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Commentary

Reflections on STAT3, STAT5, and STAT6 as fat STATs

James E. Darnell, Jr.

The Rockefeller University, 1230 York Avenue, New York, NY 10021

The current issue of the *Proceedings* contains an article entitled "Defective STAT signaling by the leptin receptor in *diabetic* mice" by Ghilardi *et al.* (1). The reported results suggest how leptin, the recently discovered weight control hormone (2), may signal cells through its cognate receptor by activation of STATs, proteins that serve the dual function of signal transducers and activators of transcription in cells exposed to signaling polypeptides (3, 4).

Mice that produce no leptin (*obese* or *ob* mutants) weigh up to 60 g instead of the usual 15–20 g for a normal mouse. The human protein is virtually identical to mouse leptin, suggesting that the control of body weight in humans may also be regulated by this hormone (2). The effect of leptin on *ob* mice is to control food intake, so that weight loss ensues (5–7). In addition, the mice exhibit increased "mouse-like" exploratory activity. Thus, the description of the first molecule in the weight control pathway opens up the chance to explore in molecular detail the control of a complex behavior.

The Ghilardi *et al.* paper (1) reports confirmatory results showing the presence in cells of widely scattered tissues, including the hypothalamus, the putative control center for feeding behavior, of a "long" and "short" leptin receptor (8–10). The leptin receptor has considerable sequence similarity to the gp130 transmembrane receptor chain that pairs as the signaling molecule with a number of other transmembrane proteins to constitute the receptor for many ligands including interleukin (IL)-6, ciliary neurotrophic factor, leukemia-inhibitory factor (11, 12). The leptin receptor appears not to function normally in the mouse mutant termed *diabetes* (*db*) because of a base change in an intron that leads to a frequent aberrant splice choice; the resulting mRNA retains a translation stop codon producing a truncated protein lacking approximately 270 amino acids of the cytoplasmic domain of the transmembrane receptor (9). The omission of these amino acids was hypothesized to prevent intracellular signaling occasioned by leptin binding to its cell surface receptor. Based on the homology between the leptin receptor and the gp130 transmembrane protein (8–12), the pathway through which the leptin receptor seemed likely to signal is the recently recognized JAK/STAT pathway (3, 4). All of the known receptors that contain gp130 have JAK kinases (tyrosine kinases) bound to their intracellular tails (11). After ligand-mediated receptor assembly, the JAKs become phosphorylated on tyrosine and thereby activated as tyrosine kinases. The intracellular tail of one or more receptor chains is then phosphorylated on one or more tyrosine residues (13–15), offering binding sites to the Src homology 2 groups of latent cytoplasmic proteins called STATs (15). The attached STATs become phosphorylated on tyrosine by the activated Jak kinases. The STATs then dimerize, translocate to the nucleus, and participate in transcriptional regulation by binding to specific DNA sites. In the mutant *db* receptor both the putative STAT-binding sites and the JAK-binding sites are missing.

There are six mouse and human STATs known at present (seven if the duplicated STAT5A and STAT5B genes are considered as two) and at least STAT1, STAT3, and STAT5 exhibit differentially spliced forms. Over 30 different polypeptides have been recorded that cause STAT activation in various mammalian cells (3, 4, 11). The most potent activation of STATs through the gp130-containing receptors is of STAT3 (15–18) and by sequence comparison the wild-type leptin receptor has potential docking sites for STAT3 molecules. Ghilardi *et al.* (1) now report success in showing STAT activation dependent on the long form of the leptin receptor. In a comprehensive set of experiments they cotransfected COS cells with either the long or short versions of the leptin receptor together with, individually, each of six mouse or human STAT proteins. Leptin treatment of the cells transfected with the long but not the short receptor resulted in activation of DNA-binding complexes containing, individually, STAT3, STAT5, or STAT6; STAT1, STAT2, and STAT4 were not detectably activated. It will now be crucial to show whether the same set of STATs is activated by leptin in the hypothalamus, which is hypothesized to be the center for weight control in the Coleman model (19). In this model the *ob* gene product, a circulating hormone, would operate by binding to a hypothalamic receptor, the *db* gene product, to regulate feeding.

