Akt modulates STAT3-mediated gene expression through a FKHR (FOXO1a)-dependent mechanism

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Summary

The PI 3-kinase/Akt pathway plays an important role in the signaling of insulin and other growth factors, which reportedly attenuate the interleukin-6 (IL-6) mediated stimulation of acute phase plasma protein genes. We investigated the effect of the protein kinase Akt on IL-6-mediated transcriptional activation. The transient expression of constitutively active Akt inhibited the IL-6-dependent activity of the α2-macroglobulin (α2-M) promoter in HepG2 cells, whereas expression of an inactive mutant of PDK1 had the opposite effect. Since Akt is known to regulate gene expression through inactivation of the transcription factor FKHR, we examined the effect of FKHR on STAT3-mediated transcriptional regulation. Indeed, the overexpression of FKHR specifically enhanced the activity of STAT3-dependent promoters, but not that of a STAT5-responsive promoter. The effect of FKHR required the presence of functional STAT3 and was abrogated by the expression of dominant negative STAT3 mutants. Furthermore, FKHR and STAT3 were shown to coimmunoprecipitate and to colocalize in the nuclear regions of IL-6-treated HepG2 cells. Our results indicate that FKHR can modulate the IL-6-induced transcriptional activity by acting as a coactivator of STAT3.

Abbreviations used in this paper:

α2-M, α2-macroglobulin; APP, acute phase protein; CBP, CREB-binding protein; CREB, cAMP response element binding protein; FKHR, forkhead-related transcription factor; FOXO1a, forkhead box transcription factor O1a; PDK1, 3-phosphoinositide-dependent protein kinase-1; PI, phosphatidylinositol; STAT, signal transducer and activator of transcription; IL-6, interleukin-6
Introduction

IL-6 is the major regulator of acute phase protein (APP) synthesis by the liver during the inflammatory response (1). It exerts its actions through binding to the receptor complex consisting of a ligand-specific IL-6R α-chain (gp80) and two signal transducing β-subunits (gp130). Activation of the gp130-associated Janus kinases Jak1, Jak2 and Tyk2, results in the tyrosine phosphorylation of several cellular substrates, including STAT3 (signal transducer and activator of transcription 3), the major mediator of IL-6-induced signaling (2,3). Phosphorylated STAT3 dimerizes and translocates to the nucleus, where it regulates the transcription of multiple target genes.

The STAT3-dependent action of IL-6 appears to be modulated by a variety of stimuli, including insulin and epidermal growth factor (EGF) that have been reported to inhibit the APP production by cultured hepatic cells (4,5). The potential inhibitory role of growth promoting signals on the IL-6-inducible Jak/STAT3 pathway corresponds well with the suppressed acute phase response in regenerating liver (6). However, the mechanisms whereby growth factors mediate this effect remained unclear. One of the major effects of signaling via the insulin receptor and other growth factor receptors is the activation of PI 3-kinase (7). Generation of PI 3-phosphorylated lipids in the plasma membrane leads to phosphorylation and activation of the serine/threonine kinase Akt (also called protein kinase B, PKB) by phosphatidylinositol-dependent kinase 1 (PDK1). Consecutively, activated Akt has been described to translocate to the nucleus (8) and to directly phosphorylate members of the forkhead family of transcription factors (9-11). Phosphorylation of FKHR or closely related FKHRL1 and AFX by Akt results in their transcriptional inactivation and retention in the cytoplasm (9,12).
In the present study, we investigated a potential crosstalk between the PI 3-kinase/Akt signaling and the IL-6-inducible Jak/STAT3 pathway. We have identified FKHR as a specific transcriptional coactivator of STAT3. This functional interaction reflects the association of both proteins and their colocalization in nuclear regions of HepG2 cells.
Experimental Procedures

Cytokines and reagents - Recombinant human IL-6 (2×10^6 B-cell stimulatory factor-2 units/mg) together with soluble human IL-6 receptor was used as described previously (13). Recombinant insulin, TGFβ and IFNγ were from Roche, R&D Systems and PeproTech; erythropoietin was a kind gift of J. Burg and K.-H. Sellinger (Roche). LY294002 was from Calbiochem. Polyclonal rabbit FKHR antiserum was generated using purified GST-FKHR as antigen.

