Is Stat1 activated catalytically within cells by the interferon-gamma receptor complex?

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Abstract

Introduction

Two distinct models of the activation of Stat1 by the interferon-gamma receptor complex attempt to explain how activated Stat1 translates to the nucleus to modify gene activity. Most analyses of Stat1 activation focus only on the amount of activated Stat1. Analysis of early time points of Stat1 activation has not been satisfactorily done, and may help refine models of Stat1 activation.

The Hypothesis

If individual receptor complexes activate many Stat1 molecules over a long period of time, then the progressive activation of interferon-gamma complexes in cells (by gradual binding of interferon-gamma to its cellular receptors) will result in a parabolic initial activation of Stat1 over time. However, if individual receptor complexes activate only a limited number of Stat1 molecules or are quickly inactivated, Stat1 activation will occur linearly with time.

Evaluation of hypothesis

The initial time-resolved activation of Stat1 was investigated in response to various concentrations of interferon-gamma in certain cell lines. Parabolic Stat1 activation was seen; lower interferon-gamma concentrations correlated with longer parabolic Stat1 activation. The values of various kinetic enzymatic parameters were estimated. The latency observed in epithelial cell lines is not consistent with progressive receptor activation, but rather with a secondary superactivation.

Conclusion

Individual receptor complexes activate many Stat1 molecules. Mathematical modelling suggests that (1) binding of Stat1 to the receptor complex limits the overall rate of cellular Stat1 activation and (2) an enzymatic superactivation of ligand-bound receptor complexes can occur. This kinetic model can be used to analyse the effects of specific inhibitors on interferon-gamma signalling in mechanistic detail, or can be adapted to other cytokine signalling systems.

Introduction

Activation of Stats by the interferon-gamma receptor complex

The interferon-gamma (IFN-γ) receptor complex, composed of the ligand-binding IFN-γR1 chain, the accessory IFN-γR2 chain and their associated Janus kinases (Jak1 and Jak2 respectively), interacts with IFN-γ to form the enzymatically active entity. The Stat proteins are the only transcription factors that are directly activated by elements of the IFN-γ receptor complex; others require Stat1-influenced gene transcription. Although, IFN-γ activates Stat1, Stat3 and Stat5 in vitro, and Stat3 can activate limited numbers of IFN-γ-sensitive genes in vitro in the absence of Stat115, whole-animal studies have supported a role for only Stat1 in IFN-γ function.

The activation of Stat1 occurs by phosphorylation of tyrosine-701 and serine-727. In response to an optimal dose of IFN-γ (over 35 U/mL or 100 pM), all the Stat1 in the cell is tyrosine phosphorylated within 10–15 min13, and nuclear migration is complete within 30 min1. Tyr-701 phosphorylation is sufficient and necessary for DNA binding5,9, while Ser-727 phosphorylation modifies the induction of many Stat1-sensitive genes5,9. The carboxyl-terminal SH2 domain of one Stat1 monomer binds phospho-Tyr-701 of another Stat1 monomer; the reciprocal interaction completes the dimer that binds specific DNA sequences10. Dephosphorylation of Tyr-701 forces its dissociation from DNA11–14.

Mechanisms of Stat nuclear translocation from the cell surface receptor complex

Two distinct mechanisms are proposed to explain how activated Stat1 migrates into the nucleus. The most commonly accepted mechanism states that activated Stat1 dimers migrate in a freely diffusible manner through the cytosol in the absence of its activating receptors, and are transported through the nuclear pore15,16. Other studies have indicated that Stat1 translocates to the nucleus only in a complex with IFN-γ and IFN-γR117–19, implying that Stat1 may not be catalytically activated by the IFN-γ receptor complex. Thus, determining whether Stat1 is catalytically activated by Jaks is important in refining mechanistic models of signalling.

The Hypothesis

Clearly, the IFN-γ receptors do not perpetually activate Stat proteins. However, if Jaks are active for an indefinite period of time, the successive but gradual activation of more receptors results in a parabolic increase
in the levels of phosphorylated Stat proteins at early time points (Figure 1, top). On the other hand, if only limited numbers of Stat proteins are activated by each receptor complex, then the successive but gradual activation of receptors will result instead in a quasi-linear increase in the levels of Stat proteins at early time points (Figure 1, bottom).

Furthermore, we speculated that we may model the time-resolved activation of Stat1 by the IFN-γ receptor complexes mathematically, by assuming that each receptor complex is an enzyme obeying Michaelis–Menten kinetics. Using this model, we can estimate kinetic parameters and begin to dissect the biochemical events that occur during the activation of the IFN-γ receptor complex. Time-resolved signalling models can measure parameters that are unobtainable in most single time-point assay systems, allowing a more thorough characterisation of modifiers of signalling.

**Evaluation of Hypothesis**

The authors have referenced some of their own studies in this hypothesis. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

**Biochemical assays**

We justify the use of the electrophoretic mobility shift assay (EMSA) as a reliable time-resolved assay system in Supplementary Text 1. Our protocol for treating cells with interferon, stopping the reactions by rapidly cooling cells, preparing the lysates, resolving the lysates by EMSA and quantitative imaging of the EMSA is described elsewhere.

