Activation of the insulin receptor (IR) by insulin and a synthetic peptide has different effects on gene expression in IR-transfected L6 myoblasts

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Title: Activation of the insulin receptor (IR) by insulin and a synthetic peptide has different effects on gene expression in IR-transfected L6 myoblasts

Short title: Insulin versus an Insulin Mimetic Peptide

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Abbreviations used: IR, insulin receptor; qRT-PCR, quantitative real-time PCR

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SYNOPSIS

Single chain peptides have been recently produced that display either mimetic or antagonistic properties against the insulin and IGF-1 receptors. We have earlier shown that the insulin mimetic peptide S597 leads to significant differences in receptor activation and initiation of downstream signaling cascades despite similar binding affinity and in vivo hypoglycemic potency. It is still unclear how two ligands can initiate different signaling responses through the insulin receptor (IR). To further investigate how the activation of the IR by insulin and S597 differentially activates post-receptor signaling, we here studied the gene expression profile in response to IR activation by either insulin or S597 using microarray technology. We found striking differences between the patterns induced by these two ligands. Most remarkable was that almost half of the genes differentially regulated by insulin and S597 were involved in cell proliferation and growth. Insulin either selectively regulated the expression of these genes or was a more potent regulator. Furthermore we found that half of the differentially regulated genes interact with the genes involved with the MAPK pathway. These findings support our signaling results obtained earlier and confirm that the main difference between S597 and insulin stimulation resides in the activation of the MAPK pathway. In conclusion we show that insulin and S597 acting via the same receptor differentially affect gene expression in cells resulting in a different mitogenicity of the two ligands, a finding which has critical therapeutic implications.

INTRODUCTION

The insulin receptor (IR) is a member of the large family of receptor tyrosine kinases and has a covalent dimeric $\alpha_2\beta_2$ – organization [1]. Activation of the IR initiates a cascade of intracellular signaling events, which regulate multiple biological processes such as glucose and lipid uptake/metabolism, gene expression/protein synthesis and cell growth, division and survival [2].
The binding of insulin to its receptor is complex and binding models for insulin integrating the available data from structural and kinetic evidence have been proposed [3;4]. The complexity of the insulin binding mechanism makes the design of IR agonists especially difficult. Investigation of phage display libraries (ranging in size from 20 to 40 amino acids) have identified several peptides that bind to the IR [5]. These peptides bound three non-overlapping hot spots on the IR (Sites 1, 2 and 3). Site 1 and 2 seem to correlate with the two insulin binding sites on the IR, whereas the site 3 IR binding peptides seem to bind somewhere near the site 2 peptides possibly influencing the binding of these peptides. Whether or not these insulin mimetic peptides actually bind to the same sites as insulin on the IR is not known, but they are located close enough to be able to compete with insulin for binding [5].
Recently homodimers and heterodimers of the site 1 and 2 mimetics have been generated to create molecules that activate the insulin receptor with high potency and specificity - possibly through a mechanism similar to the binding of insulin [6]. From this selection a heterodimer (site 2-site1 mimetic) was found to lower the blood glucose level in rats with a potency similar to insulin's. Due to the smaller size and simpler structure of these insulin mimetic peptides, they are good candidates for drug development with potential use in the treatment of diabetes as a replacement for,- or in combination with, insulin, and as templates for the design of peptidomimetics.
We have now studied such an optimized peptide (S597) in detail with regard to binding to the IR (isoform A) (manuscript in preparation) as well as the effect on IR phosphorylation and downstream signaling molecules [7]. S597 was found to bind selectively to the IR and not the closely related IGF-1 receptor. The binding of S597 to the IR is similar to insulin with respect to association rates, affinity and pH dependence of binding. However S597 dissociates very slowly from the IR, suggesting that it interacts with the receptor in a different way than insulin ([7] and manuscript in preparation). Moreover we found that binding of S597 and insulin leads to significant differences in receptor activation and initiation of downstream signaling pathways [7]. S597 phosphorylates the same number of insulin receptors as insulin but to a lower degree. S597 can fully activate the PI3K/Akt pathway compared to insulin but only weakly activates the Shc/ERK pathway [7]. Furthermore S597 is able to stimulate glycogen synthesis to almost the same extent as insulin but has a much lesser effect on cell proliferation.
It is still unclear how two ligands can initiate different signaling responses through the IR. To further investigate how the activation of the IR by insulin and S597 differentially activates post-receptor signaling, we here studied the gene expression profile in response to IR activation by either insulin or S597 using microarray technology.

EXPERIMENTAL

The peptide, S597

The insulin mimetic peptide, S597, with the sequence Ac-SLEEEWAQIECEVYGRGCPSESFYDWFERQL-amide, was synthesized by standard solid phase peptide synthesis using Fmoc chemistry and purified by RP-HPLC after formation of the disulphide bridge. Insulin was from Novo Nordisk, Denmark

Cell culture

The rat L6 myoblast cell line stably transfected with the human insulin receptor (hIR) isoform A (L6-hIR) used in this study was kindly provided by Bo Falck Hansen, Novo Nordisk, Denmark. The cells were cultured in DMEM 21885-025 supplemented with 10% Fetal Bovine Serum, 100 U/ml Penicillin, 100 ug/ml Streptomycin and 0.5 mg/ml Geneticin. The cells were grown at 37 ^{o}C in 5 % CO_2 humidified atmosphere. Cells were passaged 2-3 times a week by trypsination and resuspended in fresh media at an appropriate dilution – usually 1:6 (all from Gibco, USA).

Preparation of cells, RNA and microarrays

L6-hIR cells were harvested, seeded out in 12 large Petri dishes and allowed to recover for 3 days. The cells were then starved for 24 hours and stimulated for 3 hours with 100 nM insulin, S597 or left unstimulated. RNA isolation, reverse transcription and cRNA synthesis, labeling and hybridization with Affymetrix Rat genome 230 2.0 GeneChip® array (Affymetrix Microarray Suite; Affymetirx, Santa Clara, CA) were conducted following the standard affymetrix protocol (available at http://www.affymetrix.com/support/technical/manual/expression_manual.affx). In summary four GeneChip microarrays were hybridized with cells from the same passage for each experimental condition (insulin-, S597- or non-stimulated cells).

Microarray analysis

Quality control of the arrays was performed using the R package from Bioconductor and confirmed that the arrays were of good quality. The probe level data (CEL files) were transformed into expression measures using R (http://cran.at.r-project.org) and the gcrma package from BioConductor (http://www.bioconductor.org). Gene list comparisons were made in DChip [8] (available at http://www.dchip.org) using a P value < 0.005, a fold change > 1.5 fold or 2 fold, and a lower confidence bound of fold changes for filtering. Furthermore the threshold for absolute difference between the two group means was set to 35. The P value was chosen as it gave a false discovery rate - of max. 3.3% calculated by running 100 permutations in dChip. Transcripts preferentially regulated by insulin (i.e. the genes that met the above mentioned selection criteria for insulin treated samples compared to untreated samples but not for S597 treated samples compared to untreated) (these are the results shown in figure 2) were determined using dChip by comparing the gene expression affected more than 1.5 and 2 fold by insulin and excluding transcripts that also were affected more than 1.5 and 2 fold by S597 (compared to untreated). Transcripts preferentially regulated by S597 were determined in the same way. Transcripts responsive to both ligands were identified using dChip by only including transcripts that were affected more than 1.5 fold by both ligands compared to untreated samples. As many genes are represented on arrays by multiple probesets the number of transcripts regulated by insulin and S597 given in figure 1 and 2 and, table 1 and 2 refer to the number of probesets found on the microarray and not number of unique genes. The transcripts responsive to insulin, S597 or both ligands are listed in supplemental table 2. Functional analyses of these transcripts were subsequently generated using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com). Transcripts that met the fold change cut off of 1.5 and were associated with biological functions in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that data set is due to chance alone. The differentially regulated transcripts were identified in a direct comparison of the insulin- and S597-treated samples using dChip (listed in supplemental table 3). The transcripts regulated similarly by insulin and S597 were determined by comparing the gene expression affected by insulin and S597 compared to untreated, excluding transcripts already categorized as differentially regulated (listed in supplemental table 4).

