Chemical Genetic Identification of the IGF-Linked Pathway that Is Mediated by STAT6 and MFP2

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Summary

Insulin-like growth factor 2 (IGF2) is a potent mitogen whose deregulation plays a role in developing liver, breast, and prostate cancers. Here, we take a small-molecule approach to investigate molecular pathways that modulate IGF2 signaling, by using chromeceptin, a synthetic molecule that selectively impairs the viability and growth of IGF2-overexpressing hepatocellular carcinoma cells. Affinity purification revealed that chromeceptin binds to multifunctional protein 2 (MFP-2), a seemingly multifunctional enzyme implicated in peroxisomal β-oxidation. The small molecule-protein interaction stimulates the expression of IGF binding protein 1 (IGFBP-1) and suppressor of cytokine signaling-3 (SOCS-3), two cellular attenuators of the IGF signals, through activation of signal transducers and activators of transcription 6 (STAT6). The results underline the importance of STATs in IGF/insulin regulation, and they implicate a new pathway for STAT6 activation that is amenable to small-molecule intervention.

Introduction

Bioactive small molecules have proven to be valuable tools for exploring complex cellular processes [1–3]. Small-molecule tools are complementary to nucleic acid-based tools in that they target the gene product rather than the gene locus or mRNA and affect particular functions for defined periods in cells or organisms [4]. Identification of protein targets of such molecules is a challenging, but powerful, approach to discovering new drug targets and signaling pathways relevant to human diseases [5–8]. Herein, we take a small-molecule-initiated approach to discovering a molecular pathway that controls regulation of insulin-like growth factor 2 (IGF2).

IGF2 is a potent mitogen for a range of cell types. Cancer cells expressing higher levels of IGF2 and its recep-

tor, IGF1R, are usually more aggressive and have a stronger tendency to metastasize [9]. Overexpression of IGF2 has been observed in a number of malignancies, including hepatocellular carcinoma [10], breast cancer [11], Wilm's tumor [12], and hemangioma [13]. The involvement of IGF2 in these human malignancies suggests that the molecular analysis of IGF2 regulation with small molecules may lead to a better understanding of human cancers and possibly a new cancer therapy.

We previously discovered from a chemical library a small organic molecule that inhibits insulin-induced adipogenesis. The drug-like molecule was later shown to impair the growth and viability of IGF2-overexpressing hepatocellular carcinoma cells and was named chromeceptin (Figure 1A) [14]. Molecular biological experiments suggest that chromeceptin exerts its biological activity by blocking the autocrine loop of IGF2. However, its precise mechanism of action remained unknown. Here, we report the identity of a chromeceptin binding protein and a molecular pathway that is modulated by chromeceptin with the goal of providing new insights into the regulation of IGF2 in cancer.

Results

Chromeceptin Activates the Expression of IGFBP-1

To identify genes whose expression patterns are influenced by chromeceptin, we performed gene expression analysis with DNA microarrays consisting of duplicated spots for 1,146 best-characterized cancer-related human genes (http://www.mdanderson.org/∼genome). The microarray results showed that the non-stress-responsive gene that was statistically the most upregulated by chromeceptin among the 1,146 genes was that of IGFBP-1, a secreted IGF binding polypeptide that is known to inhibit the metabolic and mitogenic functions of IGF2 [15–18]. The upregulated expression of IGFBP-1 was further validated by RT-PCR (Figure 1B) and ELISA (Figure 1C). Incubation of HepG2 cells with IGFBP-1 resulted in inhibition of proliferation by ∼20%, while the same concentration of IGFBP-1 had no detectable effects on the growth of cells with low levels of IGF2 (SK-Hep-1). Although other genes may be involved in the chromeceptin phenotype, the clear induction of IGFBP-1 encouraged us to use IGFBP-1 as a starting point for identifying IGF-linked molecular pathways modulated by chromeceptin.

Identification of a Chromeceptin-Responsive Element in the IGFBP-1 Promoter

Given that transcription factors are usually far-downstream factors of signal transduction pathways [19, 20], identification of a chromeceptin-responsive transcription factor that activates the IGFBP-1 promoter would provide an initial step for analyzing the chromeceptin-induced signaling. To define DNA sequences that confer the responsiveness to chromeceptin, a series of IGFBP-1 promoter fragments was ligated to a SEAP reporter gene and transiently transfected to IGF2-overexpressing HepG2 cells. As shown in Figure 2A, a 190

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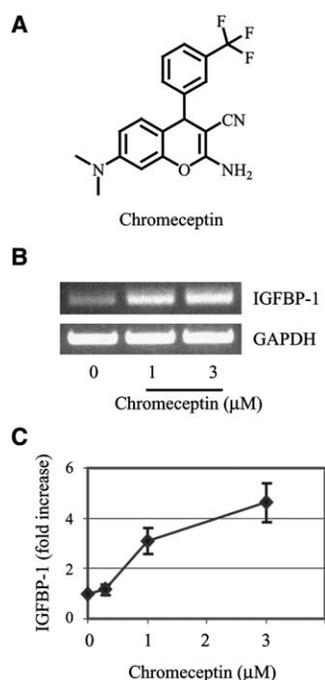
Figure 1. Chromeceptin Activates the Expression of IGFBP-1 in HepG2 Cells

(A) The chemical structure of chromeceptin.

