Insulin selectively activates STAT5b, but not STAT5a, via a JAK2-independent signalling pathway in Kym-1 rhabdomyosarcoma cells

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Abstract The STAT multigene family of transcriptional regulators conveys signals from several cytokines and growth factors upon phosphorylation by janus kinases (JAK). Activation of STAT5 is typically mediated by JAK2, but more recent data indicate a direct activation by the insulin receptor kinase. STAT5 exists in two closely homologous isoforms, STAT5a and b. We here describe the selective tyrosine phosphorylation of STAT5b in Kym-1 cells in response to insulin. Blocking insulin signalling by HNMPA-$(AM)_3$, an insulin receptor kinase inhibitor, resulted in the loss of insulin-induced STAT5b tyrosine phosphorylation, whereas the inhibition of JAK2 by the JAK selective inhibitor tyrphostin AG490 had no effect. By contrast, in the same cells, IFNγ-induced STAT5b activation was JAK2-dependent, indicating that this signal pathway is functional in Kym-1 cells. We conclude from this rhabdomyosarcoma model that STAT5b, but not STAT5a is a direct target of the insulin receptor kinase.

Key words: STAT5; Insulin; JAK2; Rhabdomyosarcoma

1. Introduction

Signal transducer and activator of transcription 5 (STAT5) belongs to the family of STAT effector molecules [1,2] downstream of cellular receptors leading to the induction of target genes upon activation by interferons, a wide range of cytokines including prolactin (PRL), erythropoietin, thrombopoietin, granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-2, IL-3, IL-5, IL-7, IL-9 and growth hormone (GH) [3–8]. STAT transcription factors play important roles in many cell types and mediate several responses, e.g. proliferation, cell cycle control, cell differentiation [7,9–12] and prevention of apoptosis [13,14]. The physiologic function of STAT5 is not yet fully elucidated [3,4]. Evidence for a multifunctional role stems from the observation that STAT5 is capable to act in concert with other transcription factors too, e.g. with the glucocorticoid receptor [9].

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Abbreviations: AP, alkaline phosphatase; GH, growth hormone; GM-CSF, granulocyte macrophage colony stimulating factor; IFN, interferon; IL, interleukin; IR, insulin receptor; IRS, insulin receptor substrate; JAK, janus kinase; PRL, prolactin; SH, src homology; STAT, signal transducer and activator of transcription; w/o, without

Recently, beside insulin receptor substrate (IRS), STAT5 has been described as an additional, direct substrate of the insulin receptor [15,16], and has been implicated to participate in the regulation of the transcriptional program of insulin responsive genes in myotubes [17]. STAT5 interacts with IRβ in the two hybrid system, gets tyrosine phosphorylated by purified IR kinase domain in vitro [15], and a direct interaction between these two signalling proteins was demonstrated by docking of STAT5 via src homology (SH)2-domains to Y960 of the insulin receptor β-chain [16]. Two STAT5 homologues, STAT5a and STAT5b, which share 96% amino acid sequence identity are known so far [18,19]. Though both STAT5 isoforms are simultaneously activated by a number of cytokines, it is not clarified yet, if one or both STAT5 isoforms are activated by insulin, and if activation via JAK2 plays a role in insulin-mediated STAT activation. While cytokine i.e. GH and PRL-induced activation of STAT5 is mediated by the janus kinase (JAK)2, [3,20–22], in insulin signalling, JAK2-dependent and independent STAT5 activation was reported [3,23–26]. We here demonstrate in Kym-1 rhabdomyosarcoma cells that insulin-induced STAT activation is restricted to the STAT5b isoform. Furthermore this activation is, in contrast to interferon (IFN)γ-induced STAT5b activation in the same cells, independent of JAK2 activation and a direct consequence of binding to and phosphorylation by IRβ kinase.

2. Materials and methods

2.1. Cell culture and transfection

The human rhabdomyosarcoma cell line Kym-1 was cultured in Clicks RPMI 1640 medium supplemented with 10% FCS. Kym-1 cells were grown to 80% confluence in tissue culture plates. Before treatment with IFNγ or insulin, Kym-1 cells were serum starved (clicks RPMI 1640, without (w/o) FCS) for 2 h and stimulated as indicated for various concentrations and times. Rat1 fibroblasts overexpressing the human insulin receptor (Rat1 HIR cl5) were a kind gift of A. Ullrich, Max Planck Institut für Biochemie, 82152 Martinsried, Germany. Rat1 HIR cl5 cells were cultured in DMEM (4.5 g/l glucose) medium supplemented with 10% FCS. Transfection of these cells was performed by lipofection (Pfx-6), using the Pfx-kit (Invitrogen) under standard conditions.