**Designing the mathematical and time-resolved model**

We used the transform scripting language within Sigma Plot 5-12 (Systat Software, Inc., San Jose, CA) to implement the time-resolved mathematical signalling model.

A mechanistic model of IFN-γ signalling can be represented by a series of reactions (i.e. ligand binds receptor, receptor binds Stat1). From these reactions, kinetic equations can be derived to predict a time-resolved model of how signalling proceeds (in this case, the pattern of Stat1 activation). These predictions can then be compared with empirical data, and the model can be refined accordingly to more closely resemble real data by modifying kinetic parameters or adding subsequent reactions.

Details concerning the modelling of IFN-γ binding to cell surface receptors can be found in Supplementary Text 2. The modelling of catalytic activation of Stat1 by the IFN-γ receptor complex is described in Supplementary Text 3.

**Previous kinetic data of Stat1 activation by the interferon-gamma receptor complex**

A few semi-kinetic analyses have been done. Stat1 activation can be seen in the levels of phosphorylated Stat proteins at early time points (Figure 1, top). On the other hand, if only limited numbers of Stat proteins are activated by each receptor complex, then the successive but gradual activation of receptors will result instead in a quasi-linear increase in the levels of Stat proteins at early time points (Figure 1, bottom).

![Diagram of Stat1 activation](image-url)

**Figure 1:** Catalytic versus noncatalytic Stat1 activation. (top) Prediction of Stat1 activation by persistently active receptors. Here each receptor activated by ligand continues to activate Stat at a constant rate (thin black lines). The successive activation of additional receptors by successive ligand-binding events (identified with labelled arrows) results in an overall parabolic increase in activated Stat1 levels (thick blue line). (bottom) Prediction of Stat1 activation by transiently active receptors. Here each receptor activates Stat proteins for only a limited time (thin black curves). The successive activation of additional receptors by individual ligand-binding events results in a virtually linear increase in total activated Stat1 levels (thick blue line).
after 1 min, is optimal after 5–10 min, persists for an hour, and then decreases. Little attention has been paid to the initial (<10 min) activation of Stat1 by the IFN-γ receptor complex. Because this is the period during which the JakS are activated and when their pharmacological manipulation will be most effective, we chose to focus on these early time periods.

Unexpectedly, the initial activation of Stat1 by high concentrations of IFN-γ in cells is rather complicated: it varies among cell types, especially within the immune system. These variations can be reproduced in epithelial cell lines by overexpressing either IFN-γR1 or IFN-γR2, suggesting that receptor chain expression controls how IFN-γ initiates signalling. When treated with 3 nM IFN-γ, no latency of Stat1 activation was observed either in HeLa cells overexpressing IFN-γR2 or in monocytic cell lines. We therefore started to characterise Stat1 signalling in these cells (hereafter we call this rapid pattern of Stat1 activation the ‘fast’ state), especially with lower, more physiological concentrations of IFN-γ (when receptor occupation is more gradual).

**Empirical data**

**Rapid Stat1 activation**

Our signalling model (in which ligand-binding results in the perpetually active IFN-γ receptor complexes capable of activating all Stat1 in the cell) predicted that at lower concentrations of IFN-γ, a latency period appears. Slower ligand binding (Supplementary Figure 1, top) correlated with an increasingly visible latency period (Supplementary Figure 1, bottom). Because the shape of the latency resembles a parabola, we called this activation parabolic. These data allow us to predict that progressive ligand binding will result in parabolic Stat1 activation.

We confirmed the prediction of the signalling model by analysing Stat1 activation initiated by lower concentrations of IFN-γ in two cell lines in which IFN-γR2 was overexpressed: in HeLa cells stably harbouring a plasmid overexpressing IFN-γR2 (Figure 2, top) and in hamster Chinese hamster ovary-27 (CHO-27) cells (CHO-derived 16-9 cells containing the chromosomal human IFN-γR1 gene, and a stably transfected plasmid ectopically expressing human IFN-γR2, Figure 2, bottom). In both cell lines, latency periods appeared in the activation rate of Stat1 as the IFN-γ concentration decreased. These data argue strongly for a catalytic activation of Stat proteins in cells by the IFN-γ receptor complex.

Concluding sentence: Notably, in both cell lines, Stat1 activation in response to 0.9 pM IFN-γ never achieved the optimal levels seen when more than 10 pM IFN-γ was used (Figure 3). Our signalling models assume that all Stat1 will eventually be phosphorylated, no matter how low the IFN-γ concentration is. The induction of mechanisms to silence Jak signalling in cells (e.g. suppressor of cytokine signalling induction, phosphatase activity, receptor downregulation) likely accounts for this discrepancy.

We analysed Stat1 activation under conditions used to characterise ligand
binding to determine the value of various kinetic parameters ($K_\text{d}$ of 305,000 Stat1 dimers/cell and $V_{\text{MAX}}$ of 165 dimers/min) that govern the activation of Stat1 by the IFN-γ receptor complexes. We describe how we obtained these values in Supplementary Text 4.