(B) HepG2 cells were treated with chromeceptin for 6 hr in serum-free medium, and IGFBP-1 mRNA levels were quantitated by RT-PCR analysis.

(C) The concentrations of IGFBP-1 were measured by ELISA after treatment with various amounts of chromeceptin for 6 hr. The results are shown as fold increase over the IGFBP-1 concentration in DMSO-treated cells.

bp segment of the IGFBP-1 promoter (−3173/−2983) is highly responsive to chromeceptin. Further deletion from either the 5′ or 3′ ends significantly reduced the reporter response to chromeceptin, suggesting that the 190 bp region contains enhancer element(s) mediating the chromeceptin-induced transactivation of IGFBP-1. To further narrow down DNA sequences that confer the responsiveness to chromeceptin, a series of 28 bp oligonucleotide duplexes was designed to cover the 190 bp segment of the IGFBP-1 promoter, and 3 tandem copies of each fragment were ligated to a SEAP reporter gene. Transfection of the reporter constructs showed that the reporter gene comprised of oligonucleotide #5 was responsive to chromeceptin (Figure 2B), indicating that oligonucleotide #5 contains a chromeceptin-responsive DNA element.

STAT6 Is a Chromeceptin-Responsive Element Binding Transcription Factor

By using the DNA sequence of oligonucleotide #5, we searched databases for transcription factor(s) that potentially bind to the chromeceptin-responsive element. Our search suggested that the sequence contains a binding site consensus for the STAT family of transcription factors (Figure 3A), which mediate cellular transcriptional responses to a variety of cytokines and growth factors [21]. Introduction of point mutations to the STAT binding site in the reporter construct abolished
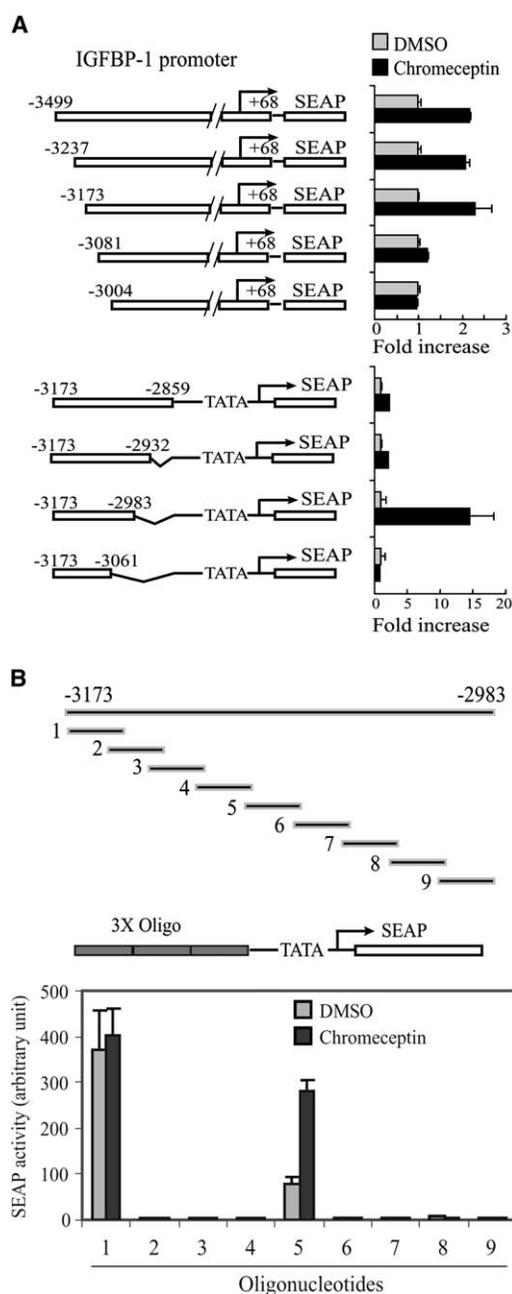
Figure 2. Identification of a Chromeceptin-Responsive Element in the IGFBP-1 Promoter

(A and B) Identification of the 190 bp IGFBP-1 promoter responsive to chromeceptin. (A) Reporter gene constructs in which a SEAP gene is controlled by various fragments of the IGFBP-1 promoter were transfected into HepG2 cells, and the effects of chromeceptin on the reporter gene expression were examined. It is evident that the promoter −3173/−2983 is highly responsive to chromeceptin (15-fold induction). All presented sequence coordinates of the promoter fragments are relative to the transcription start site. (B) Design of a series of 28 bp oligonucleotide duplexes that cover the 109 bp promoter (−3173/−2983) (top). Three tandem copies of each 28 bp fragment were linked to a SEAP reporter gene, and their promoter activities were monitored by SEAP assay (bottom). It is evident that oligonucleotide #5 confers the responsiveness to chromeceptin.