2.2. Cytokines, antibodies and reagents

Insulin from bovine pancreas was purchased from Sigma, and IFNγ was a kind gift of B. Otto, Fraunhofer Institut für Grenzflächen und Bioverfahrenstechnik, Hannover, Germany. Antibodies against insulin receptor β (C-19), STAT5a (L-20, selective for STAT5a), STAT5b (G-2, selective for STAT5b), STAT5 (C-17) and JAK2 (HR-758) were from Santa Cruz. The phosphotyrosine antibody 4G10 was from Upstate. All inhibitors of proteases and phosphatases were from Biomol. Secondary alkaline phosphatase (AP)-linked antibodies, goat anti-

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mouse IgG and IgM (H+L), and goat anti-rabbit IgG (H+L) were from Dianova. The STAT5b expression vector (pXM-mSTAT5b) was a kind gift of B. Groner, Georg-Speyer-Haus, Frankfurt/M, Germany [9]. The insulin receptor inhibitor HNMPA-$(AM)_3$ (hydroxy-2-naphtalenylmethylphosphonic acid Tris acetoxymethylester) was from Calbiochem, and the JAK2 inhibitor tyrphostin AG490 (tyrphostin B42) was from Alexis Corporation.

2.3. Cell lysis and immunoprecipitation

After stimulation the cells were washed three times with PBS (4°C) and scraped in 750 μl ice-cold lysisbuffer (50 mM Tris–HCl pH 7.4, 1% Triton X-100 (v/v), 150 mM NaCl, 5 mM EDTA pH 7.4, 1 mM NaF, 1 mM NaPP, 2 mM sodium orthovanadate, 1 mM sodium molybdate, 100 nM okadaic acid, 100 nM calyculin A, 1 mM *p*-nitrophenyl-phosphate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). After 1 h cell lysis, the lysates were centrifuged ($10\,000\times g$, 15 min, 4°C) and immunoprecipitation (1 μg/ml antibody or antiserum per sample) was performed as described [17]. Protein contents in lysates were determined with the Bio-Rad Protein Assay using BSA as a standard.

2.4. JAK2 kinase assay

The cells were lysed as described above. After immunoprecipitation of JAK2, the Protein-A pellets were washed thrice (50 mM Tris–HCl pH 7.4, 150 mM NaCl) and re-suspended in 30 μl of kinase buffer (20 mM Tris–HCl pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$). After addition of 4 μCi γ-[^{32}P]ATP (Amersham) in 10 μl kinase buffer, the samples were incubated for 15 min at 37°C. The reaction was stopped by addition of 2×reducing Laemmli buffer, the samples were separated on 7.5% SDS–PAGE, and blotted on nitrocellulose. Quantitative analysis of the kinase assays was performed with a phosphoimager (Molecular Dynamics).

3. Results

3.1. Functionality of the insulin receptor signalling, and direct association of STAT5 and IRβ

To demonstrate the functionality of the insulin receptor cascade, the tyrosine phosphorylations of the insulin receptor β-chain (IRβ), and one of its substrates, IRS-1, were analyzed. Treatment of Kym-1 cells with insulin for 5 min resulted in an induction of IRβ tyrosine phosphorylation (Fig. 1a, left panel) and increase in IRS-1 tyrosine phosphorylation (Fig. 1a, right panel), whereas insulin had no effect on protein levels. Of note, IRS-1 shows in these cells a low, but clearly discernible basal phosphorylation. As no basal IRβ activity was detectable, this could reflect activation of IRS by other endogenous signals constitutively present in these transformed cells. Direct association between the insulin receptor β-chain and STAT5 could be verified by co-immunoprecipitation analysis using STAT5 antibodies for precipitation and IRβ antibodies for detection of the Western blot (1) upon transient transfection of STAT5b in Rat1 HIR cl5 cells, (Fig. 1b, right lane) and (2) upon immunoprecipitation of endogenous STAT5 in Kym-1 cells (Fig. 1b, left lane).