Plasmids - Wt-Akt, ca-Akt (iSH2Akt) in pECE, pSVL-STAT3, pGL3-α2-M-215luc, pECE-STAT5A, pSVL-Eg-YLVLD and pGL3-β-casein-luc have been described before (14-17). Wt-PDK1 and kd-PDK1 in pCMV5 were a kind gift from Dario Alessi (Dundee, UK). pSIE-tk-luc was kindly provided by Hugues Gascan (Angers, France). STAT3F and STAT3D in pCAGGS were from Koichi Nakajima and Toshio Hirano (Osaka, Japan). The FKHR cDNA (a kind gift from Frank Rauscher, Pennsylvania, PA) was subcloned into pcDNA3.1/His C (Invitrogen) or pEGFP-C1 (Clontech). The pEGFP-FKHR expression plasmid was further digested with AccI, Van91I, BpiI or HindIII in combination with BamHI (Roche), filled-in with Klenow and religated to obtain FKHR Δ357, Δ505, Δ560 and Δ639, respectively. The FKHR point mutants (T24A, S256A, S319A) were generated by PCR-based site-directed mutagenesis. The resulting constructs were verified by sequencing.
**Transient transfections and luciferase reporter gene assays** - HepG2 cells were transiently transfected using FuGene6 reagent (Roche) or by the calcium phosphate method as described previously (16). 24 hours after transfection, cells were stimulated with cytokines for another 18 hours. Luciferase activities were determined with the Luciferase Assay System (Promega) and the data were normalized according to co-expressed β-galactosidase (pCH110) or Renilla-luciferase (pRLTK) activities. All the experiments were repeated at least three times with similar results. Shown are the means +/- s.d. of one representative experiment performed in triplicates.

**Northern blots** - Total RNA was extracted from HepG2-cells with the RNeasy kit from Qiagen. Ten µg of RNA were separated by 1% agarose gel electrophoresis and transferred onto nylon membranes (Nytran, Schleicher and Schüll). Detection with the $^{32}$P-labeled α2-M probe was performed as described previously (18) and the signals were detected using a Personal Molecular Imager FX (Biorad).

**Cell fractionation, immunoprecipitation and Western blot analysis** - Nuclear extracts were prepared as described (13) with modifications. Cells were lysed in hypotonic buffer A (10 mM Hepes/KOH pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 1 mM Na$_3$VO$_4$, 0.2 mM PMSF) for 10 min at 4°C and centrifugated at 300 g for 2 min at 4°C. The crude nuclei were incubated in lysis buffer B (1% (w/v) BRIJ-97, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM sodium fluoride, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 1 mM PMSF, 5 µg/ml aprotinin and 5 µg/ml leupeptin) for 30 min at 4°C. Short sonication and centrifugation at 14,000 g for 2 min at 4°C yielded the nuclear extracts. Total cellular
lysates were prepared by direct lysis of the harvested cells in buffer B and processed as described above. Protein extracts were incubated overnight at 4°C with polyclonal antiserum against STAT3 (C-20), SMAD2/3 (N-19), ERK2 (C-14, Santa Cruz) or monoclonal antibodies against STAT5 (MGF, Transduction Laboratories). The precipitates were later collected with Protein A-Sepharose (Amersham Pharmacia), washed three times with lysis buffer and resolved on 7.5% SDS/PAGE gels. After transfer to PVDF membranes (GelmanSciences), the blots were probed with the respective antibodies and detected for signals using the ECL system (Amersham Pharmacia).

**Akt kinase assay** - Cells were lysed lysis buffer (50 mM Hepes pH 7.6, 150mM NaCl, 10% (v/v) glycerol, 1 % (v/v) Triton X-100, 1 mM PMSF, 1 mM benzamidin, 1 mM Na3VO4, 30 mM Na4P2O7, 10 mM NaF, 1mM EDTA, 1 mM DTT, 100 nM ocadaic acid) and the Akt activity was assayed as described previously (19). The phosphopeptide spots were quantified using a Cyclone phosphorimager and the Optiquant-software (Packard).