A

-3097   -3070

wt: GGGATTCATGAATGTTCCCAGAAATGGC

mut: GGGATTCATGAATGTGCCCATACATGGC

B

IGFBP-1

-3173

-2983

C

D
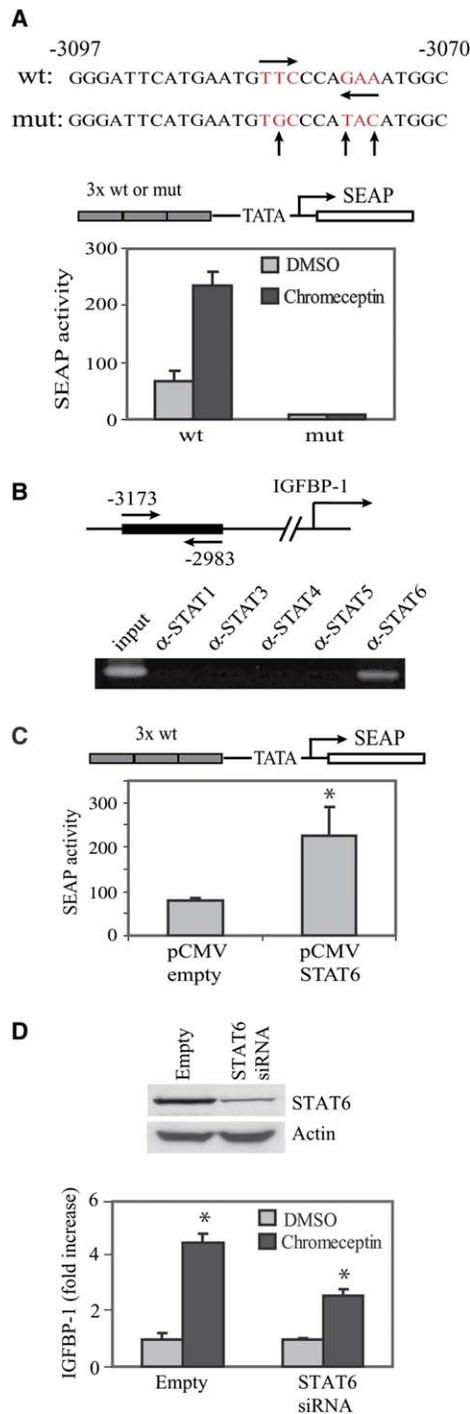
Figure 3. Identification of STAT6 as a Chromeceptin-Responsive Element Binding Transcription Factor

(A) Mutational analysis of the consensus binding site for STAT. The reporter gene driven by three tandem copies of either wild-type or mutant oligonucleotide #5 was transfected into HepG2 cells, and promoter activities were monitored by SEAP assay. The consensus binding site for the STAT family of transcription factors is highlighted in red, and the point mutations in the STAT binding site are indicated by arrows. The presented sequence coordinates of the promoter fragment are relative to the transcription start site.

(B) ChIP assays were performed with sheared chromatins from chromeceptin-treated cells. It is evident that an anti-STAT6 antibody immunoprecipitates an IGFBP-1 promoter fragment contain-

the chromeceptin responsiveness (Figure 3A), indicating that the STAT binding site is a chromeceptin-responsive element.

A total of seven members of human STATs have been described in literature: STAT1–4, -5a, -5b, and -6 [22]. To determine which STAT members bind to the chromeceptin-responsive element in HepG2 cells, we performed a chromatin immunoprecipitation (ChIP) assay. Sheared chromatins were immunoprecipitated with antibodies raised against each member of the STAT family, and purified DNA was subjected to PCR analysis to amplify an IGFBP-1 promoter sequence encompassing the chromeceptin-responsive element. As shown in Figure 3B, anti-STAT6 antibody specifically immunoprecipitated chromatin fragments containing the chromeceptin-responsive element, while those against the other STAT members showed no detectable PCR amplification of the IGFBP-1 promoter.

To confirm the binding of STAT6 to the chromeceptin-responsive element, we cotransfected HepG2 cells with an expression plasmid encoding STAT6 and a reporter gene driven by three tandem copies of the chromeceptin-responsive element. Overexpression of STAT6 enhanced the reporter gene expression, compared with when an empty expression vector was transfected (Figure 3C). We also examined if siRNA knockdown of STAT6 impairs the chromeceptin-induced expression of the endogenous IGFBP-1 gene. As shown in Figure 3D, stable downregulation of STAT6 expression led to a decrease of the chromeceptin-induced IGFBP-1 expression, as compared to that in mock-transfected cells. These results provide evidence that STAT6 is a chromeceptin-responsive element binding transcription factor.

SOCS-3 Expression Is Increased by Chromeceptin

One family of the genes that are known to be activated by STAT6 is SOCS genes (suppressors of cytokine signaling) [23, 24]. Recent animal and molecular biological studies revealed that SOCS-1 and SOCS-3 have an unexpected function besides suppression of cytokine signaling: inhibition of IGF/insulin signaling [25–29]. The relevance of SOCSs both to STAT6 and IGF led to the hypothesis that chromeceptin increases the expression of SOCS-1 or -3 as well as IGFBP-1 through activating STAT6. RT-PCR experiments showed that the expression of SOCS-3 was increased in chromeceptin-treated

ing the chromeceptin-responsive element. The primer set for amplifying the promoter fragment is indicated by arrows.