Validation of genes using qRTPCR on biological replicates

Two-step qRT-PCR was performed on 13 selected genes to validate the microarray data. Cells from four different passages were stimulated at different days with 100 nM insulin, S597 or nothing and the RNA extracted as described above. The RNA was reverse transcribed into single-stranded cDNA according to the manufacturer's protocol using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). The real-time RT-PCR assay was performed using FastStart TaqMan Probe Master (Rox) (Roche Applied Science). Probes were purchased from Universal ProbeLibrary and primer sequences designed using the ProbeFinder software (Universal ProbeLibrary, Roche). The primers used are listed in supplementary table 1 and were purchased from DNA-technologies, DK. Standard curves were generated using serial dilutions of cDNA. TaqMan PCR assays for each gene target were performed in triplicate on cDNA samples on an ABI 7900HT Prism sequence detection system (Applied Biosystems) according to manufacturers protocol (Roche Applied Science). 18S was used as an internal control and included on all plates. The data were analyzed using Sequence Detector Software (Applied Biostystems), the C_T values normalized to S18 and fold changes calculated.

RESULTS

To analyze gene expression profiles following IR activation by either insulin or S597, L6 rat myoblasts stably transfected with the human IR were stimulated with either ligand (100 nM) for 3 hours. This cell line was chosen as skeletal muscle is one of the major targets for insulin action. The same concentration was used as S597 binds specifically and with a similar affinity as insulin to the IR isoform A [7]. Cells from the same passage were stimulated and harvested in quadruples. RNA was isolated and hybrized to Affymetrix GeneChip Rat Genome 230 2.0 Arrays according to Affymetrix procedure – four arrays per condition.

Cellular and molecular functions of transcripts affected by insulin and S597

We identified transcripts (i.e. probe sets) that were regulated between the unstimulated, insulin- or S597-stimulated samples, using a P value < 0.005 and fold changes > 1.5 fold or 2 fold as selection criteria (see *Experimental* for more detail). Using these selection criteria we found that insulin affected two (> 1.5 fold change) to four (> 2 fold change) times as many transcripts as S597 (figure 1). As many genes are represented on arrays by multiple probesets, the number of transcripts regulated by insulin refer to the number of probesets found on the microarray and not to the number of unique genes. Subsequently we focused on the transcripts that were regulated by insulin and S597 compared to untreated. These transcripts were divided into three groups: transcripts preferentially regulated by insulin, by S597 or by both insulin and S597 (figure 2, all transcripts are listed in supplemental table 2). The transcripts regulated by both ligands were defined as transcripts that met the above mentioned selection criteria for both insulin and S597 treated samples when comparing with untreated. In contrast transcripts preferentially regulated by insulin were defined as transcripts that only met the selection criteria when comparing insulin treated with untreated samples and not S597 treated samples. In this way transcripts preferentially regulated by insulin are transcripts that are significant regulated by insulin compared to untreated However this does not mean that there exists a significant difference in expression between insulin and S597 samples, only that S597 is not able to induce a fold change that is significant compared to untreated. Transcripts preferentially regulated by S597 were defined in the same way. An overview of the gene lists used in this study is shown in table 1. Subsequently the five most significant molecular and cellular functions associated with the three groups were investigated by performing a functional analysis using the Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com) as described in *Experimental*. Not all the transcripts are annotated in Ingenuity and furthermore only a subset of these genes is eligible for functional analysis. The differences in significance as well as the number of unique genes associated with the cellular and molecular functions between the three groups are shown in table 2. The five most significant functions with which the transcripts preferentially regulated by insulin were associated were: Gene expression, cellular development, cellular growth and proliferation, cell cycle and cell death. Transcripts preferentially regulated by S597 were most significantly associated with: molecular transport, gene expression, amino acid metabolism, cell cycle and cell morphology. The most significant functions with which the third group, transcripts responsive to both insulin and S597, was associated were: cellular growth and proliferation, cell cycle, gene expression, cell death and cell morphology. This analysis indicates that the major functional themes associated with insulin and S597 stimulation are related to cellular growth and proliferation, gene expression and cell cycle.

Transcripts differentially regulated by insulin and S597

It is difficult to define if genes are differentially or similarly regulated by two ligands as no real biological selection criteria exist. As the aim of this study was to investigate in more detail how S597 and insulin initiate different responses through the IR we started by defining the selection criteria for the differentially regulated transcripts. We defined differentially regulated transcripts as transcripts where there is a significant (P value < 0.005) difference and a fold change in expression values of more than 1.5 fold between insulin and S597 samples. In this way we are certain that a significant difference exists between the insulin and S597 treated samples but without making assumptions about the effect compared to untreated. 131 transcripts (i.e. probesets) were found to be differentially regulated by insulin and S597. The annotated transcripts are listed in table 3 (the complete list (i.e. inclusive the ESTs) is in supplemental table 3). 77 of the differentially regulated transcripts responded (>1.5 fc) only to insulin. One transcript, an EST (affymetrix probe set 1377092_at) responded (>1.5 fc) only to S597 and was down-regulated. Using Blast (http://www.ncbi.nlm.nih.gov/) we searched for similar sequences. We found that this EST has a high sequence identity (93%) to a part of the mRNA of suppressor of cytokine signaling 3 (SOCS3) in mouse. However we could not confirm the presence of this transcript in the samples using qRT-PCR.

Accordingly 58% of the differentially regulated transcripts are transcripts regulated by insulin > 1.5 fold but not by S597. No transcripts were found to be regulated in opposite directions by insulin and S597 compared to untreated. For the remaining transcripts S597 has a similar effect as insulin compared to untreated but to a lesser extent. The differentially regulated transcripts are involved in many functions but as many as 33 of the annotated (unique) genes are involved in growth and proliferation as found by using the Ingenuity Pathway Analysis software, dChip and PubMed searches (marked in table 3 and supplementary table 3). Twenty-two of these genes are selectively regulated by insulin. The rest of the genes involved in growth and proliferation are up- or down-regulated by insulin and to a lesser extent S597 compared to untreated. Analyzing the differential regulated annotated genes (using the Ingenuity Pathway Analysis software) we see that the changes in gene expression have both positive and negative effects on cell growth and cell proliferation (shown in table 3). The number of genes of which up-or down-regulation has a positive effect on cell growth and cell proliferation is larger than the ones having a negative effect. The analysis does not, however, provide information regarding the importance of individual genes for the resulting effect (positive or negative) on the function.
Using the KEGG pathway database we also identified five genes involved in the MAPK signaling pathway: v-jun sarcoma virus 17 oncogene homolog (JUN), activating transcription factor ATF-4 (ATF4), dual specificity phosphatase 5 (DUSP5), dual specificity phosphatase 6 (DUSP6) and v-myc avian myelocytomatosis viral oncogene homolog (MYC). The proteins encoded by DUSP5 and DUSP6 are phosphatases that negatively regulate members of the MAPK superfamily (MAPK/ERK, SAPK/JNK, p38). The expression of DUSP5 is induced by VEGF and further regulated by KRAS, which both are found to be up-regulated in this study [9]. The expression of DUSP6 has been found to be regulated by MYC and IL-6 [10] which both are up-regulated by insulin and to a lesser extent S597 in this study.
The genes hexokinase 2 (HK2) and MYC are involved in the expression level of glucose-6-phosphatase, a key-enzyme in glycolysis and gluconeogenesis [11;12]. HK2 and heparin-binding EGF-like growth factor (HBEGF) are involved in the metabolism of D-glucose [13]. These genes were all more up-regulated by insulin than by S597.