**Immunocytochemistry and confocal fluorescence microscopy** - Cells grown on coverslips to subconfluence were serum-deprived for the last 12 hours, fixed and permeabilized as described before (20). For intracellular staining, polyclonal anti-STAT3 antibodies (C-20, Santa Cruz Biotechnology) or rabbit antiserum against FKHR and then secondary donkey polyclonal FITC- or Rhodamine-coupled antibodies were used (Santa Cruz Biotechnology). After mounting, fluorescence images were visualized by
confocal laser scanning microscopy (LSM 510, Zeiss, Apochromat 63× objective lens). Ar and He-Ne lasers were switched between the excitation wavelengths for fluorescein ($\lambda_{ex} = 488$ nm) and rhodamine ($\lambda_{ex} = 543$ nm) fluorescences, which were detected using a 505-530 nm band pass or 560 nm long pass emission filter, respectively.
Results

Activation of PDK1/Akt signaling inhibits the IL-6-mediated gene activation through a FKHR-dependent mechanism

In agreement with previous studies (4,5), we found that insulin inhibits the IL-6 induced expression of several acute phase plasma proteins, including α2-M in hepatoma cells. The levels of α2-M mRNA in IL-6-treated HepG2 hepatoma cells were significantly decreased in the presence of insulin (Fig. 1A). One of the well-known mediators of insulin signaling is PI 3-kinase, which further downstream activates the serine/threonine kinase Akt (7). Interestingly, the inhibitory effect of insulin on α2-M expression could be partially relieved by an inhibitor of PI 3-kinase, wortmannin (Fig. 1A). As shown in Fig. 1B, similar effects were also observed on the level of α2-M promoter activity using a fragment of the 5’ regulatory sequence of the rat α2-M gene, which is known for its high sensitivity to STAT3-mediated signaling (16). Therefore, we decided to investigate a potential crosstalk between the PI 3-kinase/Akt pathway and the IL-6-inducible Jak/STAT3 signal transduction cascade. Transfection of HepG2 cells with a constitutively active variant of Akt (ca-Akt) resulted in a significant reduction of the IL-6-mediated activation of a transfected α2-M promoter-luciferase reporter gene construct (Fig. 2A), comparable to the degree of inhibition by insulin (Fig. 1B). Consistently, overexpression of a kinase-dead variant of PDK1 (kd-PDK1) exerted the opposite effect and augmented the reporter gene activity. The effects observed using wild type forms of both kinases were less pronounced (data not shown). These results suggested that Akt kinase activity can negatively influence IL-6-dependent gene expression.
The protein kinase Akt is known to directly phosphorylate and inhibit transcription factors of the forkhead family (9,21). Therefore, we asked whether FKHR (FOXO1a), a forkhead transcription factor which has been reported to be regulated in an insulin-independent manner in hepatic cells (22), is involved in α2-M promoter regulation. Transfection of HepG2 cells with increasing amounts of FKHR cDNA led to a marked and dose-dependent increase of the IL-6-responsiveness of the α2-M promoter (Fig. 2B), without a significant effect on the promoter activity in untreated cells. In order to confirm that FKHR links Akt to IL-6 signaling, we examined whether its overexpression affects the inhibitory action of PDK1 and Akt. As shown in Figure 2C (left panel), wild type PDK1 decreased the IL-6-induced reporter gene activity by about 50% in FKHR-overexpressing cells, whereas the kinase-dead PDK1 mutant produced the opposite effect. In addition, overexpression of FKHR remarkably enhanced the effect of Akt. While wt-Akt similarly to wt-PDK1 decreased IL-6-mediated gene expression, expression of constitutively active Akt further reduced the reporter gene activity to about 25% of the control level (Fig. 2C, right panel).

The inactivation of FKHR by Akt results from a direct phosphorylation of three regulatory sites (10,23,24): T24, S256 and S319, which in turn leads to cytoplasmic retention of FKHR (9,12). In order to investigate the influence of FKHR phosphorylation on α2-M promoter activity, we compared the effects of wild-type FKHR and mutated FKHR variants lacking the first, the first two or all three phosphorylation sites (Fig. 2D). All three examined FKHR mutants were more efficient than wt-FKHR in augmenting the IL-6-induced α2-M promoter activity. Interestingly, mutation of threonine 24 to alanine was already sufficient for a near maximal effect; introduction of additional point
mutations (S256A and S319A, respectively) did not lead to a significant further increase in promoter activity. These results indicate that PDK1/Akt signaling can modulate the transcriptional activation of IL-6-responsive genes by targeting FKHR.