(C) Overexpression of STAT6 activates the chromeceptin-responsive element in HepG2 cells. A CMV-STAT6 expression vector and a reporter gene driven by three tandem copies of oligonucleotide #5 were transiently cotransfected into HepG2 cells, and promoter activities were monitored by SEAP assay. Transfection of an empty CMV vector served as a control. Values are the mean ± SD (n = 3); *, p < 0.05 versus pCMV empty.

(D) STAT6 is required for the chromeceptin-induced IGFBP-1 expression. Western blot analysis of STAT6 proteins in clonal HepG2 cell lines stably transfected with either an expression vector of STAT6 siRNA or an empty vector (top). The cell lines were treated with chromeceptin for 6 hr, and the concentrations of IGFBP-1 were measured by ELISA. The results are shown as fold increase over the IGFBP-1 concentration in DMSO-treated cells (bottom). Values are the mean ± SD (n = 3); *, p < 0.001 versus DMSO control.

A

B

C

D

Figure 4. Chromeceptin Induces SOCS-3 Expression and Downregulates the Phosphorylation of Akt

(A and B) HepG2 cells were either left untreated or treated with 1 μM of chromeceptin for indicated time periods, and then SOCS-3 levels were quantitated by RT-PCR analysis and Western blot.

(C) STAT6 is required for the chromeceptin-induced SOCS-3 expression. The cell lines were treated with 1 μM chromeceptin for 6 hr, and then total protein samples were subjected to Western blot analysis with an anti-SOCS-3 antibody.

(D) Chromeceptin downregulates the phosphorylation of Akt in HepG2 cells. HepG2 cells were plated out into a 6-well plate at a density of 5×10^5/well. After 24 hr of incubation, the cells were further incubated in serum-free medium containing either DMSO or chromeceptin for indicated time periods. Total cell lysates were subjected to Western blot analysis with an anti-phospho-Akt antibody (top), and then the blot was reprobed with an anti-Akt antibody to show equal loading of proteins (bottom).

cells compared to those in untreated cells (Figure 4A), while SOCS-1 expression was not affected at any time point analyzed (data not shown). Western blot analysis of SOCS-3 protein supports the notion that chromeceptin treatment leads to an increase of SOCS-3 expression in HepG2 cells (Figure 4B). The chromeceptin-induced SOCS-3 expression was diminished by STAT6 knockdown, suggesting a STAT6-dependent SOCS-3 expression by chromeceptin (Figure 4C).

The induction of SOCS-3 and IGFBP-1 takes about 6 hr. If the STAT6-mediated expression of SOCS-3 and IGFBP-1 is responsible for the inhibition of IGF/insulin signaling by chromeceptin, then activation of Akt in the IGF/insulin pathway should not be blocked until 6 hr af-

A

B
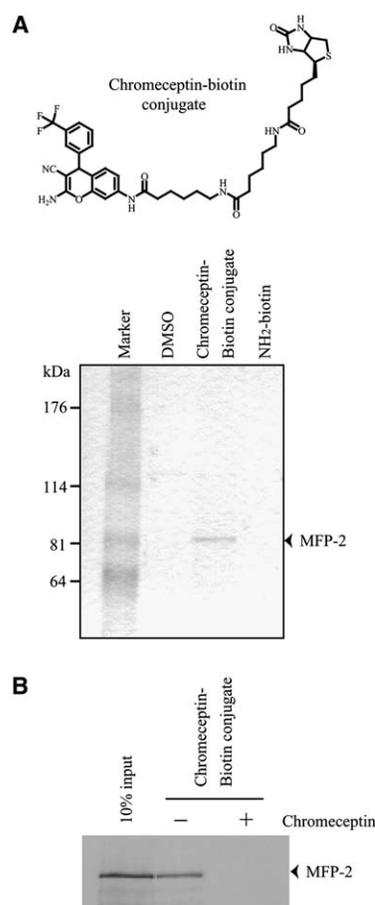
Figure 5. Isolation of a Chromeceptin Binding Protein

(A) An 81 kDa protein that binds specifically to the biotinylated derivative of chromeceptin was purified from HepG2 cell extracts and determined to be MFP-2 by microsequencing. The structure of a biotinylated derivative of chromeceptin is shown.

(B) Chromeceptin competes with the chromeceptin-biotin conjugate for MFP-2 binding. ^{35}S-Met-labeled MFP-2 protein was prepared by the TNT T7 Quick Coupled Transcription/translation system (Promega). The MFP-2 protein was incubated with the chromeceptin-biotin conjugate in the absence (−) or presence (+) of chromeceptin, and then the bound MFP-2 protein was purified by using NeutrAvidin agarose (Pierce).

ter chromeceptin treatment. Inhibition of the Akt phosphorylation was indeed observed 6 hr after chromeceptin treatment (Figure 4D), paralleling the induction of SOCS-3 and IGFBP-1. These results indicate that the expression of SOCS-3 and IGFBP-1, and perhaps that of the other genes that are controlled by STAT6, is upregulated by chromeceptin and may work together to suppress the function of IGF2 in hepatocellular carcinoma cells.