3.2. Insulin mediates STAT5b, but not STAT5a tyrosine phosphorylation

STAT5 has been recently identified as a novel substrate of the insulin receptor and a role in the physiological regulation of insulin responses has been proposed [15]. Both STAT5 isoforms, STAT5a (94 kDa) and STAT5b (92 kDa) are expressed in Kym-1 rhabdomyosarcoma cells, and could be distinguished by specific antibodies. Interestingly, only STAT5b, but not STAT5a was tyrosine phosphorylated after insulin treatment, indicating an exclusive role of STAT5b in insulin signalling in these cells (Fig. 2a). To demonstrate that insulin-induced STAT5b phosphorylation is linked to the insulin receptor kinase activity, tyrosine phosphorylation of STAT5b in presence of the insulin receptor kinase inhibitor HNMPA-$(AM)_3$ [26] was measured; a complete loss of STAT5b activation was noted under these conditions (Fig. 2b). STAT5b activation by insulin occurs in a time- and dose-dependent manner. Tyrosine phosphorylation of STAT5b is readily detectable 10 min upon insulin stimulation (Fig. 2c), appears at low insulin concentrations (Fig. 2d), and is comparable in magnitude to IFNγ-induced STAT5b phosphorylation in the same cells (Fig. 2c).

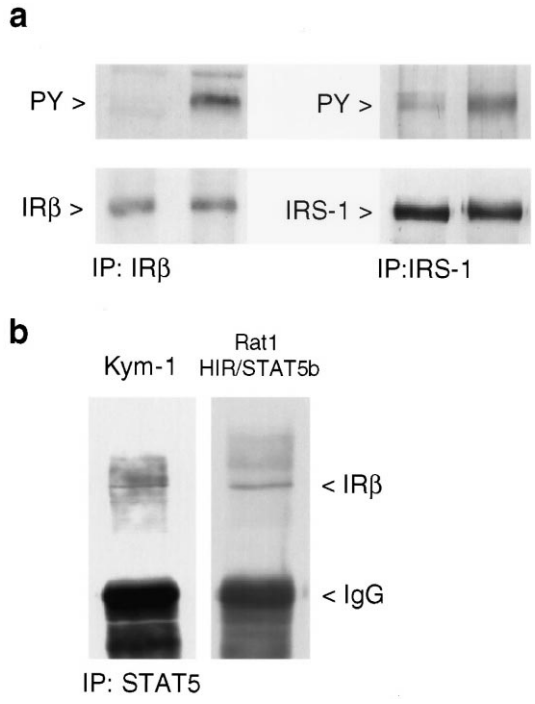

Fig. 1. a: Tyrosine phosphorylation of IRβ and IRS-1 in response to insulin. 5×10^6 Kym-1 cells were stimulated with insulin (100 nM) for 5 min. Cells were lysed in lysisbuffer, and IRβ or IRS-1 were immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose, and stained with PY specific (4G10) or IRβ/IRS-1 specific antibodies. b: Co-immunoprecipitation of IRβ with STAT5b. 5×10^6 Rat1 HIR cl5 cells, transiently transfected with STAT5b (right panel), or 5×10^6 Kym-1 cells (left panel), were lysed in lysisbuffer and STAT5 (C-17) was immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose and the blot was stained with antibodies against IRβ.

3.3. The activation of STAT5b by insulin is independent of JAK2

In Kym-1 cells JAK2 could be reciprocally co-immunoprecipitated with IRS-1 in both, untreated and insulin-stimulated Kym-1 cells (Fig. 3a), whereas JAK2 was not detectable in immunoprecipitates of IRβ (data not shown). A constitutive association with the IRS-1 signalling complex suggests a potential involvement of JAK2 in insulin-induced STAT5 activation. However, JAK2 kinase, which has been shown to be an upstream activator of STAT5b in IFNγ-induced STAT5 activation [3,27] appears not to be involved in insulin-induced STAT5b activation, which is evident from the following experiments: Upon insulin treatment, JAK2 was not tyrosine phosphorylated, in comparison to IFNγ-treated cells, which