How Do Activated Genes Change Phenotypic Behavior?

There will be intense interest by those who specialize in nutrition and metabolic diseases and eventually in neurobiology in how the activation of this set of STAT proteins and any specific target genes that can be defined contribute to such a miraculous change in the feeding behavior of animals. This goal, however, dramatically highlights a significantly large gap in our general knowledge about how polypeptide stimuli change cell behavior. First, we know far too little about which genes are activated in response to which polypeptides. Second, we know very little about how a new set of proteins or changed concentrations of pre-existing proteins brings about a new physiologic state. Take the case of the interferons, studies on which uncovered the JAK/STAT pathway (3, 4). Although we know that some genes are stimulated by interferon α (IFN-α), some by interferon γ (IFN-γ), and some by both, we do not know how formation of the new proteins causes the antiviral state or causes growth restraint, both of which responses are caused by both interferons. We do know that STAT1 and STAT2, which are activated by IFN-α, must be present in cultured cells to allow the IFN-α responses (20, 21) and that STAT1 but not STAT2 is required for IFN-γ responses (20, 21). Moreover, STAT1, which is activated by both IFN-α and IFN-γ, has been removed from mice by gene targeting (22, 23). Although the mice breed normally in germ-free conditions they cannot withstand infection by only a few virus particles or bacteria—they succumb to a normally trivial infection. Thus, the assumption is reasonable that the transcriptional changes that depend on STATs underlie the different physiologic outcomes induced by different polypeptide ligands. We review here the question of how activation among the STAT proteins may contribute to specificity in transcriptional responses to extracellular polypeptides.

Definition of the Problem of Specificity in Immediate Gene Activation by Different Polypeptide Ligands

Because the immediate transcriptional activation profiles of many different polypeptides are not defined, what are the

present expectations for such transcriptional specificity? At one extreme it might be imagined that each polypeptide ligand has a few (or at least one) absolutely specific target gene(s) on which a specific physiologic response depends, and there certainly are genes that are known to be stimulated from a very low level of transcription to a high level by individual polypeptides. At the other extreme it might be imagined that no gene is immediately responsive only to a single polypeptide ligand. Rather, a set of genes from a repertoire of say a few dozen polypeptide responsive genes (24) might be activated quantitatively differently by different ligands. In this formulation both large stimulations of previously quiescent or more modest stimulation of already active genes could be included. In this case the basis for specificity of response to different ligands would be in the degree of response within the set of activated genes. Very little is known about the lists of target genes for a wide range of ligands to try to answer this problem. Even among the genes that are known to be targets of one polypeptide (e.g., IFN-α) and not another (e.g., IFN-γ) little information exists about their potential activation by a wide range of other polypeptides. So at present, in considering specificity of polypeptide response, it seems reasonable to think of both qualitative and quantitative variations in gene responses added together to bring about a given phenotypic change.

Differential STAT Activation by Signaling Polypeptides

To assess the possible contributions of STAT gene activation to specific polypeptide responses, we need initially to consider the limitations in the type of data available. First of all, STAT activations have been detected only *qualitatively*: (*i*) by assessing the presence or absence of DNA-binding activity, often at a single time point with a single labeled DNA-binding site to which different activated STATs may bind differently and/or (*ii*) by induction of immunoreactivity of tyrosine phosphate in potentially nonquantitative immune precipitates of STAT proteins. When two or more STATs are activated by a ligand (usually examined in cultured cells) such tests are obviously not adequate to answer the relative degree of activation *between* the STATs (especially in tissues in the body). Nevertheless, some differences and some similarities in the activating profile of different ligands or classes of ligands are known. Initially, IFN-α was shown to activate STAT1 and STAT2 and IFN-γ STAT1 only and to date only IFN-α has been reported to immediately activate STAT2 (3, 4). The targeted gene removal of STAT1, which renders null animals incapable of responding acutely to infections, verified the importance of STAT1 in the innate response to infection that must be due in part to the IFN response (22, 23). Other receptors with similarities to the IFN receptors are known. For example, the IL-10 receptor has significant homology to the IFN-α receptor; both IFN-α and IL-10 activate STAT1 and STAT3, but IL-10 also activates STAT5 (25).

