 4.8        | 4.0         | 2.5       | Transport    |
| AF087434 | NF-Atc isoform a                                                             | 3.5        | 2.3         | 1.4       | Transcription|
| AW121247 | Unknown                                                                      | 2.8        | 12.7        | 2.9       | Miscellaneous |
| M88242   | gprPGHS                                                                      | 2.4        | 5.6         | 1.7       | Miscellaneous |
| AI594427 | Unknown                                                                      | 2.7        | 4.7         | 1.8       | Miscellaneous |
| AF002719 | Secretory leukoprotease inhibitor                                            | 4.0        | 2.4         | 1.3       | Miscellaneous |
| AF087687 | Calcium-binding protein S100A1                                              | 3.4        | 2.3         | 1.6       | Miscellaneous |
| AI644158 | Unknown                                                                      | 4.1        | −1.7        | −5.9      | Miscellaneous |
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EGF, transforming growth factor-α, amphiregulin, neuregulin, and betacellulin all being classed as absent by the Affymetrix software. Interestingly, the mRNA levels of epiregulin itself were also induced somewhat more by activated TIGR than TIR (3.8-fold), and epiregulin was only excluded from our list of differentially regulated genes by a narrow margin. However, TIR stimulation also induced expression of epiregulin mRNA, in contrast to the all-or-nothing response seen in the case of HB-EGF. Because the EGFR ligand detected correlates more with the expression of HB-EGF mRNA than epiregulin, it is highly likely to be HB-EGF, although a minor contributory role of epiregulin cannot be entirely ruled out.

TIGR signaling to HB-EGF transcription requires activation of MAPK—Selective inhibitors were used to elucidate the signaling pathways leading from the TIGR to HB-EGF gene transcription. Serum-starved 3T3-L1 TIR/TIGR cells were treated with NT-3 for 4 h, alone or in combination with wortmannin (an inhibitor of phosphatidylinositol 3-kinase (PI3K)) or PD98059 (an inhibitor of MAPK pathway signaling). Northern blots from three independent experiments demonstrated that wortmannin had no effect on the TIGR-mediated stimulation of HB-EGF transcription, whereas the MAPK pathway inhibitor PD98059 completely abrogated the response (Fig. 4A). Therefore, TIGR signaling to HB-EGF gene transcription appears to depend on MAPK but does not require PI3K activity.

Differences in HB-EGF mRNA regulation are not explained by differences in MAPK phosphorylation—The chimeric receptors have been shown to signal differentially to MAPK in 3T3-L1 adipocytes (11), so we studied MAPK phosphorylation in 3T3-L1 TIR/TIGR preadipocytes to test the hypothesis that differential MAPK phosphorylation could be responsible for the differential regulation of HB-EGF. We initially verified that there were no significant differences in chimeric receptor phosphorylation between the two cell lines (Fig. 4B). Chimeric receptor tyrosine phosphorylation was sustained over 24 h,
whereas MAPK phosphorylation was transient, decreasing almost back to basal levels by 15 min (Fig. 4C). Activated TIR and TIGR were both capable of causing robust stimulation of MAPK phosphorylation. Thus, although the activation of MAPK is essential for the up-regulation of HB-EGF expression, differences in the extent or time course of MAPK activation cannot explain the selective effect of the TIGR versus the TIR on HB-EGF gene transcription.

**TIGR-mediated Up-regulation of HB-EGF Gene Transcription Does Not Involve the EGFR**—It has been suggested previously (13) that the activation of MAPK by the IGF-1R is completely dependent on the cleavage and release of membrane-bound, cell surface HB-EGF with subsequent activation of EGFRs. Additionally, HB-EGF has been shown to up-regulate the transcription of its own gene (14). Thus it was possible that the IGF-1R-mediated increase in HB-EGF mRNA levels was in fact occurring via cleavage of HB-EGF already present at the cell surface and subsequent activation of the EGFR. To examine whether the EGFR itself was critically involved in the induction of HB-EGF expression by the TIGR, we used the specific EGFR kinase inhibitor PD153035. 3T3-L1 TIR/TIGR cells were incubated for 4 h with or without NT-3, in conjunction with PD153035 or in conjunction with a combination of PD153035 and the MAPK inhibitor PD98059. Northern blots from three independent experiments showed that the EGFR kinase inhibitor PD153035 had no effect on HB-EGF gene transcription, whereas the MAPK pathway inhibitor PD98059 suppressed the response of HB-EGF to TIGR signaling as expected (Fig. 5). This indicates that TIGR-mediated regulation of HB-EGF gene transcription, although requiring MAPK activation, does not require a functional EGFR.