### Slow Stat1 activation

We previously identified two conditions where the slow rate of Stat1 activation seen in the latency period does not transit into rapid Stat1 activation even when cells are treated with 3 nM IFN-γ; it takes over 45 min for optimal levels of phosphorylated Stat1 to be observed (hereafter we call this pattern of Stat1 activation the ‘slow’ state). In one condition, IFN-γ R1 is overexpressed; in the second condition, a dominant-negative receptor chain IFN-γ R2/aR2b is overexpressed. Because the excessive levels of IFN-γ R1 do not inhibit Stat1 activation when less than 100 pM IFN-γ is used, we analysed the kinetics of Stat1 activation at various concentrations of IFN-γ in HeLa cells overexpressing IFN-γ R2/aR2b.

We observed again a latency period in the Stat1 activation pattern when cells were treated with intermediate IFN-γ concentrations (Supplementary Figure 4). We hypothesise that here also the receptor complexes once bound by IFN-γ remain active. Because we observed above that Stat1 activation is inhibited after 40 min in cells, only time points earlier than 40 min were used to optimise the fit of the model to the empirical data and determine kinetic parameters for the slow state. Here we obtained values of 3.33 Stat dimers/min for $V_{\text{MAX}}$ and 49,600 Stat dimers/cell for $K_\text{d}$ (data not shown).

Possessing two distinct values of $K_\text{d}$ and $V_{\text{MAX}}$ we estimated the binding and dissociation rates, and the apparent affinity of Stat1 for the IFN-γ receptor complex. This allowed us to make some interesting observations with respect to probabilities of Stat1 being phosphorylated before dissociating from the receptor complex in the fast and slow state. See Supplementary Text 5 for details.

### Delayed rapid Stat1 activation

A latency in the Stat1 activation rate was observed when neither IFN-γ R1 nor IFN-γ R2 is overexpressed, even at 3 nM IFN-γ. This latency is clearly not related to IFN-γ binding its receptor—receptor binding is fully completed (1 min) well before the latency period ended (2 min). The shape of the latency period could not be fit using the signalling model above. We previously hypothesised that this latency was caused by a change from a ‘slow’ state to a ‘fast’ state in the IFN-γ receptor complexes. We explored this hypothesis in a time-resolved model by expanding our signalling model to account for a conversion of the ligand-bound receptor (that we assume is in the slow state) to a new enzymatic state (that we assume is in the fast state). We appended a new

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**Figure 3:** Fits of three-state modelling to Stat1 activation observed in HeLa and Raji cells. (top) Modelling the activation of Stat1. HeLa cells were treated with 25 pM IFN-γ (left) or 2940 pM IFN-γ (centre) for the indicated time points. Also, Raji cells were treated with 2940 pM IFN-γ for the indicated times (right). Predictions of only fast Stat1 activation (in black) and only slow Stat1 activation (in red) are shown in each panel. (bottom) Modelling the binding and superactivation of receptor complexes. The predicted levels of various complexes on the cell surface are displayed. The total bound receptor (in black), intermediates and products of unimolecular superactivation (in red and green, respectively) and intermediates and products of the bimolecular superactivation (in yellow and blue, respectively) are plotted.

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reaction converting the slow receptors into the fast receptors (that we call superactivation). We also assume that the \( [K_c, V_{MAX}] \) kinetic parameters identified for the slow and the fast states above apply to the states before and after superactivation.

We placed a detailed description of how we modelled the superactivation of IFN-\( \gamma \) receptor complexes in Supplementary Text 6.

We modelled a bimolecular superactivation because of our earlier hypothesis that a superactivation results from the interaction of two ‘slow state’ receptor complexes to form a dimer of ‘fast state’ receptor complexes, explaining an apparent functional tetramerisation of IFN-\( \gamma \) during signalling in epithelial cells after dimers of IFN-\( \gamma \) bind the cell surface\(^2\). We also modelled a (pseudo) unimolecular superactivation, hypothesising that a component in great excess to the 1800 receptor complexes binds and superactivate them.

Notably, a superactivation reaction properly modelled the latency period found in HeLa and Raji cells (Figure 3). This supports our hypothesis that an enzymatic change in the receptor complexes creates the latency period seen in the Stat1 activation rate. Note that both unimolecular and bimolecular superactivation reactions satisfactorily fit the experimental data obtained in HeLa cells at 25 and 2940 pM IFN-\( \gamma \) and in Raji cells at 2940 pM. Table 1 lists the superactivation rate constants from these fits.

**Discussion**

In our kinetic model of IFN-\( \gamma \) signalling, a receptor complex activated by IFN-\( \gamma \) is immediately and persistently active, activating many Stat1 monomers; thus, we can characterise the IFN-\( \gamma \) receptor complex as an enzyme. Stat1 is catalytically activated, and translocates to the nucleus as an entity free of the receptor complex. Stat1 is catalytically activated, the IFN-\( \gamma \) dimer of ‘fast state’ receptor complexes to form a dimer of ‘fast state’ receptor complexes, explaining an apparent functional tetramerisation of IFN-\( \gamma \) during signalling in epithelial cells after dimers of IFN-\( \gamma \) bind the cell surface\(^2\). We also modelled a (pseudo) unimolecular superactivation, hypothesising that a component in great excess to the 1800 receptor complexes binds and superactivate them.

