Published in final edited form as:

Sci Signal. ; 2(66): pt2. doi:10.1126/scisignal.266pt2.

Molecular Origin and Functional Consequences of Digital Signaling and Hysteresis During Ras Activation in Lymphocytes

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Abstract

Activation of Ras proteins underlies functional decisions in diverse cell types. Two molecules, Ras-GRP and SOS (Ras-guanine nucleotide-releasing protein and Son of Sevenless, respectively), catalyze Ras activation in lymphocytes. Binding of active Ras to the allosteric pocket of SOS markedly increases the activity of SOS. Thus, there is a positive feedback loop regulating SOS. Combining *in silico* and *in vitro* studies, we demonstrate that “digital” signaling in lymphocytes (cells are “on” or “off”) is predicated on this allosteric regulation of SOS. The SOS feedback loop leads to hysteresis in the dose-response curve, which may enable T cells to exhibit “memory” of past encounters with antigen. Ras activation by Ras-GRP alone is “analog” (a graded increase in activation in response to an increase in the amplitude of the stimulus). We describe how the complementary analog (Ras-GRP) and digital (SOS) pathways act on Ras to efficiently convert analog input to digital output and make predictions regarding the importance of digital signaling in lymphocyte function and development.

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A presentation from the EMBO workshop “Visualizing Immune System Complexity,” Centre d'Immunologie Marseille-Luminy, Marseille, France, 15 to 17 January 2009.

Editor's Note: This contribution is not intended to be equivalent to an original research paper. Note, in particular, that the text and associated slides have not been peer-reviewed.

Citation: A. K. Chakraborty, J. Das, J. Zikherman, M. Yang, C. C. Govern, M. Ho, A. Weiss, J. Roose, Molecular origin and functional consequences of digital signaling and hysteresis during Ras activation in lymphocytes. *Sci. Signal.* **2**, pt2 (2009).

Presentation Notes

Slide 1: Science Signaling logo

The slide show and notes for this presentation are provided by *Science Signaling* (www.sciencesignaling.org).

Slide 2: Molecular Origin and Functional Consequences of Digital Signaling and Hysteresis During Ras Activation in Lymphocytes

This talk presents a new aspect of the membrane-proximal signaling machinery in lymphocytes that may have important functional consequences. The work is the result of a very close collaboration that was initiated by Art Weiss and me in 2006 when Jeroen Roose was a member of Art's laboratory. Jeroen now has his own laboratory, and so this is now a three-way collaboration. From my laboratory, the principal contributions were made by Jayajit Das (a postdoctoral fellow), who has since started his own laboratory at Ohio State University.

Slide 3: Cells signal when stimulated

The question we have considered here can be posed quite generally. When cells are stimulated through their receptors, downstream signaling molecules can be activated. If cells are weakly stimulated—for example, by just a few ligands—the basal level of active signaling is maintained, and no new cellular response is initiated. If the stimulus increases in a continuous (or analog fashion)—for example, by increasing the number of ligands—then cells could respond in one of two ways, which are illustrated in the next slide.

Slide 4: Cells can make binary decisions

When the number of ligands is continuously increased, the cells can respond in one of two ways. The entire population of cells could continuously increase levels of signaling activity (shown in red). Such a continuously increasing response to a continuous increase in stimulus may be termed “analog.” Alternatively, beyond a threshold stimulus level, the population of cells could split into two subpopulations, one that has turned on a large amount of signaling molecules and the other that maintains a basal amount of signaling. Such a response may be termed, for comparison, “digital.” T lymphocytes have been observed to exhibit digital signaling (1). In this presentation, I briefly describe the molecular machinery that enables lymphocytes to signal in a digital fashion, as well as some potential functional consequences of this ability. The work integrates theoretical and computational studies (rooted in physics and engineering) with genetic and biochemical studies [see (2,3) for details].

