Insulin Stimulates the Serine Phosphorylation of the Signal Transducer and Activator of Transcription (STAT3) Isoform*

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Insulin stimulation of Chinese hamster ovary cells expressing the human insulin receptor and differentiated 3T3L1 adipocytes resulted in a time-dependent reduction in the SDS-polyacrylamide gel electrophoretic mobility of STAT3. The decreased STAT3 mobility initially occurred by 2 min and was quantitative by 5 min. In addition, the change in STAT3 mobility was concentration-dependent and was detectable at 0.3 nM insulin with maximal effect between 1 and 3 nM. Although both these cell types also expressed the STAT1α, STAT1β, STAT5, and STAT6 isoforms, only STAT3 was observed to undergo an insulin-dependent reduction in mobility. Immuno-precipitation of STAT1 and STAT3 from [32P]labeled cells demonstrated that only STAT3 was phosphorylated in response to insulin whereas phosphoamino acid analysis indicated that this phosphorylation event occurred exclusively on serine residues. Furthermore, treatment of cell extracts with alkaline phosphatase reversed the insulin-stimulated decrease in STAT3 mobility. Together, these data demonstrate that insulin is a specific activator of STAT3 serine phosphorylation without affecting the other STAT isoforms.

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1 The abbreviations used are: STAT, signal transducers and activators of transcription; SH2, Src homology 2; SH3, Src homology 3; CHO/IR, Chinese hamster ovary cells expressing the human insulin receptor; PVDF, polyvinylidene difluoride.

The STAT protein dimers then translocate into the nucleus and bind to specific DNA recognition sequences resulting in increased transcriptional activation of various effector genes (7, 11–16).

In addition to cytokine receptors, several growth factor tyrosine kinase receptors (epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor) as well as non-tyrosine kinase receptors (prolactin, growth hormone, and angiotensin II) also induce the tyrosine phosphorylation, dimerization, and transcriptional activation of the STAT proteins (12, 17–23). However, since insulin does not stimulate the JAK kinases or increase the tyrosine phosphorylation of the STAT proteins, it is generally accepted that insulin does not impinge upon this particular signal transduction pathway (18, 24, 25). Nevertheless, in contrast to tyrosine phosphorylation, recent studies have indicated that growth hormone, interferon α, and interleukin 6 can also stimulate the serine phosphorylation of the STAT proteins in a manner distinct from the JAK-mediated tyrosine phosphorylation (11, 26–28). Furthermore, both tyrosine and serine phosphorylation of STAT1 and STAT3 is required for maximal DNA transcriptional activity of reporter genes presumably through fostering STAT homodimerization (28, 29). Based upon these findings, we have re-evaluated the potential role of insulin on STAT phosphorylation. In this study, we demonstrate that insulin stimulation results in a rapid quantitative serine phosphorylation of STAT3 without any significant effect on STAT1α, STAT1β, STAT5, or STAT6 phosphorylation.

**EXPERIMENTAL PROCEDURES**

Materials—Monoclonal antibodies for Western blot analysis were purchased from Transduction Laboratories. Polyclonal STAT1 and STAT3 antibodies for immunoprecipitation and Protein G Plus agarose were purchased from Santa Cruz Biotechnology. [32P]Orthophosphate and enhanced chemiluminescence reagents were obtained from Amersham Corp. Diphosphorylation buffer was purchased from Boehringer Mannheim. All other reagents were purchased from Sigma.

Cell Culture—Chinese hamster ovary cells stably transfected with the human insulin receptor (CHO/IR) were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum as described previously (30). 3T3L1 pre-adipocytes were cultured in standard medium (Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 2 mM glutamine, and 10% calf serum). Adipocyte differentiation was induced 2 days postconfluence with differentiation medium (standard medium supplemented with 1.0 μg/ml insulin, 0.1 μg/ml dexamethasone, and 27.8 μg/ml isobutylmethylxanthine). Following 4 days in differentiation medium, the dexamethasone and isobutylmethylxanthine were removed by switching the cells to standard medium containing 1.0 μg/ml insulin. After an additional 4 days, the cells were changed back into standard medium and maintained for 1–7 days before use.