**DISCUSSION**

We chose to compare the effects of signaling from IR and IGF-1R intracellular domains to gene transcription using a chimeric receptor system previously developed in our group (9). The use of TIR/TIGR chimeras avoids many of the problems associated with the comparative study of holoreceptors, including background expression of endogenous receptors, cross-reactivity of ligand with non-cognate receptors, and the formation of hybrid receptors. As both chimeras are identical in their extracellular regions and bind the same ligand, any differences observed between the two will be entirely due to differences in the sequence and function of their intracellular domains. If, as has been suggested, some of the differential signaling properties of the IR and IGF-1R relate to the residence time of the ligand on the receptor, with IGF-1R having more sustained activation (15, 16), this real biological difference will not be observed in our system. However, we feel that our findings of consistent and repeatable differential biological responses using these chimeras, both herein and in previous reports (9–11), adequately justify their continued use for this purpose.

We observed a generally greater stimulatory effect of TIGR than TIR on transcription using our selection criteria, and almost all of the differentially regulated genes were up-regulated to a greater extent in TIGR cells than in TIR cells. This is in agreement with the general patterns observed in previous microarray work (17). Dupont et al. (17) analyzed gene expression in NIH-3T3 cells overexpressing IR or IGF-1R using spotted cDNA arrays, and they found 30 genes were up-regulated...
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more in IGF-1R cells, whereas 9 genes were up-regulated more in IR cells. The greater response to IGF-1R kinase signaling could be because both 3T3-L1 preadipocytes and NIH-3T3 fibroblasts are more IGF-1-responsive cell lines, in which case IR kinase signaling would be expected to cause a greater response in a more classically insulin-responsive system, such as adipocytes. Alternatively, the IGF-1R kinase may intrinsically couple more to transcriptional responses regardless of the cell system.

The set of genes revealed to be differentially regulated in our system did not overlap at all with the results of Dupont et al. (17). This may be due, in part, to differences in our selection criteria. We eliminated transcripts that were differentially expressed in unstimulated cells, whereas this was not the case in the study of Dupont et al. (17). Certain genes in our experiments followed a similar pattern to that of Dupont et al. (17) but were just outside the borders of our selection criteria (e.g. Glvr-1, Jun). In addition, there were differences in the cell system and receptor constructs employed, microarrays used, and time points studied (4 h in our study compared with 90 min chosen by Dupont et al. (17)).

Our microarray analysis revealed more similarly regulated transcripts than differentially regulated ones. This is in contrast to the work by Dupont et al. (17), who found more differentially regulated genes. However, insulin and IGF-1 do have broadly overlapping effects in cell culture, with no all-or-nothing differences being described thus far, so one might expect more similarities than differences between the two in terms of transcriptional regulation. Three genes with roles in proliferation were found to be up-regulated in a similar manner by the TIR and TIGR. Similar induction of these genes by TIR and TIGR is consistent with both receptors being capable of signaling to cellular proliferation. Several factors involved in various stages of gene expression from transcription through RNA processing and translation to protein modification and finally protein degradation were up-regulated, and these processes would also be important for the correct regulation of cell growth.

Fewer genes were down-regulated equally by TIR and TIGR signaling. Pyruvate dehydrogenase kinase (PDK4) was down-regulated by more than 4-fold. Decreasing PDK4 levels would be expected to relieve the inhibition of pyruvate dehydrogenase, thus encouraging glucose rather than fatty acid oxidation (18). It therefore seems logical that insulin, which would normally indicate abundant availability of carbohydrate in vivo, would down-regulate this gene. The purpose of IGF-1-mediated PDK4 down-regulation, however, is unclear.

Turning now to genes preferentially stimulated by activation of the TIGR, our data revealed two up-regulated transcripts involved in cell adhesion. A selective regulation of the α6 integrin subunit by IGF-1 rather than insulin has been previously demonstrated, and differentiation of neuroepithelial cells into retinal neurons was found to be dependent on expression of α6 integrin (19). Interaction of α6 integrin with laminin also promotes cell survival and proliferation (20). The selective regulation of α6 integrin is therefore consistent with the more growth- and differentiation-regulatory functions of IGF-1.

The microarray data revealed one growth factor, HB-EGF, which was up-regulated by TIGR much more than TIR. This gene was of particular interest to us, because selective regulation of a soluble growth factor by the IGF-1R but not the IR could contribute toward the differential effects the two receptors have on cell proliferation. HB-EGF was first discovered as a 22-kDa heparin-binding growth factor secreted by macrophage-like U-937 cells. It was identified as a new member of the HB-EGF family and is shown to be mitogenic for fibroblasts and smooth muscle cells. HB-EGF binding to EGFR receptors was demonstrated, and in smooth muscle cells it appeared to be a more potent mitogen than EGF (21).

Since its discovery, the mitogenic properties of HB-EGF have been implicated in several processes, including wound healing, liver regeneration, and cancer (14, 22–24). It is synthesized as a transmembrane precursor (also known as the diphtheria toxin receptor (25)) which is cleaved to release mature soluble HB-EGF. However, both the transmembrane and soluble forms of HB-EGF are thought to be active signaling molecules. Soluble HB-EGF has been proposed to act in a paracrine fashion, whereas the transmembrane form acts in a juxtacrine manner on neighboring cells (26).