Slide 5: Membrane-proximal TCR signaling

One of the earliest events following T cell receptor (TCR) stimulation is the recruitment and activation of a Src kinase family member, Lck, which then phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) of the antigen receptor complex. Doubly phosphorylated ITAMs bind the kinase ZAP-70 (in T cells) or Syk (in B cells). Active ZAP-70 phosphorylates tyrosines on an adaptor protein called LAT, which then assembles a signaling complex made up of a number of proteins. This signaling complex is assembled in a cooperative way (3). For now, it is important to note that phospholipase C- γ , a protein that is part of the LAT signaling complex and that produces diacylglycerol (DAG), activates the Ras guanine nucleotide exchange factor (GEF) Ras-guanine nucleotide-releasing protein (Ras-GRP1) in a DAG-dependent way. This GEF catalytically converts inactive guanosine diphosphate (GDP)-bound Ras proteins to the active guanosine triphosphate (GTP)-bound form. Lymphocytes have another family of GEF proteins, Son of Sevenless (SOS), which also activate Ras proteins. SOS is recruited to the LAT complex through a protein called Grb2. We were initially intrigued by the fact that lymphocytes have two Ras GEFs that couple to antigen receptor–signaling

pathways, and we wondered why two very different GEFs were used. Let's focus first on the characteristics of Ras activation by SOS.

Slide 6: SOS has an allosteric site that can bind Ras

The catalytic activity of SOS has been characterized with crystallography and biochemical analyses by the Kuriyan and Bar-Sagi groups (4–6). The catalytic domain of SOS binds GDP-associated Ras and distorts the Ras molecule, which results in release of GDP. This then allows abundantly present cellular GTP to bind to Ras. The basal catalytic activity of SOS is low. Distal from the catalytic domain, SOS has another site that also binds nucleotide-associated Ras. If Ras-GDP is bound to this site, the GEF activity of SOS becomes modestly faster. However, if Ras-GTP is bound to the allosteric site, the catalytic rate is ~75 times as fast as it would be if Ras-GTP were not bound at the distal site; i.e., if the product of the catalytic step binds to the allosteric site, the rate of catalysis is significantly enhanced. Thus, the GEF activity of SOS is regulated by a positive feedback loop.

Slide 7: A positive feedback loop

We first explored the consequences of this positive feedback loop on SOS-mediated Ras activation as if SOS were the only GEF involved in activating Ras. For this, one can construct a very simple model. Either Ras-GDP or Ras-GTP can bind to the allosteric site of SOS. The catalytic rate of Ras activation depends on which nucleotide-associated Ras is bound to the allosteric site. Ras–guanosine triphosphatase–activating proteins (Ras–GAP proteins) accelerate the hydrolysis of Ras-GTP, thereby converting it to the inactive Ras-GDP form. We first asked how much Ras is activated as a function of the amount of SOS targeted to the membrane (which is a reflection of the amount of stimulus delivered to the TCR). The answer depends on how many times each type of reaction shown in this slide occurs during a given time. Because biochemical reactions are stochastic events, the answer will be different for each cell. At first, we ignored these cell-to-cell fluctuations and obtained the average amount of active Ras molecules in a population of cells (as would be measured in a Western blot assay).

Slide 8: A deterministic model

The average number of activated Ras molecules can be calculated by describing the signaling module in the language of ordinary differential equations. The inputs to these equations are the rate parameters and numbers of molecules of Ras and Ras-GAPs that are present. Most of the rate parameters shown have been measured.

Slide 9: Bistability and sharp responses

This graph shows the calculated amount of Ras-GTP as a function of the amount of available SOS (or the stimulus level). At low levels of stimulus, the number of active Ras molecules is low; conversely, at high levels, the number of active Ras molecules is high. At intermediate levels of stimulus, the mathematical equations predict that there are three possibilities for the amount of active Ras. However, the mathematical equations also show that the points marked in blue are unstable to the smallest perturbations, which implies that they would not be observed in an experiment. So, two possible amounts of active Ras for the same stimulus level are predicted; there is a bistability. Moreover, these results suggest that SOS-mediated Ras activation could lead to a sharp jump in the number of active Ras molecules beyond a threshold stimulus (at the point labeled “A”). This qualitative behavior (bistability) is robust to variations in the unknown parameters over wide ranges.