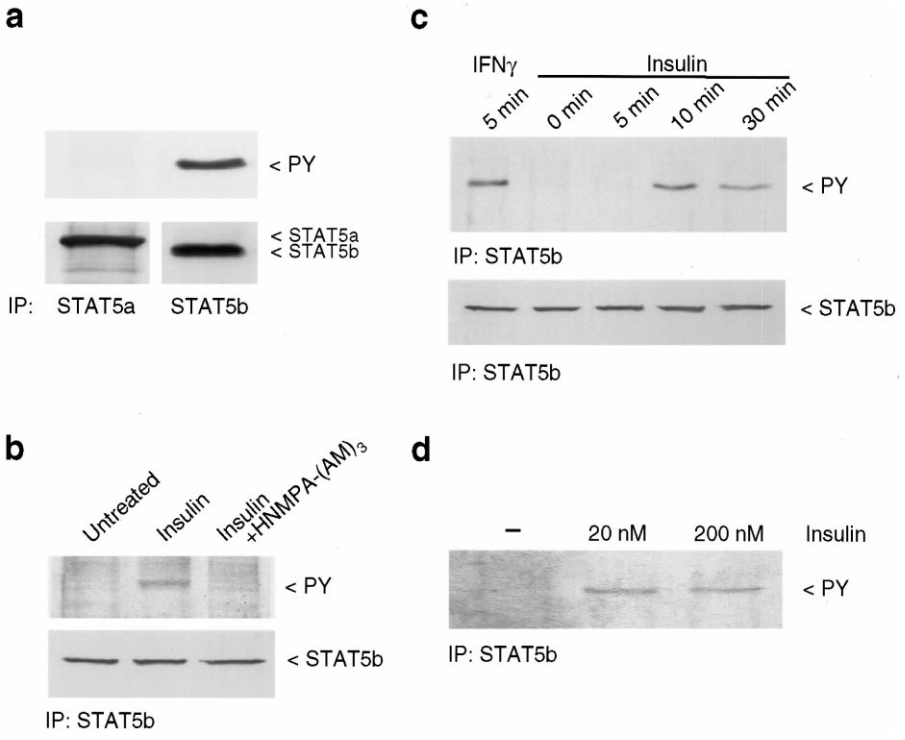

Fig. 2. a: STAT5b, but not STAT5a is phosphorylated after insulin treatment in Kym-1 cells. 5×10^6 Kym-1 cells were stimulated with insulin (100 nM) for 10 min. Cells were lysed in lysisbuffer and STAT5a (L-20, selective for STAT5a) or STAT5b (G-2, selective for STAT5b) were immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose and stained with PY (4G10), STAT5a, or STAT5b specific antibodies. b: Inhibition of STAT5b activation with HNMPA-$(AM)_3$. 5×10^6 Kym-1 cells were serum starved for 2 h in presence of 10 μM HNMPA-$(AM)_3$ and stimulated with insulin (100 nM) for 10 min. Cells were lysed in lysisbuffer and STAT5b (G-2) was immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose and stained with antibodies against PY (4G10) or STAT5b (G-2). c,d: Time and dose dependence of insulin-induced tyrosine phosphorylation of STAT5b. 5×10^6 Kym-1 cells were stimulated with insulin (10 nM) for the indicated times (50 ng/ml IFNγ served as positive control), or stimulated with indicated insulin concentrations for 10 min. Cells were lysed in lysisbuffer and STAT5b (G-2) was immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose and stained with PY (4G10) or STAT5b (G-2) specific antibodies.

served as a positive control (Fig. 3b). We further demonstrate in JAK2 kinase assays (Fig. 3c) that JAK2 was not activated in response to insulin, whereas it was activated in response to IFNγ. Pre-incubation of the cells with the specific JAK2 inhibitor tyrphostin AG490 [28] had no effect on insulin-induced STAT5b phosphorylation, whereas under the same conditions it blocked IFNγ-mediated STAT5b phosphorylation. Further, the IR kinase inhibitor HNMPA-$(AM)_3$ fully abrogated insulin-mediated STAT5b activation (Fig. 2b). Together, these results show that in Kym-1 cells STAT5b is a direct substrate of the insulin receptor kinase and that insulin and IFNγ mediate STAT5b activation by different mechanisms.