Transcripts regulated similarly by insulin and S597

We defined transcripts regulated similarly by insulin and S597 as transcripts of which expression was affected in the same direction *and* to a similar extent by both ligands compared to untreated samples (as described in *Experimental*). In this way only 139 transcripts were similarly regulated out of the 176 transcripts found above to be affected by both insulin and S597. The annotated transcripts (44 unique genes) are listed in table 4 (the complete list (i.e. inclusive the ESTs) is in supplemental table 4). The expression of the gene heme oxygenase 1 (HMOX1) was found to be much more up-regulated by insulin (14.84 fold) than by S597 (4.65 fold). Heme oxygenase is a microsomal enzyme which catalyzes the oxidation of heme to biliverdin and carbon monoxide [14]. The reason that it is not excluded from the similarly regulated genes in the microarray analysis is that the P value was not below 0.005. However, the fold changes were validated using qRT-PCR and showed that HMOX1 is indeed differentially regulated, with insulin being a much more potent inducer of HMOX1 expression than S597 (figure 4). The role of heme oxygenase in skeletal muscle is still unclear but it is involved in cellular protection against oxidative stress and regulation of heme turnover [15].
The MAPK signaling pathway is one of the major signaling pathways initiated after activation of the IR and is highly associated with cellular proliferation and differentiation and we have recently shown that S597 is not able to activate the MAPK pathway to the same extent as insulin [7]. Using the KEGG pathway database (http://www.genome.jp/kegg/pathway.html) we found two upregulated genes that are involved in the MAPK signaling pathway: RAS p21 protein activator 1 (RASA1) and Kirsten rat sarcoma viral oncogene homolog (KRAS).
Using dChip, Ingenuity Pathway Analysis and PubMed Central searches we furthermore found that many of the transcripts that were regulated similarly by insulin and S597 are involved in growth and proliferation. Using the Ingenuity software it is possible to distinguish if the changes in expression influence a given function in a positive and negative sense. In many instances however is the effect of a specific gene unclear or unknown. Analyzing the similarly regulated transcripts we saw that the changes in gene expression have both positive and negative effects on cell growth and cell proliferation (shown in table 4). The analysis does not, however, provide information regarding the importance of individual transcripts for the resulting effect on the function.

Interactions between genes involved in the MAPK pathway

To investigate if the lack of activation of the MAPK pathway when stimulating with S597 [7] could explain some of the differences in gene expression induced by insulin and S597 we took a closer look at the similarly and differentially regulated genes that were involved in the MAPK pathway to see how they interact with the rest of the genes. We imported the similarly regulated genes KRAS and RASA1 as well as the differentially regulated genes JUN, ATF4, DUSP5, DUSP6 and MYC into the Ingenuity Pathway Analysis software as they were identified to belong to the MAPK pathway (using the KEGG database). Using the Ingenuity Pathway Analysis software we analyzed for interactions between these genes and the rest of the similarly or differentially regulated genes. The interactions are shown in figure 3. Only two of the similarly regulated genes (KRAS and RASA1) belonged to the MAPK pathway and only KRAS interacts (indirectly) with one similar regulated gene (PLAGL1). In contrast the five differentially regulated MAPK pathway genes (JUN, ATF4, DUSP5, DUSP6 and MYC) interact with 21 of the rest of the differentially regulated genes. These genes were either only regulated by insulin or insulin was a more potent regulator than S597 indicating that insulin is more potent in affecting the MAPK pathway than S597.

Validation of microarray data using quantitative real-time PCR

Quantitative Real-Time PCR (qRT-PCR) was performed on 13 genes using four biological replicates (cells from different passages stimulated and harvested at different days) to confirm the microarray data. The C_T values were normalized to the internal control of 18S and fold changes calculated (figure 5). The direction (up- or down- regulated) of the expression of all 13 genes compared to untreated were the same obtained with qRT-PCR and microarray analysis. However due to variation between the biological replicates it is unclear whether S597 and insulin induces the expression of TRIB3 and MYC to a different or similar degree.

DISCUSSION

The molecular mechanisms responsible for how different ligands activating the same receptor can initiate different biological responses in the same cell are still not completely known. Selectivity in IR signaling has been suggested to be gained by differences in binding properties as well as signal transduction from different cellular compartments [16;17]. This is consistent with our recent findings that activating the IR with the slow-dissociating and non-internalizing synthetic peptide S597 and insulin led to significant differences in receptor activation and initiation of downstream signaling pathways and biological responses [7]. We have in this study further investigated how the activation of the IR by insulin and S597 differentially activates post-receptor signaling by studying the gene expression profile in response to IR activation by either insulin or S597. Using microarray technology we have found striking differences in the effect of these ligands on gene expression.

Out of more than 30,000 transcripts present on the microarrays, a total of 738 transcripts were regulated by one or both ligands (insulin and S597). The majority of these transcripts were regulated in the same direction by insulin and S597. 131 transcripts were differentially expressed in response to either insulin or S597. No genes were found to be regulated in opposite directions by insulin and S597 compared to untreated.

Microarray analysis of insulin-induced gene expression in IR-transfected cells has previously been done, however in these studies different cell models (that were not classical insulin target cells) and different stimulation and analysis modalities were used. Dupont et al. [18;19] used NIH-3T3 cells transfected with IR and stimulated for 90 min. Mulligan et al., 2002 [20] used 3T3 fibroblasts transfected with a TrkC/IR chimeric receptor and stimulated for 4 hours. Pandini et al. [21] used IGF-I receptor-deficient murine fibroblasts transfected with the human IR. In this study they stimulated the cells with 10 nM insulin for 30 min, 3 or 8 hours. All of these studies gave different lists containing genes responsive towards insulin stimulation. The different setups make it difficult to compare the results between these and our studies. However, the insulin responsive genes that are found in both these and this study are regulated in the same direction between studies. These are: early growth response 1 (EGR1), zinc transporter 1 (SLC30A1), HBEGF, cyclin G2 (CCNG2), Kruppel-like factor 9 (KLF9), JUNB and IL-6. Pandini et al. [21], also found many genes involved in proliferation, cell cycle and apoptosis up-regulated by insulin as we do. Other genes found in this study to be insulin-responsive have also previously been shown to respond to insulin using other methodologies; EGR1, Vascular Endothelial Growth Factor (VEGF), GLUT1, Basic Helix-loop-helix domain B (BHLHB2) [22-

25]. This concordance together with the qRT-PCR validation experiments on biological replicates indicates that the microarray data generated here are a valid picture of the gene expression profile.

To investigate if insulin and S597 induced different gene expression profiles we used fold changes and P values as a way to define transcripts that were differentially regulated by the two ligands. In this way the genes lists obtained with similarly and differentially regulated genes are not two discrete biological classes defined by biological criteria but opposite ends of a continuum, in which assignment is very dependent on arbitrary cut-offs set for fold change and statistical significance. Furthermore we chose very stringent selection criteria. It is therefore likely that the number of differentially regulated genes is higher. There is no good way of defining selection criteria for when genes are similarly regulated. We chose to focus on genes that were significantly regulated by both insulin and S597 compared to untreated and then exclude all genes that also fulfilled the selection criteria we made for differentially regulated genes. The transcripts included as similarly regulated are thereby not included based on statistically significant similarity but lack of statistically significant difference. This is why some of the genes included in the similarly regulated genes have very different fold changes (like HMOX1) and in reality are differentially regulated. Which genes and how many genes are included in these lists will thereby change accordingly to different cut offs.

The genes induced by insulin and S597 were involved in many different regulatory functions. As the definition of these classes is somewhat arbitrary it is difficult to make clear conclusions when comparing the cellular and molecular functions of genes in different regulatory classes. It was, however striking that almost half of the genes differentially regulated by insulin and S597 were involved in cell proliferation and growth. Insulin either selectively regulated the gene expression of these genes or was a more potent regulator than S597. Using the Ingenuity software we found that many of the changes in gene expression induced (selectively or more potently) by insulin had a positive effect on cell growth and proliferation. This is consistent which the recent finding that S597 was less potent than insulin in stimulating cell proliferation using a thymidine incorporation assay [7]. Furthermore we find that half of the (annotated) differentially regulated genes interact directly or indirectly with the genes involved with the MAPK pathway (figure 3). This fits with the earlier finding that S597 stimulates activation of Shc and ERK which are members of the MAPK pathway [7] much more weakly than insulin, and supports the idea that the main difference between insulin and S597 stimulation is the lack of activation of the MAPK pathway (14). How much the different signaling pathways contribute to the proliferation effect of insulin is not completely clear, but the MAPK pathway has been shown to play a major role in inducing the mitogenic response of insulin and the lack of activation of this pathway in our study is most likely the main reason why S597 is less potent than insulin in stimulating cell proliferation [7]. This is further supported by studies that show that activation of genes like EGR1 (that is massively up-regulated by insulin compared to S597 and interacts with the MAPK pathway genes) is critical for insulin-inducible DNA synthesis [26].
The difference in mitogenicity of insulin and S597 results most likely from different activation of the IR and not simply from activation of IGF-1 receptors or hybrid receptors by insulin. Insulin has been shown to have a low affinity for the IGF-1 receptor and hybrid receptors [27;28]. Therefore the effect of insulin through the IGF-1 receptor or receptor hybrids should be minimal at the concentration of 100 nM insulin used in the microarray studies. This is further supported by the finding that insulin only had a small effect on phosphorylation of the IGF-1 receptor and proliferation in untransfected L6 cells which express only few IRs, while S597 had no effect [7]. This supports that it is not cross-talk with IGF-1 receptors that results in the higher mitogenicity of insulin.