STAT4 is activated, so far as has been recorded, only by IL-12 (26, 27), which acts in the development of the T-helper 1 cell response. The initial reported effects in STAT4$^{-/-}$ mice is in fact lack of development of Thl cells in the animal that otherwise develops normally (28). Until the report of Ghilardi *et al.* in this issue of the *Proceedings*, that leptin activates STAT6, only IL-4 had been reported to activate STAT6 (29). Likewise STAT6$^{-/-}$ animals don't develop Th 2 cells with the consequent impairment in making circulating antibodies, but again the mice breed and develop normally (30–32).

In addition to these restricted patterns of STAT activation, classes of receptors preferentially activate particular STATs. For example, all of the large group of ligands that utilize gp130 in their receptors activate STAT3 (11, 12, 14). The gp130 receptor class also illustrates a point that likely has physiological relevance. Low concentrations, for example of IL-6, activate only STAT3, whereas high concentrations also activate STAT1 (16, 17). Some of the ligands that use gp130 (e.g., leukemia-inhibitory factor) appear to only activate STAT3 (18). STAT2, STAT4, and STAT6 are not activated by the gp130 family of receptors. Thus, the IL-12 and leptin receptors that are in the gp130 family but not identical with gp130 might have been expected to activate STAT3 and do. But each of these two activate a STAT, STAT4, for IL-12 and STAT5 and STAT6 for leptin, not activated by the commonly used gp130 receptor. There is another case of concerted activity by similar receptors: the growth hormone and prolactin receptors and erythropoietin and megakaryocyte growth factor receptors are quite similar and all four of these form a subfamily. These four ligands all strongly activate STAT5 in apparent preference to other STATs (4, 11). Finally, less has been published about the receptor tyrosine kinases but several are known to activate STAT1 and STAT3 but not STAT2, STAT4, and STAT6 (3, 4).

Two variations in STAT-containing complexes broaden their potential role. First, STAT1, STAT3, and STAT5 all have at least one protein variant produced by differential splicing. In each case the transactivating potential of the short form is less than the long form and could have dominant negative effect for some genes (33). Second, STAT1 and STAT2 and STAT1 and STAT3 can form heterodimers and it is possible that other pairs of proteins might also interact (4). Little is known about what governs heterodimer formation, but obviously such complexes could differ in transcriptional roles.

To summarize, there are documented differences of STAT activation patterns after cells are treated with various cytokines and classes of cytokines. Of course, with only six (or seven) known STATs and many dozens of ligands that activate one or more of the STATs there cannot be a one-to-one correlation between individual ligands, individual STAT activation, and specific patterns of activation.

Possible Bases for Quantitative Differences in STAT Activation

In addition to qualitative differences in ligand activation of the STAT proteins in response to specific polypeptides, there may be quantitative differences in responses to different ligands even though the same pattern of STATs might be activated. Both the length of time a signal lasts and the "strength" of the signal (number of STAT molecules activated) from a given ligand-receptor interaction may differ between receptors that activate qualitatively the same STATs. The number of receptors of a given variety, the time before internalization and deactivation of a ligand-receptor complex, and the relative affinity of the STAT docking sites on an activated receptor for the various STAT substrates could all play a role in the strength of signal emanating from a particular activated receptor. Also, phosphotyrosine-specific phosphatases (PTPases) are known to be associated with cytokine receptors (11) and to have physiologically demonstrable effects on the outcome of ligand-induced events. For example, *moth-eaten*, a single gene defect in mice that causes early leukemia, is mutant in a phosphatase (hematopoietic cell phosphatase) that is associated with a number of cytokine receptors (34, 35). In addition, a mutation in the human erythropoietin receptor that removes the SHPTPase I-binding site leads to overproduction of red blood cells (36). Thus, the possibility of differential action of phosphatases on different receptors and/or JAK kinases could clearly affect the number of STAT molecules activated at a given receptor.