Slide 10: Stochastic simulations

Given that this possibility of a digital response for Ras was robust to parameter variations, we explored the implications for Ras activation when the SOS signaling module is integrated into

the lymphocyte signaling network. Because stochastic cell-to-cell variations can be important, we accounted for them by solving the Master equations corresponding to the signaling network using a Gillespie algorithm (7). Several replicate simulations are run—each simulation is analogous to assaying a single cell in a population. The results for the amount of Ras activated in each simulation are represented in the form of a histogram. This is analogous to the way one displays results of a FACS (fluorescence-activated cell sorting) experiment, combining the results of many cells, such as many lymphocytes, in one histogram. Because many of the parameters required to simulate the signaling network depicted in the figure are unknown, we performed an extensive parameter-sensitivity analysis (2). This analysis suggested that the qualitative phenomena presented next are robust to variations of most parameters. The necessary conditions for this robustness are as follows: (i) the GEF activity of SOS is subject to positive feedback regulation, which is true (4); (ii) Ras-GAPs deactivate Ras in a catalytic fashion, which is also true (8); and (iii) the GEF activity of Ras-GRP is not so high that it converts all of the Ras proteins to active Ras before the SOS feedback loop is engaged.

Slide 11: Simulations and experiments show bimodal responses

SOS must be targeted to the membrane in order to remove inhibition of its catalytic domain. We begin our description of the qualitative phenomena by showing results of calculations that simulate a situation where different amounts of the catalytic domain of SOS (SOS_{cat}) are present. The top panels show the amount of active Ras in many *in silico* cells. When SOS_{cat} is low, the population of cells exhibits small amounts of active Ras (left). Beyond a threshold amount of SOS_{cat} , the population splits into two subpopulations, with one having many active Ras molecules and the other exhibiting basal levels (middle). Thus, the bistability shown in slide 9 is manifested as digital Ras activation. This prediction was tested by FACS experiments assaying single Jurkat T cells in a population into which varying amounts of SOS_{cat} were transfected. The results (bottom panels) mirror the computational predictions. Because an antibody for assaying Ras-GTP directly is not available, the experiments measured either the abundance of CD69 (a marker of lymphocyte activation) or the abundance of phosphorylated extracellular signal-regulated kinase (ERK) (pERK). The numbers in the corners represent the percentage of cells above and below the “threshold” line.

Because these are downstream targets of Ras activation, one could argue that the bistability observed in experiments is a consequence of signaling modules downstream of Ras activation. We carried out many complementary computational and experimental studies to examine whether SOS-mediated Ras activation predicated the measured digital response of downstream markers (2); a few examples of such investigations are presented in the next slides.

Slide 12: Ras-GRP primes the SOS feedback loop

One indication of the importance of allosteric regulation of SOS-mediated Ras activation is revealed by studying the effects of Ras-GRP deficiency. In *in silico* and *in vitro*, Ras-GRP-deficient cells exhibit bimodal responses only when the stimulus is high, as compared with wild-type cells that showed a bimodal response at the intermediate stimulus. The computational studies suggest that this is because Ras-GRP is not subject to feedback regulation. Therefore, Ras-GRP activates the first Ras proteins, which then bind to the allosteric site of SOS to prime its feedback loop. From this, we can predict that introduction of an exogenous form of Ras that binds to the allosteric pocket of SOS, but fails to mediate downstream signaling, should restore bimodal signaling in Ras-GRP-deficient cells at the lower-level stimulus.

Slide 13: Allosteric regulation predicates digital response

A Ras mutant with such properties is available. One mutation allows it to function like Ras-GTP in binding to the allosteric site of SOS, and another prevents it from binding Raf (a target of active Ras). Introducing this Ras mutant exogenously into Ras-GRP1-deficient cells restores

bimodal signaling at the same stimulus as that which produced bimodal signaling in the wild-type cells (left). Note that bimodal signaling is not restored if this Ras mutant is added in a system where the allosteric pocket of SOS has been mutated to prevent binding of Ras (right). These data suggest that digital signaling is predicated on feedback regulation of GEF activity of SOS. But one could argue that this is because feedback regulation of SOS is necessary to obtain large amounts of active Ras, which then prime downstream feedback loops where digital signaling originates (such as that seen for pERK). We have done a number of studies to examine whether digital signaling in lymphocytes is only predicated on the GEF activity of SOS or controlled by it.