We verified the differential regulation of HB-EGF by Northern blot in both the original 3T3-L1 TIR/TIGR system and in NIH-3T3 cells overexpressing whole IR/IGF-1Rs. Replication of the data in NIH-3T3 cells demonstrates that the specific regulation of HB-EGF in 3T3-L1 TIR/TIGR cells is not simply due to chimera-specific or clone-specific effects. Some increase in HB-EGF transcription was observed in NIH-3T3 IR cells, in contrast with 3T3-L1 TIR cells which showed no significant change. This could be due in part to insulin acting through endogenous IGF-1Rs in the NIH-3T3 IR cells, thus demonstrating the advantages of using the chimeraic system for detection of IR/IGF-1R specificity.

We found TIGR regulation of HB-EGF to be a primary transcriptional response. This agrees with previous work showing that HB-EGF is an immediate early gene up-regulated following EGFR stimulation (27). We further demonstrated that MAPK is needed for TIGR signaling to HB-EGF, a requirement also observed in the IGF-1R-selective stimulation of Twist and VEGF transcription (28, 29). In our study, MAPK was capable of being activated by both chimeric receptors, whereas other studies (11)2 showed greater phosphorylation of MAPK in response to TIGR signaling. However, the relative differences in MAPK phosphorylation seen in those studies are unlikely to be sufficient to account for the all-or-nothing response we have observed.

2 A. Parmar and K. Siddle, unpublished data.
observed in terms of HB-EGF transcription. In our system, MAPK phosphorylation was transient as has been shown previously (30) in other cell types, despite the long term chimeric receptor phosphorylation. Thus, although MAPK activation is essential for the effects of the IGF-1R on HB-EGF expression, the extent and duration of MAPK activation are unlikely to underlie the major differences between the IR and IGF-1R in their respective abilities to increase HB-EGF mRNA levels. We hypothesize that specificity is achieved by activation of a pathway in addition to MAPK phosphorylation, for instance a stimulatory signal involving the IGF-1R-specific residues Tyr-1250 to Tyr-1251 and/or Ser-1280 to Ser-1283 already implicated in mitogenesis and transformation (31, 32).

The secretion of active HB-EGF into culture medium by NT-3-stimulated 3T3-L1 TIGRs, but not TIRs, was detected by HaCat cells (chosen for their abundance of EGFRs). We observed mitogenic activity in the culture medium in terms of both MAPK phosphorylation and thymidine incorporation in HaCat cells, with almost exact correlation between the two. Inhibition of this activity by the EGFR kinase inhibitor PD153035 demonstrated that the mitogenic activity in the medium was an EGFR ligand. Our microarray data indicate that only one other EGF family member, namely epiregulin, was detectable in 3T3-L1 TIR/TIGR cells. However, HB-EGF expression correlates far better with both MAPK activation and the mitogenic activity in the culture medium in terms of HB-EGF mRNA. We observed mitogenic activity in the culture medium in terms of both MAPK phosphorylation and thymidine incorporation in HaCat cells, with almost exact correlation between the two. Inhibition of this activity by the EGFR kinase inhibitor PD153035 demonstrated that the mitogenic activity in the medium was an EGFR ligand. Our microarray data indicate that only one other EGF family member, namely epiregulin, was detectable in 3T3-L1 TIR/TIGR cells. However, HB-EGF expression correlates far better with both MAPK activation and proliferation selectively induced by conditioned medium taken from NT-3-stimulated TIGR versus TIR cells. Therefore, the mitogenic effects observed are likely to result mainly from the specific production of HB-EGF in response to TIGR stimulation, although at this stage we cannot rule out a minor contribution from epiregulin.

If selective induction of HB-EGF mRNA is indeed able to cause differential cell growth, why did previous work in the same cell system show no specificity in terms of proliferative signaling from the two chimeric receptors (9)? Western blotting indicates that these cells have few EGFRs, with MAPK being poorly phosphorylated in response to HB-EGF, whereas NIH-3T3 cells showed much stronger HB-EGF-stimulated MAPK phosphorylation (data not shown), and do show a differential effect of insulin and IGF-1 on proliferation (4). Therefore, HB-EGF may act in an autocrine or juxtacrine/paracrine fashion, depending on its cellular origin. A juxtacrine role for HB-EGF has already been demonstrated in the process of liver regeneration (24).

Roudabush et al. (13) reported that IGF-1 receptor signaling to Shc, but not IRS-1, depends on IGF-1-induced cleavage of pro-HB-EGF and subsequent phosphorylation of EGFRs. Rather than considering HB-EGF as an end point of the IGF-1 signaling pathway, as suggested by our work, Roudabush et al. (13) assigned a role to HB-EGF as an integral part of the IGF-1 signaling cascade, is also selectively induced by IGF-1 at the level of transcription. Selective regulation of this gene could be responsible, at least in part, for the greater effects of IGF-1 on cell proliferation.

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