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Lymphocyte signaling : beyond knockouts

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Abstract

The analysis of lymphocyte signaling was greatly enhanced by the advent of gene targeting, which allows the selective inactivation of a single gene. Whereas this gene ‘knockout’ approach is often informative, in many cases the phenotype resulting from gene ablation might not provide a complete picture of the function of the corresponding protein. If a protein has multiple functions within a single or several signaling pathways, or stabilizes other proteins in a complex, the phenotypic consequences of a gene knockout may manifest as a combination of several different perturbations. In these cases, gene targeting to ‘knockin’ subtle point mutations might provide more accurate insight into protein function. However, to be informative, such mutations must be carefully designed based on structural and biophysical data.

The study of lymphocyte signal transduction has changed greatly over the last 25 years. In the 1980’s, the main approach was protein biochemical analysis of lymphoid cell lines or of cultured human primary lymphocytes. Genetic approaches in these systems were limited primarily to overexpression of constitutively active or dominant negative signaling proteins, usually in the presence of endogenous wild-type protein. With the advent of gene targeting technology in the 1990’s, it became possible to study primary murine lymphocytes missing individual signaling molecules (gene ‘knockouts’) and thereby to directly examine protein function without overexpression. Knockout animals also facilitated access to a broad range of lymphocyte subpopulations at distinct stages of differentiation; for example, in the T cell lineage it became possible to analyze signaling in developing thymocyte subsets, in naïve CD4⁺ and CD8⁺ TCRαβ⁺ and TCRγδ⁺ T cells, and in differentiated effector subsets including T helper type 1 (T_H1) and T_H2 CD4⁺ T cells and CD8⁺ cytotoxic T cells. The physiological role of signaling molecules could also be investigated in the context of whole animal processes (e.g. during an immune response or in autoimmunity).

One obvious limitation of straightforward gene knockouts is that if a molecule is essential for antigen receptor signaling, deletion of the gene often results in a developmental block, because of the requirement for antigen receptor or pre-antigen receptor signals during the transition of cells through differentiation checkpoints. For example, inactivation of the ZAP70 tyrosine kinase or of the LAT and SLP76 adaptor proteins results in a strong block in thymic development and an absence of mature T cells^{1–5}. Thus it is not possible to study the role of these molecules in T cell biology using standard gene knockout technology.

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Furthermore, even if a mutation allows development of mature lymphocytes, it is possible that phenotypes observed in these cells are due to changes occurring during development in the thymus or bone marrow, and may not accurately reflect the acute requirement for a given signaling molecule in a mature lymphocyte. Finally, in some cases where genes play important roles outside the lymphoid system, germline gene deletion results in embryonic lethality and again no analysis is possible in mature lymphocytes.

These problems can be addressed with conditional knockouts, using the Cre-loxP system to limit deletion of the target gene to specific cell lineages (e.g. lymphoid cells), or stages of development (e.g. starting during the CD4⁺CD8⁺ stage of thymocyte development)⁶. For example germline deletion of the gene encoding the Rac1 GTPase causes early embryonic lethality, which can be circumvented using a conditional loxP-flanked allele and lymphoid-restricted expression of the Cre recombinase^{7–9}. While most use of the Cre-loxP system in lymphocytes has taken advantage of lineage-restricted or inducible transgenes expressing Cre, more recently it has become possible to deliver Cre to mature lymphocytes in a variety of ways, including a cell-permeable Cre or by adenoviral gene transfer^{10–12}.

However, even with careful spatial and temporal control of gene deletion, the phenotype of gene knockouts can give incomplete or paradoxical results. In some cases, a protein containing several different domains may exert multiple biochemical functions on a single signaling pathway, or may serve as a cross-talk point linking several signaling pathways. Complete deletion of such a protein would preclude dissection of its distinct functions. Even proteins with only one biochemical function may influence multiple physiological processes. As the phenotype resulting from complete ablation of the protein will represent the aggregate consequence of disrupting all physiological processes in which the protein is involved, effects on individual signaling pathways and processes may be obscured. In contrast, partial loss-of-function mutations could separate these different roles. In addition, distinct proteins constituting parts of a complex of proteins may stabilize each other. Removal of one member of the complex may cause the other partner proteins, which might exert distinct biochemical activities, to be degraded. Lastly, complete removal of a protein may allow a related family member to take the place of the deleted protein, thereby wholly or partially masking the functional impact of the deleted protein.