4. Discussion

Several signalling pathways leading to STAT activation are mediated via JAKs. IFNγ and GH receptors initiate STAT5 signalling by activation of JAK2 ([3,22,27], Fig. 3b,c), which in turn phosphorylates and activates STAT5 [27]. Recently it was shown that STAT5b can be activated and translocated to the nucleus in a JAK2-independent (src kinase-dependent) way [29], and it was reasoned that JAK2 and STAT phosphorylation events can be mediated by independent pathways [30]. Since in some tissues JAK2 was described to be rapidly activated after insulin infusion [24], and STAT5 was reported to be activated upon re-feeding experiments in skeletal muscle in vivo [15] as well as in myotubes in vitro [17], our aim was to investigate the insulin-induced activation mechanism of STAT5 in the rhabdomyosarcoma cell line Kym-1 with respect to involvement of JAK2 and potential STAT isoform selectivity. We here clearly demonstrate for this cell line, using insulin concentrations, which do not cross-activate the IGF-1 receptor (data not shown) that JAK2 is not involved in the insulin-induced activation of STAT5, as evident from lack of JAK2 kinase activity in insulin-treated cells (Fig. 3b,c) and abrogation of STAT5 phosphorylation only by an inhibitor of IRβ (Fig. 2b), but not by a JAK2 specific kinase inhibitor (Fig. 3d). Similarly, we have recently shown insulin-induced STAT5 activation in a murine myoblast line (pmi28) upon differentiation into multinucleated myotubes [17]. In these myotubes, like in Kym-1 cells, insulin failed to induce JAK2 kinase activity (P.S., unpublished data). In accordance with the view of differential signal pathway usage for STAT5 activation is our finding with Kym-1 cells that in this cell line IFNγ-induced STAT5 activation is JAK2-dependent (Figs. 2c,3b,d). Together, the data presented here confirm results from cell-free kinase assays with STAT5 proteins, demonstrating direct phosphorylation by the insulin receptor kinase [15]. Further, our data extend these findings by defining an isoform-selective activation, namely STAT5b. The direct interaction [16], and co-immunoprecipitation of IRβ and STAT5b from intact cells (Fig. 1b) further argue for a direct activation of STAT5 by the insulin receptor in vivo.