**IL-6 does not induce Akt activity and FKHR phosphorylation in HepG2 cells**

IL-6 has been reported to activate Akt in Hep3B and different cells from myeloma patients (25,26). In this case, IL-6 stimulation should result in nuclear translocation of activated STAT3 and concomitant repression and nuclear exclusion of FKHR. However, while insulin induced a robust and sustained activation of Akt, IL-6 did not significantly stimulate Akt kinase activity over its basal level in HepG2 cells (Fig. 3A). The low Akt activity after IL-6 stimulation was reflected by the lack of its phosphorylation on two critical regulatory residues, T308 and S473 (Fig. 3B). This also correlated with the low phosphorylation status of T24 and S256, two residues of FKHR, which become directly targeted by Akt (Fig. 3C). In contrast, Akt and FKHR were both strongly phosphorylated after treatment of the cells with insulin and their activation was not significantly affected by the presence of IL-6. However as expected, IL-6 strongly induced tyrosine-phosphorylation of STAT3, the major transcription factor mediating IL-6-dependent signal transduction (2,3) (Fig. 3D). The pattern of Akt-, FKHR- and STAT3-phosphorylation in HepG2 cells after 16 hours of IL-6 and/or insulin treatment was qualitatively similar although the phosphorylation of STAT3 was less prominent (data not shown). We conclude that in HepG2 cells, IL-6 cannot induce Akt activity and consequently does not repress FKHR.
Functional interaction between FKHR and STAT3 in transcriptional regulation

In order to clarify the role of FKHR for the regulation of the α2-M promoter, we investigated whether FKHR modifies the function of STAT3, the crucial mediator of IL-6-signaling (2,3). Coexpression of STAT3 and FKHR in HepG2 cells indeed synergistically increased the responsiveness of the α2-M promoter to IL-6 stimulation (Fig. 4A). In contrast, FKHR expression had no effect on the transcriptional activation of the β-casein promoter by STAT5, another member of the STAT family of transcription factors (Fig. 4B). Likewise, FKHR did not enhance the activity of transcription factors unrelated to STATs such as Smads, the mediators of TGFβ signaling (data not shown). Hence, FKHR appears to be a specific transcriptional coactivator of STAT3-responsive promoters.

The stimulatory effect of FKHR on the α2-M promoter activation essentially depends on the presence of functional STAT3, since overexpression of dominant negative STAT3 factors (STAT3F or STAT3D, respectively) (28) almost completely abrogated the FKHR-mediated induction of α2-M promoter activity (Fig. 4C). This is also consistent with the observation that expression of FKHR efficiently upregulates IL-6-responsiveness of an artificial promoter, comprising of a tandem of isolated STAT3 consensus binding sites (Fig. 4D). These results suggest, that the transcriptional effects of FKHR are indirect and result from an enhanced activity of STAT3.
The complete C-terminus of FKHR is crucial for the coactivation of IL-6-dependent gene expression

The PAX3-FKHR fusion protein from alveolar rhabdomyosarcoma was shown to possess a strong transactivation domain localized in its C-terminal FKHR-derived part (29,30). We found that the complete C-terminal part of FKHR was also required for its effect on the α2-M promoter activation (Fig. 4E, F). FKHR was recently shown to interact in HepG2 cells with the coactivator p300/CREB-binding protein (CBP), which is also essential for its transcriptional activity (31). STAT3 is also capable of recruiting p300/CBP, but this interaction and the level of transactivation are relatively weak in comparison with other STATs like STAT2 (32). It is widely accepted, that activating proteins like the STATs most often do not act alone, but in combination with other site-specific or more general DNA binding proteins as well as with coactivators of transcription (33,34). Therefore, the transcriptional function of STAT3 might be further enhanced by FKHR acting as an accessory factor which directs coactivator complexes to STAT3-sites in the promoter.

FKHR and STAT3 colocalize in the nuclei of HepG2 cells

In order to estimate if both proteins may interact in vivo, we assessed the localization of endogenous STAT3 and FKHR in HepG2 cells. We found by indirect immunofluorescence and confocal microscopy that in unstimulated cells the localization of FKHR (Fig. 5, right panels) was predominantly nuclear, whereas STAT3 (Fig. 5, left panels) was distributed equally in the nuclear and cytoplasmic regions. Noteworthy, although STAT3 was present in the nuclei of untreated HepG2 cells, it did not bind DNA
as measured in gelshift experiments (data not shown). Consistent with our previous observations, IL-6 stimulation did not affect FKHR localization but induced STAT3 migration to the nucleus, so that both factors accumulated in the nuclear regions of IL-6-treated HepG2 cells. In contrast, insulin treatment with or without IL-6 costimulation led to partial nuclear exclusion of FKHR. However, this effect was significantly weaker in HepG2 cells than in HeLa cells used as positive controls ((27), data not shown). Taken together, these results suggest that FKHR might associate with STAT3 in the nuclei of IL-6 stimulated HepG2 cells.