Insulin Stimulation—CHO/IR and differentiated 3T3L1 adipocytes were washed two times with phosphate-buffered saline, pH 7.4, and incubated for 3–4 h in standard medium in the absence of serum. The cells were then treated with various concentrations of insulin (0–100 nM) and times (0–60 min) as indicated in the individual figure legends. Insulin stimulation was terminated by two washes of ice-cold phosphate-buffered saline, pH 7.4, removal of excess liquid by aspiration, and addition of liquid nitrogen to the tissue culture plates. The snap-frozen cells were placed at −80 °C until harvested.

Whole Cell Detergent Lysates—CHO/IR and differentiated 3T3L1 adipocytes were extracted in ice-cold lysis buffer (50 mM HEPES, 1% Triton X-100, 2.5 mM EDTA, 100 mM NaF, 10 mM Na3VO4, pH 7.8) containing 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 1 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin A by rotation for 10 min at 4 °C. Insoluble material was separated from the soluble extract by...
Results and Discussion

Insulin Stimulation Results in a Reduction in STAT3 Electrophoretic Mobility—Previous studies have reported that although several growth factors can stimulate the tyrosine phosphorylation of STAT proteins (12, 17, 19–23), insulin does not induce STAT tyrosine phosphorylation or activation of JAKs (18, 24, 25). However, recent studies have also demonstrated that STAT1α and STAT3 are also serine-phosphorylated, which appears to enhance their transcriptional activity in concert with tyrosine phosphorylation (29). To evaluate the potential role for insulin in mediating the serine phosphorylation of STAT proteins, CHO/IR and 3T3L1 adipocytes were incubated with and without insulin, and cell extracts were subjected to STAT Western blotting (Fig. 1). Using STAT isoform-specific antibodies both cell types were found to express STAT1α, STAT1β, STAT3, STAT5, and STAT6. In contrast, we were unable to detect any significant levels of STAT2 or STAT4 (data not shown). The absence of STAT4 is consistent with the observation of its limited tissue distribution with expression limited to the testis, thymus, and spleen (31). However, the absence of STAT2 was somewhat surprising as it has been observed in several tissues and cultured cell lines (4).

Nevertheless, insulin stimulation had no effect on the apparent mobility of STAT1α, STAT1β, STAT5, and STAT6 (Fig. 1, A, C, and D). In contrast, insulin treatment for 5 min of either CHO/IR or 3T3L1 adipocytes resulted in a marked reduction of SDS-polyacrylamide gel electrophoretic mobility of STAT3 (Fig. 1B). This insulin-stimulated decrease in STAT3 SDS-polyacrylamide gel electrophoretic mobility was a typical characteristic of post-translational serine/threonine phosphorylation similar to that observed for a number of intracellular signaling proteins including STAT3 (32–34).

Insulin-Stimulated Reduction in STAT3 Mobility Is Time and Concentration-dependent—To determine whether this modification of STAT3 was an early or late event in insulin action, we next examined the time dependence of STAT3 gel shift in CHO/IR and 3T3L1 adipocytes (Fig. 2). In both cell types, insulin stimulation for 2 min resulted in a small but detectable reduction in STAT3 mobility, which was fully shifted by 5 min (Fig. 2, A and B). The insulin-stimulated decreased mobility of STAT3 in CHO/IR cells was persistent for up to 60 min. In contrast, following 60 min of insulin stimulation in 3T3L1 adipocytes there was a partial recovery of STAT3 mobility back toward its basal state. This difference in kinetics probably reflects the greater amount of insulin receptors expressed in the CHO/IR cells compared with the endogenous levels in 3T3L1 adipocytes. In any case, the effect of insulin on STAT3 was quantitative since the entire immunoreactive population of STAT3 was gel shifted between 5 and 30 min. Similarly, stimulation of CHO/IR cells with 3 nM insulin was sufficient to induce a complete gel shift of STAT3 with the half-maximal response occurring at approximately 0.3 nM (Fig. 2C).
Insulin-stimulated STAT3 Serine Phosphorylation

STAT3 Is Serine-phosphorylated in Response to Insulin—Post-translational modification of proteins by phosphorylation is commonly associated with changes in SDS-polyacrylamide gel electrophoretic mobilities (11, 30, 33, 34). To determine the basis of the insulin-stimulated reduction in STAT3 mobility, we first examined this phenomenon in unstimulated and insulin-stimulated CHO/IR lines (1995) Trends Biochem. Sci. 19, 222–227.

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Insulin-stimulated STAT3 Serine Phosphorylation

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