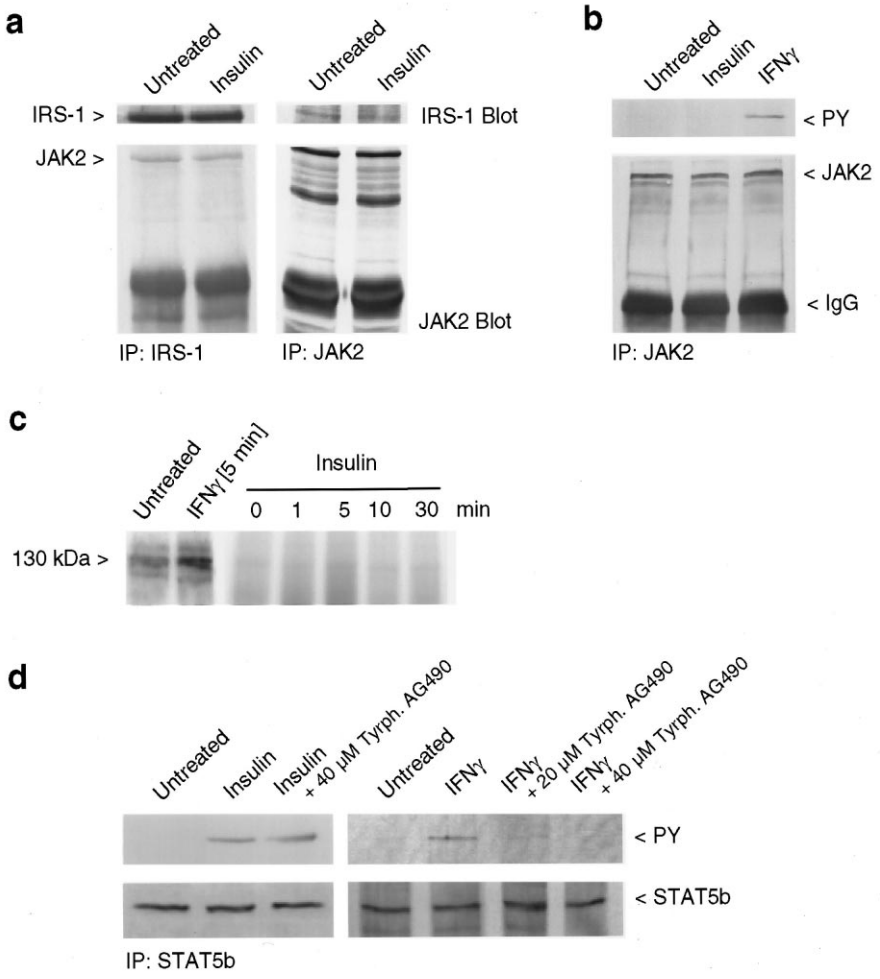

Fig. 3. JAK2 is co-immunoprecipitated with IRS-1, but not activated by insulin treatment. a: Co-immunoprecipitation of IRS-1 and JAK2. 15×10^6 Kym-1 cells were serum starved for 2 h and left untreated, or stimulated with insulin (100 nM) for 10 min. Cells were lysed in lysisbuffer and IRS-1 (left panel) or JAK2 (right panel) were immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose, and stained with antibodies against IRS-1 and JAK2. b: 5×10^6 Kym-1 cells were serum starved for 2 h and stimulated with insulin (50 nM) or IFNγ (50 ng/ml, positive control) for 5 min. Cells were lysed in lysisbuffer and JAK2 was immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose, and stained with antibodies against PY (4G10), or JAK2. c: 15×10^6 Kym-1 cells were serum starved for 2 h and stimulated with insulin (100 nM, right panel) for indicated times, or IFNγ (50 ng/ml, 5 min, left panel). Cells were lysed in lysisbuffer and JAK2 was immunoprecipitated. A JAK2 kinase assay was performed and the samples were separated on SDS–PAGE, transferred to nitrocellulose, and visualized by autoradiography d: STAT5b activation by insulin is independent of JAK2. 5×10^6 Kym-1 cells were serum starved for 2 h in presence of tyrphostin AG490 (as indicated) and stimulated with insulin (100 nM), or IFNγ (50 ng/ml) for 5 min. Cells were lysed in lysisbuffer and STAT5b (G-2) was immunoprecipitated. The samples were separated on SDS–PAGE, transferred to nitrocellulose and stained with antibodies against PY (4G10).

The JAK2 independence of insulin-induced STAT5 activation despite constitutive association of JAK2 with IRS-1 in Kym-1 cells (Fig. 3a) is of note and illustrates the complexity of this multifunctional signalling module. The function of JAK2 in the IRS-1 complex is, at present, not fully understood, but a role in insulin-independent phosphorylation of IRS-1 is evident from various studies. JAK1 or JAK2 display a different phosphorylation pattern of IRS-1 compared to the IRβ [31], and thus are likely to initiate a different spectrum of downstream pathways [20,31–33]. For example, using JAK2 deficient cell lines, or expressing a dominant negative mutant of JAK2, Yamauchi et al. showed that JAK2 is required in GH- and PRL-dependent IRS-1 and IRS-2 tyrosine phosphorylation [34]. JAK2 is also implicated in cell proliferation and the co-precipitation (Fig. 3a) with as well as the basal phosphorylation of IRS-1 in Kym-1 cells (Fig. 1a) could reflect a constitutive signalling towards mitogenic pathways or PI 3-kinase activation [34] via autocrine acting factors produced by the tumor cells such as GH or IGFs.

Recently, distinct functional roles for both STAT5 isoforms were described [3,35]. The phenotypes of mice with deleted STAT5a and STAT5b genes demonstrate some redundant but also individual functions for the two STAT proteins, and the loss of either one cannot be compensated by the other isoform [18,36–38]. A selective engagement of the two isoforms by distinct extracellular stimuli becomes increasingly apparent. Thus, it was shown that upon GM-CSF stimulation of blood monocytes a selective, JAK2-dependent activation of STAT5a occurs [35]. The data presented here with Kym-1 rhabdomyosarcoma cells, showing that insulin selectively activates, in a JAK2-independent manner, STAT5b, but not STAT5a provides further experimental evidence for a differential functional role of these two closely related isoforms. In conclusion, our data show that in myosarcoma (this study)

and muscle cells [17] the insulin-induced activation of STAT5b is a direct IRβ-mediated event and suggest a specific function of the STAT5b isoform in insulin-regulated gene induction.

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