Slide 14: Intermediate levels of Ras-GRP for efficient digital responses

The computational results shown in this slide indicate that, as the amount of Ras-GRP is increased, digital Ras activation occurs at lower stimulus levels. But they also predict that if the amount of Ras-GRP is too high, Ras activation will be analog in character. It is interesting that the most efficient digital Ras responses occur when the amount of the GEF that mediates analog signaling (Ras-GRP) is at an intermediate level. From the standpoint of the importance of allosteric regulation of SOS in digital responses of lymphocytes, the more relevant prediction is that stimulation of lymphocytes through the Ras-GRP pathway alone should exhibit analog Ras activation.

Slide 15: Stimulation engaging only Ras-GRP (using PMA) does not lead to bimodal response even at high doses

We have performed computer simulations and experiments with receptor stimulation of lymphocytes (T cells and B cells) (2). The time courses for Ras activation (in simulations) and phosphorylated ERK (pERK) (in experiments) for wild-type systems showed a bimodal distribution of active signaling molecules at intermediate time points (not shown). In contrast, the computer simulations for a system without SOS showed the temporal evolution of a single peak to higher amounts of active Ras (red histograms). If the cells are stimulated through the Ras-GRP pathway alone to produce large amounts of Ras signaling, and the downstream signaling markers exhibit analog signaling, this would indicate that downstream signaling modules do not translate large amounts of active Ras into digital outputs. Indeed, the phospho-ERK response (gray histograms) that results from stimulation of T cells with phorbol 12-myristate 13-acetate (PMA), which only engages the DAG-dependent Ras-GRP pathway, results in an analog response. The numbers are the results of a statistical test to determine whether the response is unimodal or not.

Slide 16: Analog signaling without SOS

Although I do not present other experimental results suggesting that feedback regulation of GEF activity of SOS is a dominant factor in digital signaling observed for markers downstream of Ras, these results are summarized. These results show that digital signaling in the DT40 B cell line (9) is abrogated in the corresponding SOS^{-/-} mutants. Primary T cells also exhibit digital signaling upon receptor stimulation, but not when stimulated using PMA.

Slide 17: Hysteresis in the dose-response curve

Next, I describe a phenomenon that is a somewhat less anticipated consequence of bistability that can be directly tested by population-level assays of Ras activation and that may have an interesting functional consequence. The phenomenon is illustrated by the same graph displayed earlier showing the characteristics of the SOS module. If we increase stimulus, the dose-response curve should exhibit a sharp jump at the point labeled A, but, if a cell is first strongly stimulated, then reducing the stimulus is predicted to lead to a different dose-response curve with the sharp jump down occurring at the point labeled B. Thus, the dose-response curve

depends on whether it is obtained by stimulating cells with increasing doses of stimulus or whether it is measured by reducing the stimulus after robust stimulation. This behavior is termed hysteresis and is a direct consequence of bistability due to feedback regulation of the GEF activity of SOS. The biochemical reason for this is clear. When “naïve” cells are stimulated at intermediate doses of antigen, most SOS proteins do not have Ras-GTP bound to the allosteric site, but, when previously stimulated cells are subjected to lower stimulus doses, many SOS molecules will already have Ras-GTP bound to the allosteric site. Thus, for the same stimulus dose, one should observe a larger amount of active Ras because the catalyst, SOS, is more active. Of course, this effect only manifests itself over a finite period of time.

Slide 18: Simulations confirm hysteresis in the dose-response curve

This graph demonstrates that computer simulations of the signaling network shown in slide 5 do show hysteresis. This prediction from the computer simulations has been tested in two ways, one of which is described in more detail because it directly pertains to a possible functional consequence.

Slide 19: Testing the prediction of hysteresis

Jeroen and Art designed a way to test for hysteresis with a drug called PP2, which inhibits kinases of the Src family and so acts on the signaling network upstream of Ras activation. By fixing the stimulus level to one that leads to robust Ras activation, one can change the PP2 dose to produce different effective stimulus levels. PP2 can be added with the stimulus—in order to obtain the dose-response curve “on the way up”—or PP2 can be added after cells have first been robustly stimulated (after 3 min)—in order to obtain the dose-response curve “on the way down.” A hysteretic response would imply that the same dose of PP2, depending on whether it is added initially or after robust stimulation, would lead to different amounts of active Ras.