Notably, a superactivation reaction properly modelled the latency period found in HeLa and Raji cells (Figure 3). This supports our hypothesis that an enzymatic change in the receptor complexes creates the latency period seen in the Stat1 activation rate. Note that both unimolecular and bimolecular superactivation reactions satisfactorily fit the experimental data obtained in HeLa cells at 25 and 2940 pM IFN-\( \gamma \) and in Raji cells at 2940 pM. Table 1 lists the superactivation rate constants from these fits.

**Conclusion**

The discovery of a superactivation reaction required a time-resolved model, and opens up new modes to inhibit or modify IFN-\( \gamma \) signalling. It will be interesting if specific signalling pathways (e.g. PKC, MAPK, PI3K) require superactivated or only initially activated receptor complexes.

We also expect that a time-resolved model of catalytic Janus kinase signalling within IFN-\( \gamma \) receptors can be easily applied to other signalling networks, whether initiated by other cytokines or interleukins, orothertyrosine kinases.

**Acknowledgment**

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**Abbreviations list**

CH2-27, Chinese hamster ovary-27; EMSA, electrophoretic mobility shift assay; IFN-\( \gamma \), interferon-gamma; Jak, Janus kinase.

**Table 1 Superactivation rate constants, in three different conditions**

<table>
<thead>
<tr>
<th>Superactivation rate constant</th>
<th>25 pM, HeLa</th>
<th>2940 pM, HeLa</th>
<th>2940 pM, Raji</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unimolecular (min(^{-1}))</td>
<td>0.05</td>
<td>0.08</td>
<td>0.11</td>
</tr>
<tr>
<td>Bimolecular (( \times 10^{-4} ) (RC/cell)-1 ( \times ) min(^{-1}))</td>
<td>1.2</td>
<td>0.6</td>
<td>0.8</td>
</tr>
</tbody>
</table>

**References**

Supplementary Text 1. Ground conditions to use electrophoretic mobility shift assay to detect and quantitate activated Stat1

First, we needed to be satisfied that measuring the level of DNA bound by Stat1 in an electrophoretic mobility shift assay (EMSA) was directly related to the amount of Stat1 activated in cells (here, by IFN-γ). So, we ensured that far more radiolabelled DNA is present than it can be bound by all the activated DNA-binding proteins in the cellular extract. Next, we determined that the amount of Stat1 bound to DNA was linearly proportional to the amount of cellular lysate added. Others demonstrated that Stat1 and Stat5 bind DNA only when they are phosphorylated, and no longer bind DNA when dephosphorylated. Finally, the crystal structure of a tyrosine-phosphorylated Stat1 dimer and DNA showed that the complex of phospho-Stat1 and DNA forms in the absence of other proteins. Therefore, for every two Stat1 molecules phosphorylated, one molecule of radiolabelled DNA is bound, and migrates more slowly, and all Stat1 molecules in the lysate will bind DNA if activated.

Because the amount of DNA bound by Stat1 in a lane of an EMSA can be calculated based on phosphorimaging, and the number of cells lysed can be estimated, one can measure the number of Stat1 molecules activated per cell. We estimated that 80,000 Stat1 dimers/cell can be activated by IFN-γ (data not shown).

Supplementary Text 2. Ligand binding to and activation of the cell surface receptors

Modelling ligand binding to the receptor complex

We first reproduced the first stage of cellular activation: the binding of IFN-γ to receptors on U937 cells at 37°C. Because the levels of IFN-γ in solution greatly exceed the numbers of receptor complexes on the cell surface of all the cells, the binding reaction proceeds without a significant reduction in the levels of ligand. Thus we modelled IFN-γ (called L) binding to the receptor (called R) to form the bound receptor complex (called RL) as a pseudo-unimolecular reaction. Because dissociation of IFN-γ from its receptor complex has been measured, we modelled the receptor binding to be reversible, thus we have reaction (A).

\[ R + L \rightleftharpoons RL \] (A)

Rate constants link the molar concentrations to time. We define rate constants of \( k_1 \) and \( k_{-1} \) for the forward and reverse reactions, respectively. The constant \( k_i \) is the arithmetic product of the true bimolecular rate constants \( k_f \) and \([L]\), the IFN-γ concentration, that is, \( k_i = k_f[L] \). The kinetic equation derived from reaction (A), in which the numbers of ligand-bound receptor complexes can be calculated at any time (RL) is given by Equation (1). Here \( t \) is measured in minutes and \( R_0 \) is the total number of receptor complexes on the cell (1800/cell).

\[
RL_t = \frac{k_1R_0}{k_1 + k_{-1}} \left[1 - e^{-\left(k_i + k_{-1}\right)t}\right]
\]