**Physical association of FKHR and STAT3**

Since FKHR specifically contributes to the activation of STAT3-dependent promoters, we investigated whether this effect reflects a physical interaction between these two proteins. Western blot analysis indicated that FKHR was expressed in HepG2 cells at a low level and could be visualized only in highly concentrated total cellular lysates (compare Fig. 3C and Fig. 3D). In contrast, FKHR was easily detectable in immunoprecipitates obtained with anti-STAT3 antibodies from total cell lysates of HepG2 cells (Fig. 6A), indicating the association of both proteins. The reverse immunoprecipitation experiments performed using antiserum against FKHR yielded similar results and led to coprecipitation of STAT3 from total cellular lysates confirming the previous results (Fig. 6A). To further consolidate the specificity of the interaction between FKHR and STAT3, we carried out several additional control immunoprecipitations. As shown in Fig. 6B, FKHR did not associate with STAT5 or other unrelated signaling molecules like SMAD2/3 or Erk2. This allows us to conclude
that the functional interaction of FKHR and STAT3 reflects a specific binding of both proteins. Although STAT3 and FKHR were found to be associated also in total cell lysates from untreated cells, stimulation with IL-6 strongly enhanced the binding of both factors in the nuclear fraction of HepG2 cells (Fig. 6C). Furthermore, in consistence with our results on insulin-mediated inhibition of α2-M gene expression, costimulation with insulin abrogated the IL-6-induced interaction between STAT3 and FKHR in the nucleus. Therefore, the negative regulation of IL-6-dependent gene expression by the activation of the PI3K/Akt pathway appears to result from the loss of cooperation between STAT3 and its transcriptional partner FKHR and depends on the subcellular localization of both proteins.
Discussion

*FKHR interacts with STAT3 and augments IL-6-dependent gene expression*

The major finding of the present study is that FKHR (FOXO1a), a member of the forkhead family of transcription factors can augment IL-6-dependent transcriptional activity by interacting with STAT3. This conclusion is based on three lines of evidence. First, FKHR expression enhanced the IL-6-dependent activation of the α2-macroglobulin promoter in HepG2 cells and showed a synergistic action together with STAT3. Second, we observed the physical association of both factors in nuclear extracts of HepG2 cells, which could be further enhanced by IL-6 stimulation and abrogated in the presence of insulin. Third, STAT3 showed similar to FKHR a nuclear distribution in IL-6-treated HepG2 cells.

Our results suggest that FKHR can act as a coactivator in STAT3-mediated transcriptional activation of acute phase protein genes. Since FKHR did not significantly induce basal activity of the α2-M promoter and its positive influence on gene expression was dependent on the presence of activated STAT3, we conclude that FKHR indirectly augments IL-6-induced transcriptional activation. To our knowledge this is the first report demonstrating the cooperation between both factors. However, it is widely accepted, that activating proteins like the STATs most often do not act alone, but in combination with other site-specific or more general DNA binding proteins as well as with coactivators of transcription (33,34). Examples of such a cooperativity include Stat1 and Sp1 (35), Stat5 and the glucocorticoid receptor (36) as well as Stat3 and c-Jun (37). The cooperation between Stat3 and c-Jun has been well documented for a number of different genes (34) but the initial observation concerned their interaction on the α2-M
promoter (37). Therefore, it would be of interest to assess if c-Jun could also participate in the interaction between STAT3 and FKHR. A recent report (38) documented that STAT3 can act in concert with hepatocyte-nuclear factor 1 (HNF-1) to enhance the HNF-1-mediated transactivation of hepatic gene expression in HepG2 cells and murine livers. It seems conceivable that this finding represents a more general mechanism whereby tissue-specific and inducible transcription factors cooperate in response to external signals. The results of our study suggest a different scenario, where FKHR transcription factors, closely related to the liver-specific HNF-3 (39) support the STAT3-dependent gene expression.

FKHR was originally identified in human rhabdomyosarcomas as a fusion protein composed of the transactivation domain of FKHR combined with the intact DNA binding domain of the transcription factor PAX3 (40). The C-terminal half of FKHR turned out to be very potent in transcriptional activation even though PAX3-FKHR proteins showed impaired DNA-binding (41). Our results indicate that the C-terminal part of FKHR was also required for its effect on the α2-M promoter activation, but in this case already the lack of already the 16 last amino acid residues significantly inhibited the coactivating function of FKHR. This finding implies that the complete C-terminus of FKHR is required for an efficient cooperation with STAT3.