MFP-2 Is a Target of Chromeceptin

Identification of proteins that are targeted by bioactive small molecules has always provided new avenues for an understanding of cell signaling. To identify a target protein of chromeceptin, we chemically synthesized a biotinylated derivative of chromeceptin (Figure 5A). The structure-activity relationship of 65 chromeceptin analogs showed that the dimethylamino group has
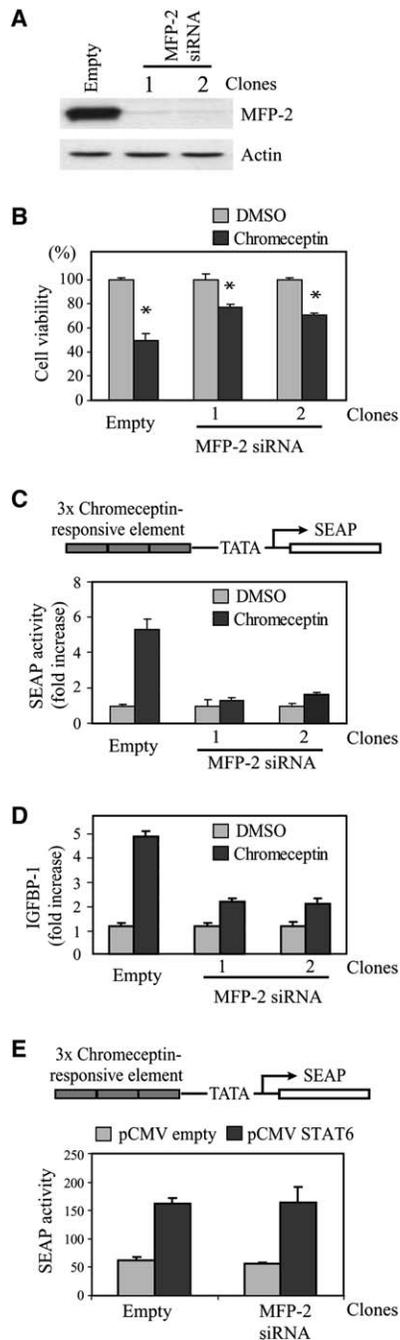
Figure 6. Analysis of MFP-2

(A) Western blot analysis of MFP-2 proteins in two clonal HepG2 cell lines stably transfected with an expression vector of MFP-2 siRNA (pSuper/MFP-2).

(B) Silencing of MFP-2 expression renders the cells less sensitive to chromeceptin in a cell viability assay. The cell lines were treated with 0.03 μM chromeceptin for 4 days, and the cell viability was determined by WST assays in triplicate (n = 3). *, p < 0.0001 versus DMSO control.

(C) MFP-2 is required for activation of the chromeceptin-responsive element. The cell lines were transfected with a SEAP reporter gene driven by the chromeceptin-responsive element, and they were then treated with 1 μM chromeceptin for 6 hr.

(D) MFP-2 is required for chromeceptin-induced IGFBP-1 expression. The cell lines were treated with 1 μM chromeceptin for 6 hr, and then the concentrations of IGFBP-1 were measured by ELISA.

limited roles in the selective biological activity (K.M. and M.U., unpublished data), suggesting the position of the dimethylamino group as a good biotinylation site. Affinity chromatography with avidin agarose beads purified a protein that binds specifically to the biotinylated derivative of chromeceptin, but not to a control biotin conjugate or avidin agarose beads (Figure 5A). Microsequencing of the protein showed three peptide sequences that matched the amino acid sequence of human MFP-2 (multifunctional protein-2). Competition assay with ^{35}S-labeled MFP-2 revealed that chromeceptin indeed competes with the chromeceptin-biotin conjugate for MFP-2 binding (Figure 5B). These results suggest that MFP-2 is a target of chromeceptin.

MFP-2 is a seemingly multifunctional enzyme implicated in peroxysomal β-oxidation. However, its precise functions and natural substrates remain unclear, and its role in relation to IGF regulation has never been investigated. To examine if MFP-2 is required for the IGF2-suppressing phenotype of chromeceptin, we generated stable cell lines that constitutively express an MFP-2 siRNA. Western blot analysis confirmed selective silencing of MFP-2 expression in the stably transfected clones (Figure 6A). The knockdown cells, which exhibited as much proliferation as mock-transfected cells, were less sensitive (0.5-fold) to chromeceptin-induced growth arrest (Figure 6B), suggesting the requirement of MFP-2 for full activity of chromeceptin. The MFP-2 knockdown also rendered the chromeceptin-responsive element essentially unresponsive to chromeceptin (Figure 6C) and diminished the chromeceptin-induced activation of IGFBP-1 (Figure 6D).

If MFP-2 is upstream of STAT6 in the pathway, then STAT6 overexpression should still induce the chromeceptin-responsive reporter gene in the MFP-2-deficient cells that are unresponsive to chromeceptin. As shown in Figure 6E, transfection of a STAT6 expression vector in MFP-2-deficient cells stimulated the chromeceptin-responsive reporter gene as much as it does in control cells. These results collectively suggest that the effects of chromeceptin are mediated, at least in part, through regulation of MFP-2 functions, and that MFP-2 is essential for activation of STAT6 by chromeceptin.