Supplementary Figure 1: Correlation of latency period with decreasing IFN-γ concentrations. (Top) IFN-γ receptor occupancy kinetics in response to 2940 pM (1000 U/mL, blue), 25 pM (8.5 U/mL, green), 2.94 pM (1 U/mL, red) and 0.294 pM (0.1 U/mL, black) IFN-γ. (bottom) Stat1 activation in response to the same concentrations of IFN-γ. The coloured lines in the two panels are intentionally colour-matched. The \( K_M \) and \( V_{MAX} \) values used for the bottom graph are 305,000 Stat dimers/cell and 165 Stat dimers/min, respectively.
Neither $k_r$ nor $k_s$ were determined at 37°C; thus, we estimated them from a system of two equations with both constants as unknowns. The observed association rate ($k_{obs}$, 0.065 min$^{-1}$) at 25 pM is the net result of the true association rate and the competing dissociation rate, thus $k_{obs} = k_i + k_s = k_L + k_{-s}$, yielding the first equation $0 = 2.94 \times 10^5 k_s - k_i$, yielding values of 0.0068 min$^{-1}$ for $k_s$ and 2.33 $\times 10^5$ M$^{-1}$min$^{-1}$ for $k_i$, obtained. These values agree reasonably well with the measured constants at 24°C: 0.0027 min$^{-1}$ for $k_s$ and (using 0.04 min$^{-1}$ at 40 pM for $k_{obs}$) 0.932 $\times 10^6$ M$^{-1}$min$^{-1}$ for $k_i$, assuming that an increase of 13°C more than doubles the rates of both reactions.

From the unimolecular decay rate $k_s$, one can calculate average lifetime ($\tau$) of the complexed state (in this case, the residence time of IFN-γ on its receptor) by taking the reciprocal of the rate constant (i.e. $\tau_{RL} = 1/k_s = 1/0.068$ min$^{-1}$) yielding 147 min. Thus there is less than a 20% chance that the ligand will dissociate from a receptor complex in 50 min; by that time, secondary inhibitory mechanisms have already begun in cells. Therefore, it is likely that once a ligand binds a receptor complex, it remains bound for the entire lifetime of the signalling cycles of that receptor complex.

(Not) Modelling receptor complex activation after ligand binding

Although conformational changes (initiated after the interaction of IFN-γ with its receptor, transmitted through the receptor chains, through the plasma membrane, to the cytoplasmic domains and ultimately to the tyrosine kinases to be activated), phosphorylations of the Jak and phosphorylation of the Stat1 recruitment site on IFN-γ-R1 can be modelled as a series of unimolecular reactions (i.e. RL $\rightarrow$ RL$^*$ $\rightarrow$ RL$^1$ $\rightarrow$ RL$^{1*}$ $\rightarrow$ RL), we assumed that the receptor complex (RL) is catalytically active immediately after ligand binding, not only to simplify the calculus of the model but also because (1) Stat1 activation occurs without a latency period at 3 nM IFN-γ, where half the receptors are bound by IFN-γ in 5.9 s; (2) maximal tyrosine phosphorylation of cellular IFN-γ-R1 and Jak1 occurs by 15–60 s at 750 pM IFN-γ

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According to Western Blot; under these conditions, half of the cellular receptors are bound by ligand in about 25 s and (3) we do not know how many identifiable intermediates there may be. Finally, including reactions with an unknown but rapid rate (completion in seconds at most) will not greatly influence our model of Stat1 activation, which occurs on the order of minutes.

**Supplementary Text 3. Algorithm calculating the catalytic activation of the Stat proteins by the IFN-γ receptor complex**

It is easier to model perpetual activation of Jaks than to model highly limited activation of Jaks. To model the catalytic activation of Stat1 (called S) by the IFN-γ receptor complex to form tyrosine-phosphorylated Stat1 (called P), we adapt the Michaelis–Menten model of substrate binding and conversion to product by a stable recycled enzyme. The relevant reaction is reaction (B):

\[ \text{RL} + S \rightleftharpoons \text{RLS} \rightarrow \text{RL} + P. \]  

(B)

Rate constants of \( k_s \) and \( k_{-s} \) are the association and dissociation rate constants of Stat1 to the IFN-γ receptor complex. \( V_{\text{MAX}} \) is the rate constant governing the rate at which Stat1 is phosphorylated and rapidly dissociates from the receptor complex. This reaction system assumes that rebinding of phospho-Stat1 to inhibit catalysis does not occur; this assumption appears to be valid. In fact, it has been shown that tyrosine-phosphorylated Stat1 readily forms SH2-mediated dimers; thus, we include the following reaction P → P₂ to remove the product and create the species that translocates to the nucleus and retards the migration of DNA in the EMSA, but we do not model it numerically. We also assume that each IFN-γ receptor complex behaves as an enzyme, immediately and always active, and that each receptor complex has the same specific activity.

**Supplementary Figure 3:** An extreme case of the Michaelis–Menten equation. Equation (6), that assumes exponential rise of product to a defined value, is fit to data obtained from HL60 cells.

The rate at which Stat1 is phosphorylated is given by Equation (2):

\[ V = \frac{d[S]}{dt} = \frac{V_{\text{MAX}}[S]}{K_M + [S]} \]  

where \( V \) is the observed rate of reaction. \([S]\) is the amount of substrate remaining at a given time. \( K_M \), that equals the combination of kinetic constants \( (V_{\text{MAX}} + k_{-s})/k_s \), is the Michaelis–Menten constant and reflects the apparent affinity of the enzyme for the substrate. \( V_{\text{MAX}} \) is the maximal reaction rate of the substrate conversion by the enzyme, which under steady-state conditions reflects the substrate turnover number per enzyme molecule. Because Equation (2) cannot be defined in closed form to yield substrate levels in a time-dependent manner, we cannot model substrate or product levels without resorting to numerical methods.