Plausible mechanisms of the costimulatory action of FKHR on STAT3-mediated gene expression can include: (1) enhanced STAT3 activation, (2) facilitated nuclear migration of STAT3, and (3) recruitment of additional coactivator proteins to the STAT3 transcriptional complex. Enhanced STAT3 activation does not seem likely, since gelshift experiments did not reveal an effect of FKHR on DNA-binding activity of STAT3 (data
not shown). Our immunofluorescence results do not support the second possibility of facilitated translocation of STAT3 to the nucleus in the presence of FKHR. We did not observe any effect of insulin on IL-6-induced nuclear STAT3 staining. Another possibility could be that FKHR augments IL-6-mediated signaling by recruiting additional coactivator molecules to the STAT3-containing transcriptosome. In HepG2 cells FKHR has recently been shown to interact with the coactivator p300/CREB-binding protein (CBP), of the constitutive transcription machinery (31). Interestingly, STAT3 has also been found to associate with p300/CBP via its carboxy-terminus. This interaction as well as the level of transactivation are relatively weak compared to other STATs such as STAT2 (32). Therefore, the transcriptional function of STAT3 might be reinforced by FKHR acting as an accessory factor directing coactivator complexes to STAT3 binding sites in the promoter of respective target genes.

**Activation of PDK1/Akt signaling modulates IL-6-mediated gene expression**

The present results suggest a novel mechanism of the modulation of IL-6-dependent gene expression by the PI3K/Akt signaling pathway. We have demonstrated that downregulation of Akt activity by a kinase-defective mutant of PDK1 and by constitutive activation of Akt induce opposite effects on the IL-6-responsive α2-M promoter in human HepG2 hepatoma cells. Moreover, these effects could be associated with the expression level and the phosphorylation status of FKHR transcription factors. FKHR has been reported to be regulated by insulin in several cell lines of hepatic origin including SV40-transformed murine hepatocytes (22), rat hepatomas (42) as well as human HepG2 hepatoma cells (43). Recently, also IL-6 has been shown to activate Akt
in a significant proportion of multiple myeloma cell lines (26) as well as in human Hep3B hepatoma cells (25,44). In both cell types activation of the PI 3-kinase/Akt pathway was suggested to play a role in IL-6-dependent protection against apoptosis. The activation of Akt signaling should result in the direct phosphorylation of three regulatory sites (T24, S256 and S319) in FKHR and subsequent transcriptional inactivation and relocation to the cytoplasm (10,23,24). However, it is not known if the reported transient activation of Akt in multiple myeloma and Hep3B hepatoma cells is sufficient for the permanent exclusion of FKHR from nuclei of treated cells. We found that IL-6 neither induced kinase activity of Akt nor did it stimulate its phosphorylation on the critical regulatory residues T308 and S473 (7). Correspondingly, phosphorylation of FKHR on T24 and S256 was not increased during IL-6 treatment of HepG2 cells for up to 16 hours (Fig. 3C and data not shown). Our initial observation that expression of kd-PDK1 (Fig. 2) and treatment with wortmannin (Fig. 1B) can enhance the IL-6-induced α2-M promoter activity, may result from an inhibition of the basal, IL-6 independent Akt activity. These results were further corroborated by immunofluorescence data showing no change in nuclear localization of endogenous FKHR as well as of overexpressed GFP-FKHR upon stimulation of HepG2 cells with IL-6 (Fig. 4 and data not shown). Therefore, we conclude that FKHR during IL-6 treatment remains active and may participate in IL-6-induced gene expression.

Phosphorylation of FKHR by Akt on T24, S256 and S319 was reported to attenuate its nuclear import possibly by binding of FKHR to 14-3-3 proteins (9,12,27). Several groups demonstrated the resistance of FKHR threonine-serine mutants to both Akt-mediated phosphorylation and PI 3-kinase stimulated nuclear export (23,24). This
could well explain the enhanced potency of the mutated FKHR variants T24A, T24A/S256A and T24A/S256A/S319A to augment IL-6-induced α2-M promoter activity as a result of nuclear retention of these proteins. Our observations indicate that the single mutation of T24 is sufficient for maximal transcriptional stimulation. They are in agreement with previous reports on the crucial role for T24, a residue which lies within a 14-3-3 consensus motif, involved in the interaction with 14-3-3 proteins (9,45).