One of the other genes involved in cell proliferation that was dramatically regulated by insulin (32.73 fold down-regulated) compared to S597 (3.95 fold down-regulated) is cyclin G2 (CCNG2). This massive fold change was also validated using qRT-PCR. This gene has also earlier been found [21], using microarray analysis and qRT-PCR, to be down-regulated by insulin in IGF-I receptor-deficient murine fibroblasts transfected with the human IR. Cyclin G2 is an unconventional cyclin highly expressed in postmitotic cells. Unlike classical cyclins that promote cell cycle progression, cyclin G2 blocks cell cycle entry [29-31]. It has been shown that ectopic expression of cycling G2 inhibits proliferation of several cell types [32-37] and that stable down-regulation of CCGN2 by short interfering RNAs resulted in enhanced cell growth. So the big difference in CCNG2 expression induced by insulin and S597 may play a critical role in the difference in mitogenicity of the two ligands. This is currently being investigated further in our laboratory. However the expression of this gene has been shown not to be affected by inhibition of ERK in human breast cancer cells [37] and this suggests that other signaling pathways that the MAPK pathway may be affected differently by S597

and insulin. Furthermore it has been shown that CCGN2 dysregulation may play an important role in epithelial transformation and the early stages of human oral cancer development [38]. Development of insulin mimetic peptides like S597 that do not lead to dysregulation of such genes could turn out to have profound therapeutic implications as enhanced mitogenicity associated with some insulin analogues designed for optimized therapy of diabetes has been a matter of major concern [39]

The finding that two ligands activating the IR give different mitogenic responses is reminiscent of earlier studies investigating the effect of insulin and IGF-2 on the IR, isoform A [21;40-42]. Like in this study, the two ligands (insulin and IGF-2) bind with a similar affinity to the IR but result in different biological effects. Insulin led primarily to metabolic effects (measured by glucose uptake), whereas IGF-2 led primarily to mitogenic effects (measured by 3H-thymidine incorporation assays). These differences in the biological effects were associated with differential recruitment and activation of intracellular substrates as well as selective changes in gene expression. However, in our present work, it is the noncognate ligand that is much less mitogenic than insulin.

The molecular mechanisms responsible for how different ligands activating the same receptor can initiate different biological responses in the same cell are still not completely known. Selectivity in insulin signaling has been suggested to be gained by differences in binding properties as well as signal transduction from different cellular compartments [16;17].
Previous work showed that insulin analogues dissociating very slowly from the IR have an increased mitogenic activity, indicating that an increased duration of the insulin signal at the receptor level result in a shift towards a more mitogenic insulin response [39;43-45]. This is in contrast to what is found in this study as S597 dissociates slowly from the IR but has a much lower mitogenic potency compared to insulin. However, we have currently shown that S597 phosphorylates the same number of insulin receptors as insulin but to a lower degree. This suggests that even though S597 is dissociating much more slowly than insulin it is not able to activate the insulin receptor to the same degree as insulin. As the duration of the phosphorylation is similar to insulin stimulation, it seems that it is not the duration that is responsible for the differences in biological properties of S597 and insulin. We believe that the reason for the differential signaling is due to this lesser activation of the insulin receptor as well as lack of internalization of the S597/insulin receptor complex seen in the study, which in the end results in full activation of the PI3K/Akt pathway and almost no activation of the Shc/ERK pathway (explaining the lower mitogenicity)[7]. The lesser activation of the MAPK pathway is most likely the main reason for the differences observed in the insulin/S597 stimulated gene expression in this study. The low phosphorylation degree of the IR is most likely part of the reason for the lack of internalization as internalization is dependent on phosphorylation of several of the tyrosines residues on the IR [46]. That selective signaling transduction occurs from different cellular compartments has been suggested by several studies (reviewed by [16;17]). It has been shown that activation of Shc is more dependent on internalization of the IR then IRS1 [47;48]. These studies support the general concept, that insulin/IGF-1 receptors initiate metabolic signaling from the plasma membrane whereas mitogenic signaling originates from inside the cells following internalization of activated receptors. Recently another study was published that investigates how different signaling cascades in beta cells can be activated through the IR [49]. The authors showed that selective activation of the glucokinase and c-fos genes was achieved through the IR-B by signaling from different cellular compartments. This supports our findings in this study that in insulin-target cells i.e. muscle cells and through the IR isoform A we can obtain differential signaling and effects on gene expression through the same receptor. That S597 stays on the cell surface for much longer than insulin is likely an important part of the reason for the differences in signaling, gene expression profiles and mitogenicity.

Activation of the MAPK pathway and Akt signaling pathway seemingly differ in the dependence of the activation degree of IR [7]. It is likely that gene-regulatory events also are differently dependent on the level of activation of signaling pathways. Thus differences in the biological responses (signaling, internalization, gene expression) could be due to different dependences for receptor activation rather than distinct signaling mechanisms. If this is the case weak agonists might be the more selective therapeutic agents, in terms of balancing metabolic and mitogenic effects.
Our work adds to a growing body of evidence suggesting that activation of the IR is not simply a matter of flipping an on/off switch, and that qualitatively as well as quantitatively the biological consequences of receptor activation are dependent on the nature, binding kinetics and spatial localization of the activating ligand.

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REFERENCE LIST

1 De Meyts, P. and Whittaker, J. (2002) Structural biology of insulin and IGF1 receptors: implications for drug design. Nat. Rev. Drug Discov. **1,** 769-783

2 Taniguchi, C. M., Emanuelli, B. and Kahn, C. R. (2006) Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell. Biol. **7,** 85-96

3 Schäffer, L. (1994) A model for insulin binding to the insulin receptor. Eu. J. Biochem. **221,** 1127-1132

4 De Meyts, P. (1994) The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling. Diabetologia **37 Suppl 2,** S135-S148

5 Pillutla, R. C., Hsiao, K. C., Beasley, J. R., Brandt, J., Østergaard, S., Hansen, P. H., Spetzler, J. C., Danielsen, G. M., Andersen, A. S., Brissette, R. E., Lennick, M., Fletcher, P. W., Blume, A. J., Schäffer, L. and Goldstein, N. I. (2002) Peptides identify the critical hotspots involved in the biological activation of the insulin receptor. J. Biol. Chem. **277,** 22590-22594

6 Schäffer, L., Brissette, R. E., Spetzler, J. C., Pillutla, R. C., Østergaard, S., Lennick, M., Brandt, J., Fletcher, P. W., Danielsen, G. M., Hsiao, K. C., Andersen, A. S., Dedova, O., Ribel, U., Hoeg-Jensen, T., Hansen, P. H., Blume, A. J., Markussen, J. and Goldstein, N. I. (2003) Assembly of high-affinity insulin receptor agonists and antagonists from peptide building blocks. Proc. Natl. Acad. Sci. U.S.A.. **100,** 4435-4439

7 Jensen, M., Hansen, B., De Meyts, P., Schäffer, L. and Ursø, B. (2007) Activation of the insulin receptor by insulin and a synthetic peptide leads to divergent metabolic and mitogenic signaling and responses. J. Biol. Chem. **282**, 35179-35186

8 Li, C. and Wong, W. H. (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. U.S.A. **98,** 31-36

9 Fu, Q., McKnight, R. A., Yu, X., Callaway, C. W. and Lane, R. H. (2006) Growth retardation alters the epigenetic characteristics of hepatic dual specificity phosphatase 5. FASEB J. **20,** 2127-2129