Although Equation (2) is commonly applied to an entire reaction, we shall instead calculate \( V \) on a per enzyme basis, especially because the binding of ligand to the receptor directly dictates whether enzymatic activity is present. To react the substrate using a numerical approximation, we utilised Equation (3):

\[ S_\omega = VRL \Delta t \]  

Here \( S_\omega \) represents the amount of substrate converted to product in a reaction during a period of time \( \Delta t \). It is calculated by multiplying the reaction rate per ligand:receptor complex (calculated from Equation (2)), the number of ligand:receptor complex.

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comple ct of reacted with the receptor complex and turnover rate in live cells, we ran the kinetics of Stat1 activation at 25 pm IFN-γ in U937 cells, the same conditions used for ligand binding and optimised the fit of the signalling model to these data. Fortunately, the kinetics of Stat1 activation by 25 pm IFN-γ in CHO-27 cells overlapped well with the data we obtained from U937 cells, so we fit the model to both sets of data simultaneously, averaging data where a given time point has data from both cell lines. Because we did not have any empirical clues to the magnitude of $K_m$ or $V_{MAX}$, we chose a series of values for each parameter and created a $[K_m, V_{MAX}]$ matrix of predicted outputs of the model. To obtain statistically valid numbers from fitting the algorithm to the real data, a deviation minimisation method must be employed. Because product curves for each $[K_m, V_{MAX}]$ pair are generated point-wise through time, we chose to minimise the summed squared deviations between the real product levels from the datapoints ($P_{i, act}$ obtained at $n$ time points $t_1, t_2, t_3,... t_n$) and the calculated product datapoints from the theoretical model ($P_{i, theo}$) at the same time points $t_1, t_2, t_3,... t_n$. A deviation, $D$, at each relevant time point is calculated with Equation (5a). A total deviation $\Sigma D$ at a given $[K_m, V_{MAX}]$ pair is calculated with Equation (5b). $\Sigma D$ evaluates the fit of the curve at any $[K_m, V_{MAX}]$ pair to the real data. No weighting of data was employed.

$$D = (P_{i, theo} - P_{i, act})^2 \quad (5a)$$

$$\Sigma D(K_m, V_{MAX}) = \sum_{i=1}^{n} D_i \quad (5b)$$

$\Sigma D$ was plotted as function of both $K_m$ and $V_{MAX}$ simultaneously to form a three-dimensional surface plot; the minimum of the surface indicates lowest total deviation of the theoretical curve to the real data, which we assumed to be the best fit (Supplementary Figure 2A).

A global minimum in the surface deviation plot (Supplementary Figure 2B) was apparent. The shape of the surface was a parabolic valley with a nearly flat diagonal trough with respect to the axes (Supplementary Figure 2C). To obtain an accurate value for the absolute minimum of this valley, two additional matrices with $[K_m, V_{MAX}]$ ranges focused about the global minimum were performed to obtain a higher resolution image.
of the minimum of the \( \Sigma D \) surface plot and obtain a smooth minimum. Using this approach, the values of \( V_{\text{MAX}} \) and \( K_s \) that yielded the minimum deviation produced a Stat1 activation pattern that most accurately fit the real data according to this model. The trough itself has a global minimum value where \( K_s = 305,000 \) Stat1 dimers/cell and \( V_{\text{MAX}} = 165 \) Stat1 dimers/min. From this global minimum, the total deviation slowly ascends as \( K_s \) increases in value and rapidly ascends as \( K_s \) decreases in value (Supplementary Figure 2D).

An unbiased deviation-minimising optimisation routine like this is necessary to determine accurate values from this algorithm. Because the deviation surface had a nearly flat diagonal trough, if one were to subjectively choose a \( K_s \) value significantly beyond the optimal value, a local minimum can be determined for a particular \( V_{\text{MAX}} \) value whose \( \Sigma D \) is not much higher than that from the curve giving the optimised \( [K_m, V_{\text{MAX}}] \) values; the converse applies. A series of curves defined from the various local minima and the absolute minima appear too similar to determine the values of the parameters visually (Supplementary Figure 2E). In these curves, \( K_s \) varied 19-fold and \( V_{\text{MAX}} \) varied 12-fold, so that a subjective guess of parameters is highly error-prone. Even at the minimum of the trough, the values of \( K_s \) and \( V_{\text{MAX}} \) could vary by as much as 60% (Supplementary Figure 2D).