Discussion

STAT6 Activation by Chromeceptin

Small molecules can be used as tools for the discovery of new molecular pathways through the identification of their responsive transcription factors, as found in the discovery of the CREB pathway by using cAMP. Investigation into the chromeceptin-induced IGFBP-1 expression led to the identification of STAT6 as a chromeceptin-responsive element binding transcription factor.

The results are shown as fold increase over the IGFBP-1 concentration in DMSO-treated cells. Values are the mean ± SD (n = 5).

(E) Overexpression of STAT6 activates the chromeceptin-responsive element in MFP-2 knockdown cells. The CMV-STAT6 expression vector and a reporter gene driven by three tandem copies of oligonucleotide #5 were transiently cotransfected into clonal HepG2 cells, and promoter activities were monitored by SEAP assay. Transfection of an empty CMV vector served as a control.

STAT6 is known to be a latent cytoplasmic transcription factor, whose nuclear translocation and transcriptional activity are regulated primarily by IL-4 and IL-13 [30, 31].

In a number of tumor-derived cell lines, however, STAT6 is translocated constitutively into the nucleus, even in the absence of cytokine stimulation [32, 33]. In HepG2 cells, STAT6 appears to be present in the nucleus and bind constitutively to the chromeceptin-responsive element in the IGFBP-1 promoter. The following preliminary observations support this hypothesis and suggest that chromeceptin activates STAT6 by a mechanism distinct from that of IL-4 (Y.C., D. Jung, and M.U., unpublished data): (1) STAT6 is constitutively phosphorylated to some extent in HepG2 cells at Tyr641, the residue known to be phosphorylated by IL-4 stimulation, even in the absence of IL-4 or chromeceptin treatment, and chromeceptin does not induce Tyr641 phosphorylation; (2) Western blots of nuclear extracts showed no significant change in the amounts of STAT6 in the nucleus before and after chromeceptin treatment; (3) chromeceptin had no detectable effects on the amounts of the IGFBP-1 promoter immunoprecipitated with an anti-STAT6 antibody; (4) the STAT6 reporter gene exhibited low levels of gene expression, even in the absence of chromeceptin, and the expression was further activated by chromeceptin. It remains to be elucidated how nuclear STAT6 is further activated by chromeceptin. Western blots of STAT6 detected a mobility shift of the STAT6 band on a SDS gel 2–6 hr after chromeceptin treatment, suggesting posttranslational modifications of STAT6 other than Tyr641 phosphorylation (Y.C., D. Jung, and M.U., unpublished data). Detailed mechanistic studies are currently underway.

SOCS-3 Induction by Chromeceptin
Identification of STAT6 as a chromeceptin-responsive transcription factor permitted the detection of chromeceptin-induced SOCS-3 expression. The SOCS-3 induction was not detected by the initial gene expression analysis with DNA microarrays. In the analysis of microarray results, expression changes of low-expressing genes such as SOCS-3 tend to be underrepresented due to their low-intensity signals and lower statistic-confidence values [34–36]. The reliable measurement is more achievable for highly expressed genes including IGFBP-1, which served as a starting point to decipher the signals of related genes with lower expression levels.

The SOCS-3 protein has recently been shown to suppress insulin-induced tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) and Akt activation in HepG2 cells [25]. The observation in cell culture was further confirmed in mice by Ueki et al. [28, 37], showing that increased SOCS-3 protein in the liver causes systemic insulin resistance through inhibition of tyrosine phosphorylation of IRS proteins. Our results provide further support for the role of SOCS-3 in IGF/insulin signaling.

Chromeceptin appears to suppress the function of IGF2 through inducing the expression of SOCS-3 and IGFBP-1, and most likely those of other STAT6-responsive genes (Figure 7). Chromeceptin indeed blocks the activation of Akt in the IGF/insulin pathway (Figure 4D), and the inhibition of the Akt phosphorylation parallels
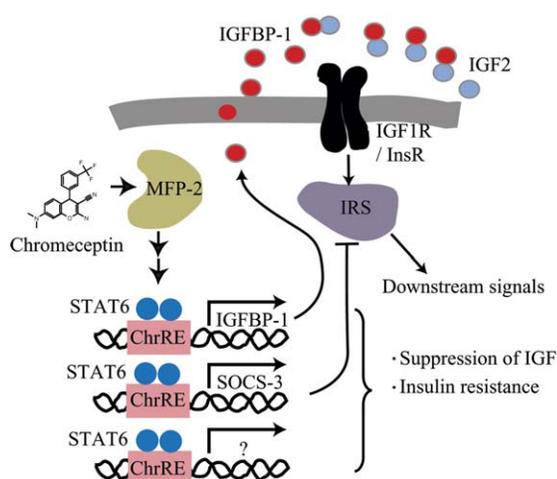
Figure 7. Functional Links among MFP-2, STAT, and IGF2 Signals
ChrRE represents a chromeceptin-responsive element.

the induction of IGFBP-1 and SOCS-3 by chromeceptin. It would be interesting to know what other STAT6-responsive genes mediate the inhibition of IGF2 in hepatocellular carcinoma.