10 Zhao, Y. and Zhang, Z. Y. (2001) The mechanism of dephosphorylation of extracellular signal-regulated kinase 2 by mitogen-activated protein kinase phosphatase 3. J. Biol. Chem. **276,** 32382-32391

11 Hansen, P. A., Marshall, B. A., Chen, M., Holloszy, J. O. and Mueckler, M. (2000) Transgenic overexpression of hexokinase II in skeletal muscle does not increase glucose disposal in wild-type or Glut1-overexpressing mice. J. Biol. Chem. **275,** 22381-22386

12 Riu, E., Bosch, F. and Valera, A. (1996) Prevention of diabetic alterations in transgenic mice overexpressing Myc in the liver. Proc. Natl. Acad. Sci. U.S.A. **93,** 2198-2202

13 Robey, R. B., Ma, J., Santos, A. V., Noboa, O. A., Coy, P. E. and Bryson, J. M. (2002) Regulation of mesangial cell hexokinase activity and expression by heparin-binding epidermal growth factor-like growth factor: epidermal growth factors and phorbol esters increase glucose metabolism via a

common mechanism involving classic mitogen-activated protein kinase pathway activation and induction of hexokinase II expression. J. Biol. Chem. **277,** 14370-14378

14 Maines, M. D. (1988) Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. FASEB J. **2,** 2557-2568

15 Vesely, M. J., Exon, D. J., Clark, J. E., Foresti, R., Green, C. J. and Motterlini, R. (1998) Heme oxygenase-1 induction in skeletal muscle cells: hemin and sodium nitroprusside are regulators in vitro. Am. J. Physiol. **275,** C1087-C1094

16 Foti, M., Moukil, M. A., Dudognon, P. and Carpentier, J. L. (2004) Insulin and IGF-1 receptor trafficking and signalling. Novartis Found. Symp. **262,** 125-141

17 Leof, E. B. (2000) Growth factor receptor signalling: location, location, location. Trends Cell. Biol. **10,** 343-348

18 Dupont, J., Khan, J., Qu, B. H., Metzler, P., Helman, L. and LeRoith, D. (2001) Insulin and IGF-1 induce different patterns of gene expression in mouse fibroblast NIH-3T3 cells: identification by cDNA microarray analysis. Endocrinology **142,** 4969-4975

19 Dupont, J., Dunn, S. E., Barrett, J. C. and LeRoith, D. (2003) Microarray analysis and identification of novel molecules involved in insulin-like growth factor-1 receptor signaling and gene expression. Recent Prog. Horm. Res. **58,** 325-342

20 Mulligan, C., Rochford, J., Denyer, G., Stephens, R., Yeo, G., Freeman, T., Siddle, K. and O'Rahilly, S. (2002) 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. J. Biol. Chem. **277,** 42480-42487

21 Pandini, G., Medico, E., Conte, E., Sciacca, L., Vigneri, R. and Belfiore, A. (2003) Differential gene expression induced by insulin and insulin-like growth factor-II through the insulin receptor isoform A. J. Biol. Chem. **278,** 42178-42189

22 Miele, C., Rochford, J. J., Filippa, N., Giorgetti-Peraldi, S. and Van Obberghen, E. (2000) Insulin and insulin-like growth factor-I induce vascular endothelial growth factor mRNA expression via different signaling pathways. J. Biol. Chem. **275,** 21695-21702

23 Zelzer, E., Levy, Y., Kahana, C., Shilo, B. Z., Rubinstein, M. and Cohen, B. (1998) Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. EMBO J. **17,** 5085-5094

24 Keeton, A .B., Bortoff, K. D., Bennett, W. L., Franklin, J. L., Venable, D. Y. and Messina, J. L. (2003) Insulin-regulated expression of Egr-1 and Krox20: dependence on ERK1/2 and interaction with p38 and PI3-kinase pathways. Endocrinology **144,** 5402-5410

25 Shen, M., Yoshida, E., Yan, W., Kawamoto, T., Suardita, K., Koyano, Y., Fujimoto, K., Noshiro, M. and Kato, Y. (2002) Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. J. Biol. Chem. **277,** 50112-50120

26 Gousseva, N., Kugathasan, K., Chesterman, C. N. and Khachigian, L. M. (2001) Early growth response factor-1 mediates insulin-inducible vascular endothelial cell proliferation and regrowth after injury. J. Cell. Biochem. **81,** 523-534

27 Kristensen, C., Andersen, A. S., Hach, M., Wiberg, F. C., Schäffer, L. and Kjeldsen, T. (1995) A single-chain insulin-like growth factor I/insulin hybrid binds with high affinity to the insulin receptor. Biochem. J. **305 (Pt 3),** 981-986

28 Soos, M. A., Field, C. E. and Siddle, K. (1993) Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. Biochem. J. **290 (Pt 2),** 419-426

29 Horne, M. C., Donaldson, K. L., Goolsby, G. L., Tran, D., Mulheisen, M., Hell, J. W. and Wahl, A. F. (1997) Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J. Biol. Chem. **272,** 12650-12661

30 Bennin, D. A., Don, A. S., Brake, T., McKenzie, J. L., Rosenbaum, H., Ortiz, L., DePaoli-Roach, A. A. and Horne, M. C. (2002) Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. J. Biol. Chem. **277,** 27449-27467

31 Martinez-Gac, L., Marqués, M., Garcia, Z., Campanero, M. R. and Carrera, A. C. (2004) Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. Mol. Cell. Biol. **24,** 2181-2189

32 Horne, M. C., Donaldson, K. L., Goolsby, G. L., Tran, D., Mulheisen, M., Hell, J. W. and Wahl, A. F. (1997) Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. J. Biol. Chem. **272,** 12650-12661

33 Yue, L., Daikoku, T., Hou, X., Li, M., Wang, H., Nojima, H., Dey, S. K. and Das, S. K. (2005) Cyclin G1 and cyclin G2 are expressed in the periimplantation mouse uterus in a cell-specific and progesterone-dependent manner: evidence for aberrant regulation with Hoxa-10 deficiency. Endocrinology **146,** 2424-2433

34 Bennin, D. A., Don, A. S., Brake, T., McKenzie, J. L., Rosenbaum, H., Ortiz, L., Paoli-Roach, A. A. and Horne, M. C. (2002) Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. J. Biol. Chem. **277,** 27449-27467

35 rachchige-Don, A. S., Dallapiazza, R. F., Bennin, D. A., Brake, T., Cowan, C. E. and Horne, M. C. (2006) Cyclin G2 is a centrosome-associated nucleocytoplasmic shuttling protein that influences microtubule stability and induces a p53-dependent cell cycle arrest. Exp. Cell. Res. **312,** 4181-4204

36 Liu, J., Cui, Z.S., Luo, Y., Jiang, L., Man, X. H. and Zhang, X. (2004) Effect of cyclin G2 on proliferative ability of SGC-7901 cell. World J. Gastroenterol **10,** 1357-1360

37 Le, X. F., rachchige-Don, A. S., Mao, W. Q., Horne, M. C. and Bast, R. C. (2007) Roles of human epidermal growth factor receptor 2, c-jun NH2- terminal kinase, phosphoinositide 3-kinase, and p70 S6 kinase pathways in regulation of cyclin G2 expression in human breast cancer cells. Mol. Cancer Ther. **6,** 2843-2857

38 Kim, Y., Shintani, S., Kohno, Y., Zhang, R. and Wong, D. T. (2004) Cyclin G2 dysregulation in human oral cancer. Cancer Res. **64,** 8980-8986

39 Kurtzhals, P., Schäffer, L., Sørensen, A., Kristensen, C., Jonassen, I., Schmid, C. and Trub, T. (2000) Correlations of receptor binding and metabolic and mitogenic potencies of insulin analogs designed for clinical use. Diabetes **49,** 999-991005

40 Sciacca, L., Mineo, R., Pandini, G., Murabito, A., Vigneri, R. and Belfiore, A. (2002) In IGF-I receptor-deficient leiomyosarcoma cells autocrine IGF-II induces cell invasion and protection from apoptosis via the insulin receptor isoform A. Oncogene **21,** 8240-8250

41 Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I. D., Belfiore, A. and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol. Cell. Biol. **19,** 3278-3288