Conclusions

In the present study, we have investigated the potential crosstalk between the PI 3-kinase/Akt and Jak/STAT3 signaling pathways at the level of transcriptional regulation. Our results reveal a novel function of FKHR, suggesting that it acts as a specific coactivator of STAT3. This functional interaction correlates with the physical association of both proteins and their colocalization in the nuclear regions of human HepG2 hepatoma cells. Taken together, our data demonstrate that activation of the PI 3-kinase/Akt pathway can modulate IL-6-signaling by targeting and inactivating FKHR, what can apply to APP genes like the α2-macroglobulin gene which are negatively regulated by insulin and growth factors.
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Figure Legends

Fig. 1. Insulin inhibits the IL-6-induced α2-M gene expression. (A) Northern blot analysis of HepG2-cells treated over night with IL-6 (200U/ml), insulin (1 µM) and wortmannin (0.1 µM) in serum free medium. Ten µg of RNA were separated by agarose gel electrophoresis and blotted onto nylon membranes. α2-M mRNA was detected with a 32P-labeled specific cDNA probe. An ethidium bromide staining of the RNA is shown as a loading control. (B) α2-M promoter activity in HepG2 cells treated as above with IL-6 (200U/ml), insulin (1 µM) and wortmannin (0.1 µM). Shown are the means +/- s.d. of a representative experiment performed in triplicate.

Fig. 2. Activation of PDK1/Akt signaling inhibits the IL-6-mediated activation of the α2-M promoter through a FKHR-dependent mechanism. (A) Effect of constitutively active Akt (ca-Akt) and kinase-dead PDK1 (kd-PDK1) on the α2-M promoter activity in HepG2 cells. Cells were transiently transfected with plasmids encoding ca-Akt or kd-PDK1 and incubated with IL-6 (black bars) or left untreated (open bars). The IL-6-induced promoter activity of the mock transfected controls was set as 100% in all experiments. (B) Dose-dependent effect of expressed FKHR-cDNA on the α2-M promoter activity in HepG2 cells. (C) Effect of PDK1 (left panel) and Akt (right panel) on the α2-M promoter activity in HepG2 cells overexpressing FKHR. Cells were transfected with combinations of expression plasmids encoding FKHR and PDK1 (wild-type or kinase-dead) or Akt (wild-type or constitutively active) and with reporter plasmids as before. (D) The effect of
FKHR T24A-, S256A- and S319A mutations on the α2-M promoter activity. HepG2 cells were transfected with reporter plasmids as above together with the expression vectors encoding wild-type or point-mutated variants of GFP-tagged FKHR (FKHR-T24A, FKHR-T24A/S256A, FKHR-T24A/S256A/S319A) or with an empty vector and the relative luciferase activity was determined (left panel). The right panel shows a Western blot with total cell lysates (10 µg/lane) probed with anti-FKHR antibody to control the expression level of the different constructs. The upper arrow indicates a non-specific band, whereas the lower arrow labels a band at 80 kDa presumably corresponding to endogenous FKHR.

Fig. 3. IL-6 does not induce Akt activity and FKHR phosphorylation in HepG2 cells. (A) Stimulation of HepG2 cells with IL-6 does not affect Akt activity. Cells were kept in serum free medium and stimulated for 15 minutes with either insulin (1 µM), IL-6 (200 U/ml) or both in combination. The data show the quantitation of incorporated $^{32}$P into the GSK-3 substrate peptide of an in vitro kinase assay. Shown are the means +/- s.d. of one representative experiment performed in triplicate with the basal Akt activity set as 100%. (B-D) Determination of the phosphorylation state of critical regulatory residues in Akt, FKHR and STAT3 following stimulation with either insulin, IL-6 or both in combination as before. 100 µg of total cell lysate were subjected to SDS-PAGE, blotted onto PVDF membranes and probed with specific antibodies. (B) Phosphorylation of Akt on residues T308 and S473. (C) Phosphorylation of FKHR on residues T24 and S256, (D) Phosphorylation of STAT3 on Y705. Single representative blots are shown.
**Fig. 4.** Subcellular localization of STAT3 (left panel) and FKHR (right panel) in response to IL-6 and insulin in HepG2 cells. Cells were serum-starved for 24 hours and incubated in the presence of LY294002 for the last hour. After starvation cells were washed extensively and stimulated for 10 minutes with either insulin (1µM), IL-6 (200 U/ml) or both in combination, fixed and analysed by confocal laser scanning microscopy. The green fluorescence corresponds to anti-STAT3- and the red fluorescence to anti-FKHR-staining. A single representative confocal section is shown, the scale bar is 10 µm.