The \( K_s \) (305,000 Stat dimers/cell) of receptors in the fast state is significantly higher than the total amount of Stat available for reaction (80,000 Stat dimers/cell). Though the following assumption is not valid in this scenario, if one assumes that \( K_s >> [\text{Stat}] \) (this assumption often applies when catalytic activity is rapid, when substrate dissociation from the enzyme is rapid or when substrate association with the enzyme is poor), then Equation (2) can be reduced to

\[
V = \frac{-d[S]_v}{dt} = \frac{V_{\text{MAX}}[S]}{K_m} \quad \text{that can be easily integrated over time to yield the exponential decay function or, rewritten in terms of product formed, Equation (6):}
\]

\[
[P]_k = [S]_v(1 - e^{-(V_{\text{MAX}}/K_m)t})
\]

This equation predicts an exponential decay of substrate and the resulting exponential rise of product in the cell over time. Reaction progress curves that fit Equation (6) generally reflect conditions where very limited substrate is present or accessible relative to the amount of enzyme or when the affinity of the enzyme for the substrate is very poor. Product activation curves in \textit{in vitro} jak assays follow this pattern. Indeed, this approximation fits the accumulation of Stat protein with rapidly saturating IFN-\( \gamma \) concentrations in cells exhibiting ‘fast’ IFN-\( \gamma \) receptors (Supplementary Figure 3). Though extraction of \( V_{\text{MAX}}/K_m \) from the cellular assay is not accurate, the fact that the data fit reasonably well to the simpler equation suggests that Stat1 availability to the IFN-\( \gamma \) receptor complex limits the cellular rate of Stat1 phosphorylation after IFN-\( \gamma \) binds to the receptor complex.

**Supplementary Text 5. Estimating and interpreting the in vivo affinity of Stat1 for the IFN-\( \gamma \) receptor complex**

We can estimate the affinity of Stat1 for the IFN-\( \gamma \) receptor complex. For both the fast and slow states, we have distinct \( V_{\text{MAX}} \) and \( K_s \) values. \( K_s \) is defined as a combination of kinetic rate constants as shown in Equation (7):

\[
K_s = \frac{V_{\text{MAX}} + k_{-s}}{k_s}
\]

For the fast state, Equation (7) evaluates to 610,000 per cell = (310 min\(^{-1}\) + \( k_{-s} \))/\( k_s \) and for the slow state, Equation (7) evaluates to 99,200 per cell = (6.66 min\(^{-1}\) + \( k_{-s} \))/\( k_s \). Assuming the substrate-binding site (the Janus kinase) is the same for both states, we can combine both equations and obtain values of 5.94 \times 10^{-4} \text{ cell min}^{-1} for \( k_s \) (Assuming a cellular volume of 2 pl, this equates to 0.7155 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}) and 52.25 \text{ min}^{-1} for \( k_d \). The affinity constant for Stat1 binding to the Jak (\( K_s \)) can be calculated by Equation (8):

\[
K_s = \frac{k_{-s}}{k_s}
\]

The obtained value is 87,962 Stat molecules/cell, equivalent to a concentration of 73 nM. This value is higher than the values obtained for Stat1 binding to IFN-\( \gamma \)R1 (137 nM) and much higher than that observed for Jak5-binding peptides \textit{in vitro} (1–20 \muM). The increase in effective affinity may be mediated by a dual contact of the Stat1 SH2 domain to phospho-IFN-\( \gamma \)R1 and of the Stat1 transactivation domain (where tyrosine-701 is located) to the active site of the jak.

As done in Supplementary Text 2, one can calculate average lifetime (\( \tau \)) of the various complexes that have Stat1 bound by taking the reciprocal of rate constants that destroy the bound state (i.e. \( \tau_s = 1/k_{-s} \times V_{\text{MAX}} = 1/V_{\text{MAX}} \times \tau_{\text{VMax}} = 1/V_{\text{MAX}} \times \tau_{\text{VMax}} \); conversion from dimers to monomers and from minutes to seconds follows. An inspection of ‘bound’ constants reveals that the average Stat1 would remain bound to the IFN-\( \gamma \)R1 COOH terminus and/or the Jak kinase for \( \tau_s = 1.15 \text{s} \times 1/52.25 \text{ min}^{-1} \times 60 \text{ s/min} \), Stat1 would remain unphosphorylated while bound to ‘fast’ state receptors for \( \tau_{\text{VMax}} = 0.181 \text{s} \times 1/165 \text{ dimers/min} \times 60 \text{ s/min} \times 1 \text{ dimer/2 monomers} \), and Stat1 would remain unphosphorylated while bound to ‘slow’ state receptors for \( \tau_{\text{VMax}} = 9.09 \text{s} \times 1/3.3 \text{ dimers/min} \times 60 \text{ s/min} \times 1 \text{ dimer/2 monomers} \). On average, almost eight Stat1 monomers will bind and dissociate from the COOH terminus before one gets phosphorylated in the ‘slow state’. On the other hand,
six of every seven Stat1 monomers bound to IFN-γR1 will get phosphorylated in the ‘fast’ state before they dissociate from the receptor complex, on average. The two states may exist to ensure that either an efficient activation of Stat1 or a very inefficient activation of Stat1 will occur.