42 Morrione, A., Valentinis, B., Xu, S. Q., Yumet, G., Louvi, A., Efstratiadis, A. and Baserga, R. (1997) Insulin-like growth factor II stimulates cell proliferation through the insulin receptor. Proc. Natl. Acad. Sci. U.S.A. **94,** 3777-3782

43 Drejer, K. (1992) The bioactivity of insulin analogues from in vitro receptor binding to in vivo glucose uptake. Diabetes Metab. Rev. **8,** 259-285

44 De Meyts, P. and Shymko, R. M. (2000) Timing-dependent modulation of insulin mitogenic versus metabolic signalling. Novartis Found. Symp. **227,** 46-57

45 Hansen, B. F., Danielsen, G. M., Drejer, K., Sørensen, A. R., Wiberg, F. C., Klein, H. H. and Lundemose, A. G. (1996) Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. Biochem. J. **315 (Pt 1),** 271-279

46 Wilden, P. A., Siddle, K., Haring, E., Backer, J. M., White, M. F. and Kahn, C. R. (1992) The role of insulin receptor kinase domain autophosphorylation in receptor-mediated activities. Analysis with insulin and anti-receptor antibodies. J. Biol. Chem. **267,** 13719-13727

47 Ceresa, B. P., Kao, A. W., Santeler, S. R. and Pessin, J. E. (1998) Inhibition of clathrin-mediated endocytosis selectively attenuates specific insulin receptor signal transduction pathways. Mol. Cell. Biol. **18,** 3862-3870

48 Chow, J. C., Condorelli, G. and Smith, R. J. (1998) Insulin-like growth factor-I receptor internalization regulates signaling via the Shc/mitogen-activated protein kinase pathway, but not the insulin receptor substrate-1 pathway. J. Biol. Chem. **273,** 4672-4680

49 Uhles, S., Moede, T., Leibiger, B., Berggren, P. O. and Leibiger, I. B. (2007) Selective gene activation by spatial segregation of insulin receptor B signaling. FASEB J. **21,** 1609-1621

Gene lists used in this study	
Comparison of insulin- and S597- treated samples to untreated (supplemental table 2):	
Regulated by both ligands (176 transcripts)	Transcripts that are significantly regulated by insulin *and* S597 compared to untreated.
Preferentially regulated by insulin (465 transcripts)	Transcripts that are *only* significantly regulated by insulin compared to untreated. (Most of these transcripts are not (significantly) differently expressed when compared directly to S597 samples)
Preferentially regulated by S597 (97 transcripts)	Transcripts that are *only* significantly regulated by S597 compared to untreated. (None of these transcripts are (significantly) different expressed when compared directly to insulin samples)
Direct comparison of insulin- and S597- untreated samples (supplemental tables 3 and 4):	
Differentially regulated (131 transcripts)	Transcripts where there is a significant difference in expression values between insulin and S597 samples.
Similarly regulated (139 transcripts)	Transcripts that are significantly regulated by insulin *and* S597 compared to untreated (as above) *excluding* transcripts where there is a significant difference between insulin and S597 samples

Table 1. **Definitions of the gene lists used in this study.**
Significantly regulated is defined as: P value < 0.005 and a difference in expression fold change > 1.5

Molecular and cellular function	Significance	Number of genes
Genes preferentially regulated by insulin (465 transcripts in total -189 unique genes eligible for functional analysis)		
Gene expression	1.37E-11– 4.42E-03	64
Cellular development	1.30E-11 – 4.42E-03	56
Cellular growth and proliferation	1.74E-07 – 4.42E-03	73
Cell cycle	5.72E-07 – 4.64E-03	33
Cell death	1.18E-06 – 4.62E-03	60
Genes preferentially regulated by S597 (97 transcripts in total - 42 unique genes eligible for functional analysis)		
Molecular transport	4.53E-04 – 4.75E-02	7
Gene expression	2.79E-03 – 3.51E-02	11
Amino acid metabolism	3.24E-03 – 4.68E-02	5
Cell cycle	3.24E-03 – 4.53E-02	4
Cell morphology	3.24E-03 – 4.92E-02	7
Genes responsive to both insulin and S597 (176 transcripts in total- 66 unique genes eligible for functional analysis)		
Cellular growth and proliferation	1.88E-10 – 6.63E-03	33
Cell cycle	8.61E-10 – 7.18E-03	25
Gene expression	1.72E-08 – 5.12E-03	29
Cell death	5.27E-08 – 7.75E-03	28
Cell morphology	7.25E-08 – 6.10E-03	27

Table 2. **Molecular and cellular functions.** Comparison of the P values for the most significant functions associated with transcripts preferentially regulated by insulin, S597 or by both ligands. The five most significant functions associated with each of the three groups were identified using the Ingenuity Pathway Analysis software. Several of top five functions where the same between the three groups (eight different functions were found in total). The significance (P value) as well as the number of unique genes associated with a given function for each of the three groups is shown. See text for more details.

Accession	Synonym	Gene description	Fold change		Resulting effect of insulin stim. on:	
			UTvsInsulin	UTvsS597	growth	prolife-ration
Genes regulated by both insulin and S597:						
AI408309	*CCNG2*	*cyclin G2*	-32.73	-3.95		+
NM_013221	HBP1	HMG-box containing protein 1	-7.29	-2.34		
BI281702	MAP1B	microtubule-associated protein 1b^	2.91	1.50		
AF313411	*NID67*	putative small membrane protein NID67	4.55	2.00		
AI175732	*VEGF*	*vascular endothelial growth factor*	4.69	2.06	+	+/-
NM_013043	*TSC22D1*	*transforming growth factor beta 1 induced transcript 4*	6.24	3.03	–	
NM_012603	*MYC*	*v-myc avian myelocytomatosis viral oncogene homolog*	6.95	3.69	+/-	+/-
BI303379	*TNFRSF12 A*	*tumor necrosis factor receptor superfamily, member12a*	7.83	3.17	+/-	+
AA901341	*GLUT3*	*solute carrier family 2, member3*	8.00	4.15		
AI548856	*PVR*	*poliovirus receptor*	11.51	4.83		+
NM_031530	*CCL13*	*chemokine (C-C motif) ligand 2*	24.73	4.70		+
BI294137	*HK2*	*hexokinase 2*	30.12	9.32		
NM_012953	*FOSL1*	*fos-like antigen 1*	32.87	7.64	+	+
NM_012551	*EGR1*	*early growth response 1*	37.72	12.92	–	+/-
AI169756	*ERRFI1*	*ERBB receptor feedback inhibitor 1*	37.76	7.71		
Genes regulated only (significantly) by insulin:						
BI275570	*MYCN*	*v-myc myelocytomatosis viral related oncogene*	-3.12	-1.55	+/-	–
NM_053352	*CMKOR1*	*chemokine orphan receptor 1*	-2.91	-1.49		–
NM_019334	*PITX2*	*paired-like homeodomain transcription factor 2*	-2.79	-1.56		
AA859235	*CDCA7*	*cell division cycle associated 7*	-2.33	-1.23		
NM_133317	*TOB1*	*transducer of ERBB2, 1*	-2.28	-1.07	+	+
AW524563	*KLF9*	*kruppel-like factor 9*	-2.24	1.05		–
AI009074	*OGT*	*O-linked N-acetylglucosamine (GlcNAc) transferase*	-1.63	1.01	–	
BG378885	HMGA1	*h igh mobility group AT-hook 1*	1.78	-1.09	+/-	
NM_021835	*JUN*	*v-jun sarcoma virus 17 oncogene homolog (avian)^*	1.79	1.06	+/-	+
NM_021846	MCL1	myeloid cell leukaemia sequence 1	2.05	1.15		
AI071665	*CSF1*	*colony stimulating factor 1(macrophage)*	2.06	1.15	+	+/-
NM_024152	ARF6	ADP-ribosylation factor 6	2.24	1.24		
NM_024403	*ATF4*	*activating transcription factor ATF-4*	2.46	1.48	+	+
NM_023021	*KCNN4*	*potassium intermediate l, subfamily N, member 4*	2.51	1.09		+
BG372334	SLC1A5	sodium-dependent neutral amino acid transporter	2.59	1.19		
AI045767	ANK	progressive ankylosis^	2.59	1.45		
NM_013111	SLC7A1	solute carrier family 7, member 1	2.80	1.62		
BI290791	NOLC1	nucleolar and coiled-body phosphoprotein 1	2.88	1.45		
AF182714	SLC35E4	putative phosphate-phosphoenolpyruvate translocator	3.11	1.63		
NM_012589	*IL6*	*interleukin 6*	3.56	1.58	+/-	+/-
AF007789	*PLAUR*	*plasminogen activator, urokinase receptor*	3.85	1.55	–	+
AI176519	*IER3*	*immediate early response 3*	4.14	1.75	–	
NM_021836	*JUNB*	*Jun-B oncogene*	4.71	1.23	+	–
BI284218	*GLUT1*	*solute carrier family, member 1*	5.07	1.92		
NM_133578	*DUSP5*	*dual specificity phosphatase 5*	6.48	1.62	–	
AB020967	TRIB3	tribbles homolog 3 (Drosophila)^	7.14	2.69		
A1102530	*NAB2*	*EGR1 binding protein 2*	7.94	3.69		+
NM_031135	*KLF10*	*TGFB inducible early growth response*	8.01	3.23		–
NM_012945	*HBEGF*	*heparin-binding EGF-like growth factor*	9.11	3.65	+	+
NM_053883	*DUSP6*	*dual specificity phosphatase 6*	19.40	4.41		
AI169398	CH25H	cholesterol 25-hydroxylase	21.26	1.07		