**Fig. 5.** Functional interaction between FKHR and STAT3 in transcriptional regulation. (A,B) FKHR contributes specifically to the function of STAT3. (A) Synergistic effect of FKHR and STAT3 on the IL-6-induced α2-M promoter activity. HepG2 cells were transiently transfected as described in Fig. 1 together with FKHR and/or wild-type STAT3 expression vectors. (B) FKHR has no effect on the STAT5-mediated activation of the β-casein promoter. HepG2 cells were transiently transfected with a vector encoding a chimeric receptor (Eg-YLVLD) specifically activating STAT5 together with vectors encoding the β-casein promoter, FKHR and/or STAT5A. Cells were treated with EPO (black bars) or left in serum-free medium (open bars). (C,D) The effect of FKHR on the transcriptional regulation depends on the presence of functional STAT3. (C) The effect of dominant negative STAT3 mutants on FKHR-induced α2-M promoter activity. HepG2 cells were transfected with reporter plasmids together with FKHR alone or in combination with dominant negative STAT3D or STAT3F. (D) FKHR enhances the IL-6-induction of STAT3-dependent SIE-tk-luc reporter gene construct. (E, F) The complete C-terminus of FKHR is crucial for its function as a coactivator of the α2-M promoter. (E)
Schematic representation of the truncated variants of FKHR; DB – DNA binding domain, NES – nuclear export sequence. (F) HepG2 cells were transfected with vectors encoding the different FKHR mutants, the α2-M promoter reporter construct, and incubated with IL-6 (black bars) or left untreated (open bars).

**Fig. 6.** Physical association of FKHR and STAT3. (A) Coimmunoprecipitation of FKHR and STAT3. HepG2 cells were serum-starved for 24 hours and incubated in the presence of LY294002 (100 µM) for the last hour. Thereafter, cells were washed with medium and stimulated for 10 minutes with IL-6 (200 U/ml), lysed and incubated with polyclonal anti-STAT3 (left panel) or polyclonal anti-FKHR antibodies (right panel). The precipitates together with 30 µg of total cell lysates (TCL) were separated by SDS-PAGE, blotted onto PVDF membranes and detected with anti-FKHR or anti-STAT3 (upper panels). After stripping the blots were redeveloped with the antibodies used for the immunoprecipitations to control loading (lower panels). (B) FKHR does not interact with signaling molecules other than STAT3. Starved HepG2 cells were stimulated with IFNγ (1000 U/ml), TGFβ (10 U/ml) or 10% FCS, lysed and incubated with anti-STAT5, anti-Smad2/3 or anti-Erk2 antibodies, respectively. The precipitates together with a fraction of total cell lysates collected before the immunoprecipitation were separated by SDS-PAGE and analyzed as described above. Upper panel – sections of the same blot developed with anti-FKHR serum. Lower panel – control detection of the stripped membranes with the respective antibodies used for the immunoprecipitations. (C) Treatment with IL-6 enhances the association of FKHR and STAT3 in nuclear fractions of HepG2 cells. Cells were serum-starved and pretreated with LY294002 as previously
and then stimulated with IL-6 (200 U/ml) or IL-6 together with insulin (1 µM). After fractionation, nuclear extracts were incubated with polyclonal anti-STAT3 antibodies or control normal rabbit serum (NRS). The precipitates were separated by SDS-PAGE, blotted onto PVDF membranes and detected with anti-FKHR or anti-STAT3 (upper panel). After stripping the blots were redeveloped with the antibodies used for the immunoprecipitations to control loading (lower panel).
Fig. 1
Fig. 2
Fig. 3

A

Akt activity (% of control)

- - + + insulin IL-6

B

- Akt (P-Thr 308)
- Akt (P-Ser 473)
- Akt

- - + + insulin IL-6

C

- FKHR (P-Thr 24)
- FKHR (P-Ser 256)
- FKHR

- - + + insulin IL-6

D

- Stat3 (P-Tyr 705)
- Stat3

- - + + insulin IL-6
Fig. 4

A

Control

+ Insulin

+ IL-6

+ Insulin + IL-6

STAT3  FKHR
Fig. 5

A

B

C

D

E

F

Fig. 5
Akt modulates STAT3-mediated gene expression through a FKHR (FOXO1a)-dependent mechanism
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