Finally, with regard to a possibly different impact on transcription from different receptors that activate the same set of STATs, we must consider serine phosphorylation of the STAT molecules themselves (18, 33, 37). Both STAT1 and STAT3 have been shown to be phosphorylated on a single serine (residue 727) and this phosphorylation has an approximately 2- to 4-fold impact on transcriptional activation caused by the already tyrosine phosphorylated and dimerized STAT (33).

The serine phosphorylation is not an absolute requirement for transcriptional activation but rather heightens the activating capacity of the STAT protein. Furthermore, it has been shown, at least for platelet-derived growth factor and for IFN-γ, that serine phosphorylation is induced by the ligand. Thus, differential serine phosphorylation of STATs after activation of different receptors could easily have an impact on the pattern or extent of gene activation.

Another recent observation illustrates how differences may arise between stimulation from two different receptors that qualitatively activate the same STATs. Ivaskin and colleagues (L. Ivaskin, personal communication) found that treatment of cells with granulocyte/macrophage colony-stimulating factor for 1 hr aborts the ability of monocytes to activate STAT3 in response to IL-6. If this phenomenon of one receptor interfering with signaling from another were to be widespread and different for different STATs, then the presence of combinations of polypeptides could have a major impact on the ligand-induced signaling.

Thus far in this section we have considered the question of one cell type responding to two different ligands that might activate the same set of STATs. It goes without saying that in differentiated cells a different protein content (receptors, phosphatases, STATs) could change the effect of the same ligand on two different cell types.

These various considerations make it clear that the possible range of activity of the STATs in polypeptide-triggered gene activity may be quite broad both with regard to effecting qualitative and quantitative changes in transcription, which may ensue after different polypeptides bind to cells.

Need for More Extensive Promoter Analyses

What is needed to resolve the question of specificity of gene induction by various polypeptide ligands? The first need is more research to define which genes are immediately activated by which polypeptides, followed by an examination of the promoters of substantial numbers of these genes. Detailed mapping of protein- binding sites on perhaps dozens of ligand-activated genes will be required to understand the precise role of various STATs or of other cooperating proteins in immediate gene activation.

The importance of acquiring details about promoter-binding sites is clearly illustrated by the recent work of Thanos and Maniatis (38) and Grosschedl (39). They have shown that enhancers must bind several proteins simultaneously to exert maximal changes in gene activity. Furthermore, Curran and colleagues (40) have shown that in the c-*fos* promoter several binding sites, including a STAT3-binding site (17, 41), must be simultaneously present for activation of this gene in a chromosome. Thus, examining the role of different STATs in such complex promoters may greatly illuminate how important different STATs may be for different gene activations. Some promoters may well respond to several STATs, but to different degrees.

Finally, the importance of quantitative levels of transcription, not just on or off transcription, needs to be emphasized. Using mutants with diminished transcriptional activation potential, we have shown that maximal STAT1 transactivation is required in cultured cells to establish the antiviral state (42) or inhibit growth in IFN-treated cells (43). Of potentially greater significance, it has been clearly documented in *Drosophila* that balanced transcription is required for developmental decisions (44, 45). In the elegant genetic analysis of the developing receptor cells in the ommatidia of the compound *Drosophila* eye, partially defective alleles of genes in the Ras–Map kinase pathway lead to improper differentiation of R7 cells. This results presumably, at least in part, from defective serine phosphorylation of nuclear transcription factors. Mutations that change the activity of proteins in this pathway by only a factor of 2- to 3-fold are quite sufficient to disrupt development of the eye.

So to return to the case that began this discussion, the activation in a test system of STAT3, STAT5, and STAT6 by the leptin receptor (1) represents a major step forward in focusing attention on the possible regulatory pathways in weight control. But it is a daunting challenge indeed to molecular geneticists to try now to discover how the STATs, individually or collectively, participate in gene regulation that allows a mouse to balance its weight at 20 g.

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