Table 3. **Genes differentially regulated by insulin and S597.**
The fold changes with respect to the untreated samples are shown. The genes are divided in two groups based on whether or not both insulin or S597 affected the gene expression compared to untreated samples (> 1.5 fold change, P value < 0.005). Genes involved in growth and proliferation were identified using Ingenuity Systems and are written in *italic.* Furthermore the resulting effect (when data was available) of up- or down-regulation of gene expression are indicated as well. Positive effect: "+" and negative effect: "–". The genes marked with ^ were identified more than once on the chip and therefore the average fold change is listed. The differential genes were identified by comparing insulin and S597 treated samples directly. 131 transcripts in total were found when using a cut off of > 1.5 fold change, P value < 0.005. Only the annotated genes are listed here, a complete list of the 131 transcripts is available as supplementary material.

Accession	Synonym	Gene description	Fold change		Resulting effect of insulin stim. on:	
			UT*vs*Insulin	UT*vs*S597	growth	prolife-ration
U61729	PNR1	proline rich 2	-16.58	-5.83		
U30789	*TXNIP*	*upregulated by 1,25-dihydroxyvitamin D-3*	-4.15	-3.51	+	+
AI603439	ULK1	unc-51-like kinase 1 (C. elegans)	-3.71	-2.26		
NM_022958	PIK3C3	phosphoinositide-3-kinase, class 3*	-2.86	-1.86		
NM_022522	*CAS2*	*caspase 2*	-2.56	-2.04		
BF556820	*CBLB*	*Cas-Br-M ectropic retroviral transforming sequence b*	-2.33	-2.19	+	
NM_080478	APBB1	amyloid beta (A4) precursor protein-binding, family B	-2.10	-1.94		
NM_030872	PDK2	pyruvate dehydrogenase kinase, isoenzyme 2^*	-2.07	-2.11		
NM_012760	*PLAGL1*	*pleiomorphic adenoma gene-like 1*	-2.10	-2.85	+	+
BI274467	*KIF2C*	*kinesin-related protein 2*	-2.08	-1.86		
AY029335	RS21C6	RS21-C6 protein	-1.73	-1.94		
U01914	AKAP8	a kinase anchor protein 8	1.59	1.68		
BG668164	RASA1	*RAS p21 protein activator (GTPase activating protein) 1*	1.68	1.87	–	
BF390141	PPP1R12A	protein phosphatase 1, regulatory subunit 12A	1.72	2.07		
BF419336	NARS	similar to Asparaginyl-tRNA synthetase, cytoplasmic	1.71	1.70		
BF282783	*DDX20*	*DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 20*	1.76	1.80		
NM_017268	HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1*	1.82	2.39		
BM387435	ARF6	ADP-ribosylation factor 6	1.89	2.11		
AA851926	DDX5	ddx5 gene	1.92	1.97		
BI289683	TARS	threonyl-tRNA synthetase	1.94	1.81		
NM_133307	*PRKCD*	*protein kinase C, delta*	1.93	1.64	+/–	–
AI060050	GNI2	guanine nucleotide binding protein-like 2	2.02	2.07		
AI176231	*DDX5*	*DEAD (Asp-Glu-Ala-Asp) box polypeptide 5*	2.07	2.16		+
NM_053713	*KLF4*	*kruppel-like factor 4 (gut)*	2.19	2.53		–
BI275908	SRR	serine racemase	2.27	2.10		
NM_031678	PER2	period homolog 2 (Drosophila)	2.32	2.25		
AI178069	*ZFP36L2*	*mRNA for parathyroid hormone regulated sequence*	2.46	1.85		
AF158186	NIP7	Saccharomyces cerevisiae Nip7p homolog	2.48	2.08		
BE100812	*PDGFA*	*platelet-derived growth factor alpha polypeptide*	2.54	1.73		+
NM_017136	SQLE	squalene epoxidase*	2.64	2.17		
AI178019	PPAN	Peter Pan homolog (Drosophila)	2.68	1.82		
BM384448	WDR12	WD repeat domain 12	2.78	2.00		
NM_012715	*ADM*	*adrenomedullin*	2.82	2.03	–	+/–
BE109242	*KRAS*	*v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog*	2.83	3.07	+/–	+
BF416474	RAI14	retinoic acid induced 14	3.02	2.19		
BM390399	*HMGCR*	*3-hydroxy-3-methylglutaryl-Coenzyme A reductase^**	3.09	2.60		
BI274923	ERCC1	excision repair cross-complem. rodent repair deficiency	3.17	2.47		
NM_024137	HIVEP2	HIV type I enhancer binding protein 2	3.34	2.09		
AB000489	SLC20A1	solute carrier family 20, member 1	3.69	2.35		
AI179795	*SLC30A*	*solute carrier family 30 (zinc transporter), member 1*	3.85	3.54	+	
AI411375	*ETS2*	*v-ets erythroblastosis virus E26 oncogene homolog 2*	4.18	2.90	+/–	+
NM_031789	NFE2L2	NF-E2-related factor 2	4.29	3.02		
NM_012912	*ATF3*	*activating transcription factor 3*	7.03	4.32	–	
NM_012580	*HMOX1*	*heme oxygenase (decycling) 1**	14.84	4.65	–	–

Table 4. **Genes similarly regulated by insulin and S597**.
The fold changes with respect to the untreated samples are shown. Genes involved in growth and proliferation were identified using Ingenuity Systems and are written in *italic.* Furthermore the resulting effect (when data was available) of up- or down-regulation of gene expression are indicated as well. Positive effect: "+" and negative effect: "–". The genes marked with * were validated by RT PCR. The genes marked with ^ were identified more than once on the chip due to multiple probe-sets for the same gene and therefore the average is listed. Genes were found by identifying genes that were induced > 1.5 fold and with a P values < 0.005 by both insulin and S597 compared to untreated. Furthermore genes found to have >1.5 fc difference and with a P value of < 0.005 between insulin and S597 treated samples were excluded from the list. 139 transcripts in total were found. Only the annotated genes are listed here, a complete list of the transcripts is available as supplementary material.

FIGURE LEGENDS

Figure 1. **Number of regulated transcripts.** Comparison of the number of regulated transcripts in rat muscle cells in the three conditions: untreated, insulin- or S597-treated. A: The number of transcripts regulated more than 1.5 fold. B: The number of transcripts regulated more than 2 fold. As many genes are represented on arrays by multiple probesets does the number of transcripts regulated refer to the number of probesets found on the microarray and not the number of unique genes.