It may also be interesting to note that recent work suggests no significant alteration of SOCS-3 expression in B cells from STAT6-knockout mice [38]. The regulation of SOCS-3 expression may be dependent on cell types.

How MFP-2 Controls STAT6
The isolation of MFP-2 as a direct binder of chromeceptin suggests a functional link between MFP-2 and IGF signals. Although chromeceptin may bind to multiple proteins in cells, MFP-2 appears to be a target important for its STAT6 activation. It remains to be elucidated how the binding of chromeceptin to MFP-2 activates STAT6. It is possible to imagine that the binding induces or stabilizes the interaction of MFP-2 with another protein, as found in the FK506-FKBP complex interacting with calcineurin [39]. If this is the case, it will be necessary to identify proteins that interact with MFP-2 in the presence of chromeceptin.

Determination of an exact chromeceptin binding region in MFP-2 would provide insights into how chromeceptin stimulates STAT6. Our preliminary results showed that the biotin derivative of chromeceptin binds to a region corresponding to amino acids 507–736 (data not shown). This region of MFP-2 shows a sequence similarity (58%) to the sterol carrier protein 2 (SCP2). However, the role of the SCP2-like domain for the functionality of MFP-2 remains unclear. Search for the proteins that mediate STAT6 activation is currently underway.

Significance

Our results showed that binding of chromeceptin to MFP-2 stimulates the expression of IGFBP-1 and SOCS-3, two important cellular attenuators of IGF signals, through activation of STAT6 (Figure 7). These results provide chemical genetic support for the roles of the STAT-SOCS pathway in IGF regulation, and they

implicate a new, to our knowledge, pathway for STAT6 activation. Although it remains to be elucidated how binding of chromeceptin to MFP-2 triggers STAT6 activation, our chemical genetic study could provide a basis for future efforts to understand the regulatory circuit of IGF2 in hepatocellular carcinoma.

Experimental Procedures

Abbreviations
The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; MFP2, multifunctional protein-2; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; ELISA, enzyme-linked immunosorbent assay; SEAP, secreted alkaline phosphotase; ChIP, chromatin immunoprecipitation.

Oligonucleotide Microarray Analysis
HepG2 cells were treated with either DMSO or 1 μM chromeceptin for 6 hr in serum-free medium. Total RNA was extracted in a TRI reagent (Molecular Research Center) and further isolated with an RNeasy Mini Kit (Qiagen). Microarray experiments were performed by using the oligonucleotide arrays (CG11 oligoarray) with 1,146 functionally known genes, produced by Cancer Genomic Core Laboratory at M.D. Anderson Cancer Center (Houston, TX), as described previously [40–42].

IGFBP-1 ELISA
HepG2 cells were plated out in triplicate at a density of 5×10^5/well onto a 6-well plate in Minimal Essential Medium (MEM) containing 10% fetal bovine serum (FBS), sodium pyruvate, glutamate, and nonessential amino acids. After a 24 hr incubation, the cells were maintained in serum-free medium and treated with chromeceptin for 6 hr. The IGFBP-1 concentration in cell-conditioned media was measured with the Active Total IGFBP-1 ELISA kit (Diagnostic Systems Laboratories). A set of IGFBP-1 standards was used to plot a standard curve of absorbance versus IGFBP-1 concentration, and the IGFBP-1 concentrations of the samples were calculated from the standard curve.

Plasmid Construction
All human IGFBP-1 promoter fragments were amplified from genomic DNA by PCR. The primer sequences were derived from *Homo sapiens* BAC clone RP11-132L11 (GenBank number: AC091524). The DNA fragments corresponding to nucleotides −3499 to +68, −3237 to +68, −3173 to +68, −3081 to +68, and −3004 to +68 were cloned to *Kpn*I/*Hind*III sites of the pTA-SEAP vector, which contains a secreted alkaline phosphatase (SEAP) reporter gene (Clontech). The DNA fragments corresponding to nucleotides −3173 to −2859, −3173 to −2932, −3173 to −2983, and −3173 to −3061 were cloned to *Kpn*I/*Bgl*II sites of the pTA-SEAP vector, where a TATA box ensures optimal induction of the reporter gene. All presented sequence coordinates of the promoter fragments are relative to the transcription start site.

Transfection and Secreted Alkaline Phosphatase Assay
HepG2 cells were plated out in triplicate at a density of 5×10^5/well onto a 6-well plate in MEM containing 10% FBS, sodium pyruvate, glutamate, and nonessential amino acids. After a 24 hr incubation, the cells were maintained in MEM containing 1% FBS, sodium pyruvate, glutamate, and nonessential amino acids, and they were then transiently transfected with 1 μg reporter gene by using FuGENE6 (Roche). A total of 17 hr after transfection, the cells were washed twice in serum-free medium, and then treated with 1 μM chromeceptin for 6 hr in serum-free medium. SEAP activity was measured as described [43].