Slide 20: Hysteresis in Ras-GTP signaling

Experiments that directly analyze the amount of active Ras (Ras-GTP) in a population of cells are consistent with this prediction of hysteresis. Ras-GTP was pulled down, analyzed by Western blotting, and quantified in either Jurkat T cells [including those preloaded with an inhibitor of mitogen-activated protein kinase kinase (MEK, the kinase upstream of ERK)] or DT40 B cells. Note that SOS^{-/-} mutant B cells do not exhibit hysteresis. Although several aspects of these results are consistent with a hysteretic response in Ras activation, they also point to couplings of the Ras activation modules with downstream signaling modules that we do not yet understand [see (2) for details].

Slide 21: Molecular “memory” for signal integration in vivo?

The hysteretic response associated with feedback regulation of SOS-mediated Ras activation may have an interesting functional consequence. Today, two-photon imaging experiments are making vivid the interactions of T cells with dendritic cells (DCs) in lymphoid tissues of living mice. One finding that emerged from these studies is that the pattern of T cell motion has three phases. In phase one, T cells migrate rapidly in the tissue, making brief contacts with DCs (left movie, T cells are shown in green and DCs bearing antigen are shown in red). In phase 2, which occurs after a time that depends on the dose and type of antigen (10,11), T cells make extended contacts with antigen-bearing DCs (right movie). In a third phase, activated T cells egress from the tissue rapidly.

Slide 22: Signal integration in vivo?

Data from several studies (10,11) suggest that, during phase one, T cells may have a way to integrate signals from multiple interrupted encounters with antigen-bearing DCs. The evidence

supporting this argument is briefly summarized [see (10,11) for details]. However, a molecular mechanism that enables such short-term molecular memory of encounters with antigen was not known.

Slide 23: Hysteresis and bistability may enable signal integration

We suggest that the hysteresis characterizing Ras activation in lymphocytes might be the molecular mechanism enabling such short-term molecular memory. This is illustrated by computational results shown in this slide. We calculated the amount of activated Ras after robust stimulation. We then withdrew the stimulus, and the amount of active Ras began to decline. We then restimulated the *in silico* cells with a weak stimulus that does not result in robust Ras activation for cells that have not previously seen antigen. Previously stimulated cells rapidly activated large amounts of Ras in response to this second weak stimulus. The three panels show results for different periods of time when the stimulus was absent. Molecular memory effects are in place as long as the resting period is not too long. Calculations show that the amount of active Ras must not decline below the unstable points (see slide 9) corresponding to the second weak stimulus for robust and rapid Ras activation to occur. Note also that the effects shown in this slide are absent if the GEF activity of SOS was not subject to allosteric feedback regulation (not shown).

Slide 24: “Memory” due to hysteresis is SOS-dependent

Jeroen and Art tested these predictions with a technique developed by Art in a different context more than 20 years ago. Concanavalin A (ConA) is a plant lectin that robustly stimulates lymphocytes and that is inhibited by α -methyl mannoside (α -MM). Wild-type DT40 B cells are robustly stimulated by ConA, and the addition of α -MM results in a decay of the amount of active Ras. After a period during which the stimulus is absent, restimulation with a very low B cell receptor (BCR) stimulus (which does not stimulate Ras activation in previously unstimulated cells) results in rapid and robust Ras activation. This result is consistent with predictions emerging from the computational results presented in the previous slide. The left panels show that SOS^{-/-} DT40 B cells do not exhibit this short-term molecular memory, which indicates that this behavior is SOS-dependent.

Slide 25: Digital and analog signaling in T cell development

We have argued that allosteric feedback regulation of the GEF activity of SOS may underlie differences in the positive and negative selection thresholds that influence T cell development (3), but, for this lecture, this is the end.

Slide 26: Summary

The main results presented in this talk are summarized in this slide.

Acknowledgments

Slide 27: Funding: The work done in my laboratory (A.K.C.) was supported by the National Institutes of Health (NIH) through the indicated mechanisms. The work done in the Weiss lab (A.W.) was support by funds from the Howard Hughes Medical Institute and the Rosalind Russell Medical Research Center for Arthritis. Work by J.R. was supported by NIH and the Arthritis Foundation.

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