Figure 2. **Transcripts regulated by both ligands or preferentially by insulin or S597.** The number of up- or down-regulated transcripts after stimulation with insulin or S597 are listed. The overlapping region denotes the number of transcripts that are significantly regulated by *both* insulin *and* S597 compared to untreated. The non-overlapping region denotes the number of transcripts that are significantly regulated by *either* insulin *or* S597 compared to untreated (called preferentially regulated transcripts). A: the number of transcripts regulated more than 1.5 fold; B: the number of transcripts regulated more than 2 fold. As many genes are represented on arrays by multiple probesets does the number of transcripts regulated refer to the number of probesets found on the microarray and not the number of unique genes.

Figure 3. **Genes involved in the MAPK pathway (grey) and their interactions.** Using the KEGG pathway database we identified two similarly regulated genes: RASA1/KRAS involved in the MAPK signaling pathway and five differentially regulated genes: JUN/ATF4/DUSP5/DUSP6/MYC. The similarly and differentially regulated genes were imported into the Ingenuity Pathway Analysis software and analyzed for interactions with RASA1/KRAS and JUN/ATF4/DUSP5/DUSP6/MYC respectively. A: Only one similarly regulated gene (PLAGL1) was found to (indirect) interact, whereas B: twenty-one of the differentially regulated genes were found to either direct (solid line) or indirect (dotted line) interact. The location of the gene products are also indicated in the figure. Black: down-regulated genes. White: up-regulated genes.

Figure 4. **QRT-PCR on biological replicates.** Validation of microarray data by qRT-PCR using biological replicates. Foldchanges for insulin- and S597-stimulated samples compared to untreated (UT) samples were measured for A: Similarly regulated genes and B: Differentially regulated genes.

Figure 1

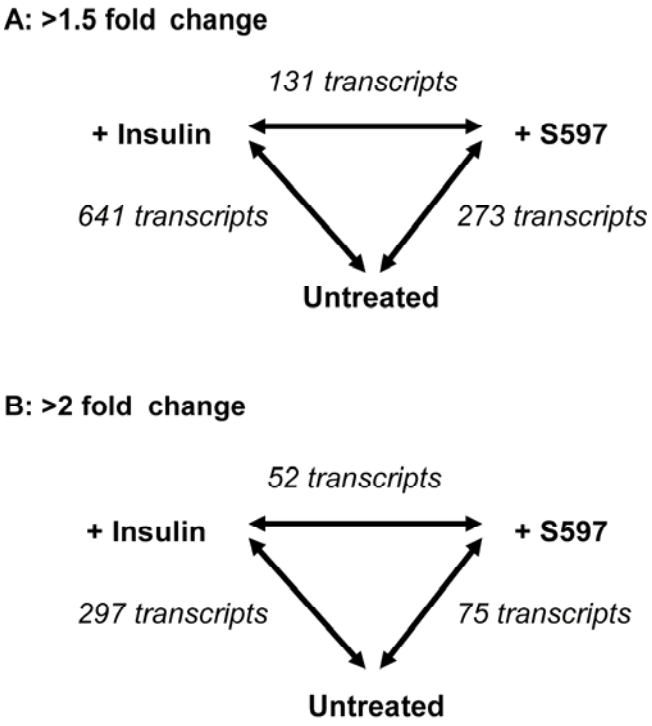

Figure 2

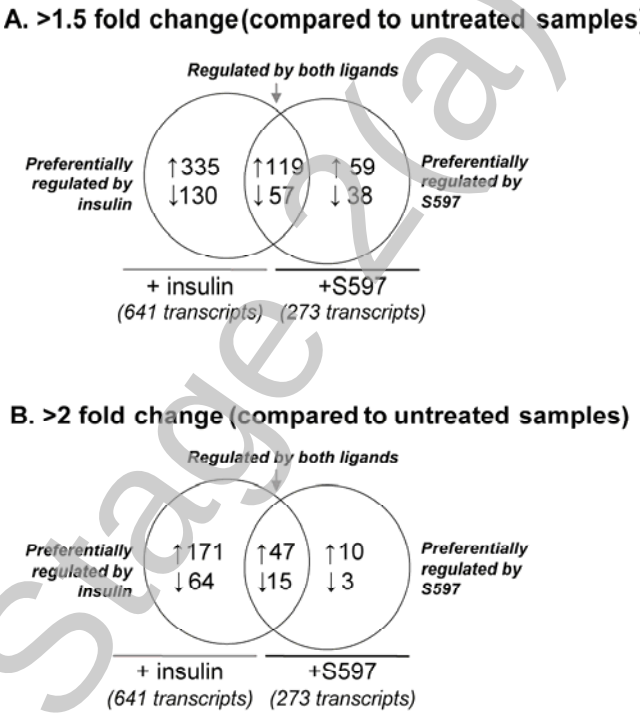

Figure 3

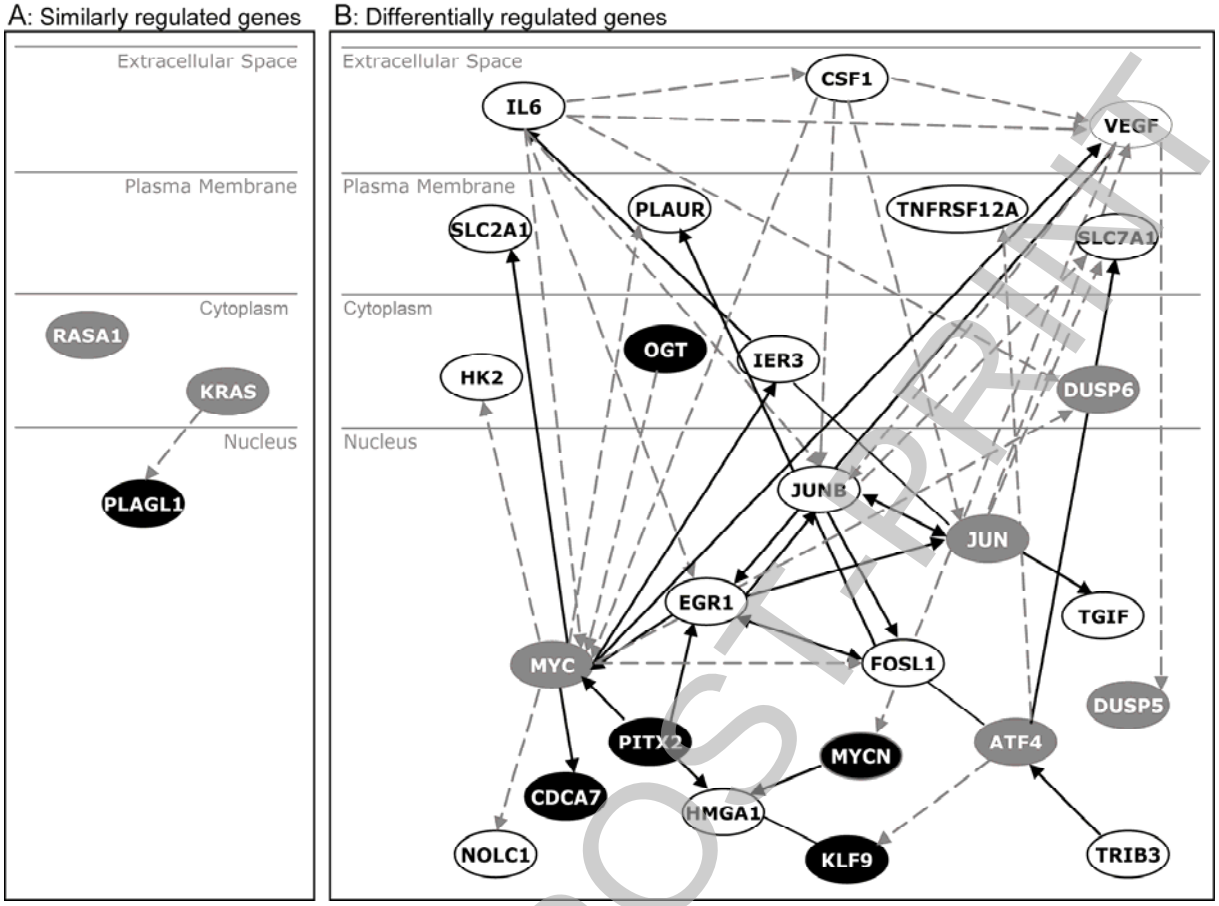

Figure 4