Reverse Transcription-PCR
Total RNA was extracted from HepG2 cells in a TRI reagent (Molecular Research Center) and further isolated with an RNeasy Mini Kit (Qiagen). The RNA sample was subjected to RT-PCR by using the Access RT-PCR System (Promega). RT-PCR reactions include total RNA, 1 μM of each primer, 0.2 mM dNTP, 1 mM MgSO$_4$, AMV reverse transcriptase (2 U), and *Tfl* DNA polymerase (2 U) in a final volume of 25 μl. The primer pairs used are as follows: 5′-TGCAGAGGC AGGGAGCCCTGAAA-3′ and 5′-TATATCTGGCAGTTATGTA-3′ for IGFBP-1; 5′-AACGGGAAGCTTGTCATCAAT-3′ and 5′-GCCAGTGA GCTTCCCGTTCA-3′ for GAPDH; 5′-TCACCCACAGCAAGTTTCC CGC-3′ and 5′-GTTGACGGTCTCCG ACAGAGATGC-3′ for SOCS-3.

Chromatin Immunoprecipitation Assay
Chromatin immunoprecipitation (ChIP) assays were performed by using the ChIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. HepG2 cells (1×10^7) were treated with 1 μM chromeceptin for 6 hr in serum-free medium, and they were then treated with formaldehyde at a final concentration of 1% for 10 min at 37°C. Lysates from the cells were sonicated with 5 sets of 10 s pulses set to 25% of maximum power to shear chromatin to lengths between 200 and 1000 bp. The sheared chromatins were incubated with 10 μg of each STAT antibody (Santa Cruz Biotechnology) at 4°C, and purified DNA was subjected to PCR analysis as follows: 1 cycle at 94°C for 4 min; 35 cycles at 94°C for 30 s, 55°C for 30 s, 68°C for 60 s; and 1 cycle at 68°C for 7 min.

Synthesis of a Biotinylated Derivative of Chromeceptin
To a solution of 2,7-Diamino-4-(3-trifluoromethyl-phenyl)-4*H*-chromene-3-carbonitrile (1.0 mg, 0.003 mmol) and diisopropylethylamine (0.003 mmol) in DMSO/CH$_2$Cl$_2$ were added a trace of 4-(dimethylamino)pyridine and a biotin-XX-NHS (3.4 mg, 0.006 mmol). The solution was stirred at room temperature for 10 days, diluted with brine, and extracted with CHCl$_3$. The combined extracts were dried over Na$_2$SO$_4$ and then concentrated in vacuum. The residue was purified by column chromatography on silica gel with CHCl$_3$/methanol mixtures to give the biotinylated chromeceptin (2.2 mg, 93%). ^{1}H NMR (CD$_3$OD, 270 MHz) δ_H 7.59 (d, J = 1.9 Hz, 1H), 7.48–7.52 (m, 4H), 7.08 (dd, J = 8.1, 2.0 Hz, 1H), 6.91 (d, J = 8.9 Hz, 1H), 4.62 (br, 1H), 4.46 (m, 1H), 4.30 (m, 1H), 3.1–3.3 (m, 5H), 2.90 (dd, J = 12.4, 4.9 Hz, 1H), 2.70 (d, J = 12.4 Hz, 1H), 2.36 (t, J = 4.6 Hz 2H), 2.15 (m, 4H), 1.3–1.7 (m, 18H); MS (ESI) Exact mass calcd for C$_{39}$H$_{48}$F$_3$N$_7$O$_5$S + H requires m/z 784.34. Found m/z 784.3.

Purification of Chromeceptin Binding Protein
HepG2 cells (2 ml of packed cell volume) were washed with PBS and collected in 15 ml PBS containing 0.5% Nonidet P-40 and 1 mM PMSF. Lysates were well shaken at 4°C for 30 min and subject to brief sonication, followed by high-speed centrifugation (100,000 $\times$ g) for 1 hr. Endogenous biotinylated proteins in supernatant were adsorbed to an Avidin agarose column (Sigma), and the flowthrough fraction was then incubated with 5 μM of the biotinylated derivative of chromeceptin for 12 hr at 4°C. The proteins bound to biotinylated derivative of chromeceptin were purified with NeutrAvidin agarose (Pierce) and eluted with SDS/PAGE sample buffer. The resulting samples were separated on an 8% polyacrylamide gel and visualized by Coomassie brilliant blue R-250 staining. The protein band was excised from the gel and microsequenced by mass spectrometry as described [44].

Knockdown of Protein Expression with Small Interfering RNA
To generate HepG2 cells that constitutively express a STAT6 siRNA, we used the GenEclipse STAT6 Vector-based RNAi kit (Chemicon). Stable downregulation of MFP-2 expression in HepG2 cells was achieved with the pSuper RNAi System (OligoEngine). The 19 nt targeting sequence derived from the MFP-2 transcript is as follow: 5′-CGGAUGACUCAGACAGUUA-3′. HepG2 cells (2×10^6) in a 10 cm culture dish were transfected with vector constructs (10 μg) by using FuGENE6 (Roche) according to the manufacturer's protocol. The cells were divided at 1:20 dilution 48 hr after transfection and further incubated in medium containing 1 mg/ml neomycin derivative (G418) before individual colonies were picked and expanded into cell lines.

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