**Supplementary Text 6. Modelling the superactivation of receptor complexes by numerical approximations of differential equations**

We decided to analyse several potential superactivation reactions. We assumed that superactivation reactions are irreversible (1) to simplify the chemical reactions, the derived differential equations and the resulting numerical approximations and (2) empirical errors in the data likely overwhelm errors caused by excluding de-superactivation. Auto-superactivation of a single activated receptor complex (i.e. RL → RL*) or interaction of a single activated receptor complex with a component that is in huge excess to the complex (i.e. RL (+ X) → RL*X) would be examples of unimolecular and pseudo-unimolecular superactivation reactions, respectively; these two reactions are indistinguishable kinetically. Interaction of one receptor complex with a different component that is in huge excess to the complex (i.e. RL → RL*Y) or of two receptor complexes with each other (i.e. RL + RL → RL*RL*) define bimolecular superactivation reactions; these two bimolecular reactions are distinguishable, but we modelled only the latter reaction. We modelled both unimolecular and bimolecular superactivation reactions, and defined $k_u$ and $k_b$ as rate constants governing the unimolecular and bimolecular superactivation reactions, respectively. Because the initially activated complex that becomes superactivated is itself the result of reaction A, we modelled the superactivation of the complexes as the second reaction of consecutive reactions. The reactions are shown as (C) and (D) in reduced form:

\[
R (+ L) \rightarrow RL \rightarrow RL^* (C) \\
2R (+ 2L) \rightarrow 2RL \rightarrow RL^* (D)
\]

To simplify the calculation of the model (and because IFN-γ remains bound to the receptor complex for so long; see Supplementary Text 2), we assume the ligand dissociation from the superactive state completely inactivates the receptor complex, resulting in the ‘R’ state. Note that the initially activated complex, RL, is an intermediate. To calculate the levels of intermediates of the (pseudo) unimolecular-unimolecular (uni-uni) and the (pseudo) unimolecular-bimolecular (uni-bi) consecutive reactions during the time course of the reaction, one must solve the differential equations describing the rate of change of levels of the intermediate that are derived from reactions (C) and (D). The respective equations are:

\[
\frac{d[RL_k]}{dt} = k_1[R]_k - k_{-1}[RL]_k - k_{2u}[RL]^*_k
\]

(9)

\[
\frac{d[RL^*_k]}{dt} = k_1[R]_k - k_{-1}[RL]_k - k_{2u}[RL^*_k]^2
\]

(10)

The amount of intermediate [RL]_k can be determined by integration of Equations (9) and (10). Integration of (9) is possible in closed form to yield an analytical solution for [RL]_k; however, integration of (10) is not possible in closed form, thus an exact solution for [RL^*_k] cannot be obtained. However, numerical point-to-point methods can give approximate solutions to (9) and (10). We tried various numerical methods to estimate the levels of the two intermediates: one of first order (Euler), three of second order (Runge-Kutta, a.k.a. midpoint, Raphson and Heun a.k.a. first-order predictor-corrector), three of third order (two of Runge-Kutta and the one by Heun) and the most commonly used of fourth order (Runge-Kutta). When comparing the three numerical methods with the exact solution of [RL]_k, we found that the fourth-order Runge-Kutta method most precisely approximated the exact solution, although the second- and third-order methods are accurate to less than 0.01% and 0.00001% deviation from the true value, when using 0.2 min time intervals, respectively (not shown). Because the order of discrepancy of the various solutions was similar in the uni-uni and the uni-bi methods (not shown), and because using smaller time intervals did not significantly change the shape or magnitude of the resultant curve; we reasoned that the fourth-order Runge-Kutta method also gave a highly accurate numerical solution to the uni-bi reactions, and used that method to determine the levels of intermediates and of superactivated complexes. The levels of superactivated complexes ([RL^*_k] and [RL^*_2k]) are determined by subtracting the levels of intermediate from the total amount of receptor bound by ligand (calculated as the total number of receptors present subtracted by the amount unbound by ligand) as shown in Equations (11a) and (11b), respectively:

\[
[RL^*_k] = \left[ R_0 - \frac{k_1 R_0}{k_1 + k_{-1}} e^{-(k_1 + k_{-1}) t} \right] - [RL]_k
\]

(11a)

\[
[RL^*_2k] = \left[ R_0 - \frac{k_1 R_0}{k_1 + k_{-1}} e^{-(k_1 + k_{-1}) t} \right] - [RL^*_k]
\]

(11b)

We assume that the receptors in the intermediate state exhibit slow-state kinetics, and that receptors in the final state exhibit fast-state kinetics. Once the numbers of intermediate and superactivated complexes are known at each time point (calculated from Equations (9) and (11a) or (11b) and (11b)), we cycle through the following steps:
through Equations (2) and (3) with Equation (11a) or with Equation (11b) using the fast-state values of $V_{MAX}$ and $K_M$, and again with Equation (9) or with Equation (10) using the slow-state values of $V_{MAX}$ and $K_M$. We then run a modified version of Equation (4), in which Stat1 is phosphorylated by both sets of complexes:

$$S_{t+\Delta t} = S_t - S_{t,\text{fast}} - S_{t,\text{slow}} \quad (12)$$

Here the appearance of the latency period is a direct consequence of the kinetic theory of consecutive reactions: in consecutive reactions, the final product as a function of time appears after a latency period if and only if the rates of the two consecutive reactions are not too different from each other. The initial appearance of final product is slower because time is needed for levels of the intermediate to build up; after the levels of this intermediate build up, the formation of the final product then proceeds at a more rapid rate.

References