Pitfalls associated with each of these cases can potentially be circumvented by the use of gene targeting to create subtle point mutations in a gene ('knockin' mutations), an approach which has become more common over the last decade. In this Perspective we discuss each of the above hypothetical cases using examples where these issues have been addressed using knockin mutations into proteins involved in T cell antigen receptor (TCR) signaling.

Proteins with multiple biochemical functions

The Vav1 protein contains 8 distinct domains (Fig. 1). Its only known enzymatic activity is as a guanine nucleotide exchange factor (GEF) for Rho-family GTPases¹³. This activity resides in the Dbl homology (DH) domain. However the presence of other domains, particularly SH2 and SH3 domains, led to the suggestion that it may also function as an adaptor protein¹⁴. Germline deletion of Vav1 indicated that Vav1 plays an important role in

signal transduction downstream of the TCR13. Absence of Vav1 resulted in impaired calcium flux, activation of the Erk MAP kinase, protein kinase D, and phosphatidylinositol-3-kinase (PI3K), and integrin activation after TCR cross-linking; these defects lead to reduced T cell activation and cytokine synthesis.

From the knockout it is not possible to tell whether the GEF activity of Vav1 is required for all, some or none of the observed consequences of Vav1 deletion. However the issue has been resolved by inserting a mutation into the DH domain of Vav1, which eliminates the GEF activity but does not affect DH domain folding or the integrity of any other domain¹⁵. Analysis of this mutant shows that GEF activity is indeed important for Vav1 function, but that Vav1 GEF activity is only required for transduction of TCR signals in a subset of Vav1-dependent pathways. The knockin mutant revealed that in the absence of Vav1 GEF activity, TCR-induced PI3K activation is defective but calcium flux and Erk activation are normal. In addition, the GEF activity of Vav1 is largely dispensable for passage through the pre-TCR checkpoint of T cell development, which is blocked in Vav1-knockout animals. Thus, signals transmitted during distinct stages of T cell development differentially 'utilize' the GEF and non-GEF functions of Vav1, a conclusion that could not have been made using a conventional knockout.

The LAT transmembrane adaptor protein contains nine conserved tyrosine residues in its cytoplasmic domain (Fig. 1). TCR signaling results in rapid phosphorylation of these tyrosine residues, which in turn act as docking sites for the recruitment of other signaling proteins. Proteins that bind either directly or indirectly to these phosphorylated tyrosine residues include phospholipase C (PLC)- γ 1, PI3K, Vav1, the adaptor proteins Grb2, Gads and SLP76, the tyrosine kinase Itk and the Cbl E3 ubiquitin ligase. Individual tyrosine residues bind different proteins *in vitro*. For example, whereas phosphorylated Tyr136 binds PLC- γ 1, phosphorylation of Tyr175 and Tyr195 generates binding sites for Grb2 and Gads, and phosphorylated Tyr235 can bind Grb216. The importance of LAT in T cell signaling is illustrated by the phenotype of LAT knockout mice, which exhibit a complete block in T cell development at the pre-TCR checkpoint⁵. Mice bearing knockin point mutations in LAT have been generated and provide some insight into the roles of the different LAT tyrosine residues. Mutation of the three distal tyrosines (Tyr175, Tyr195 and Tyr235) or of these three residues as well as Tyr136 results in the same developmental block as seen in LAT knockout mice, showing the importance of these residues for LAT function^{17,18}. In contrast, mice bearing a mutation only in Tyr136 develop lymphadenopathy and splenomegaly and show signs of autoimmunity^{19–21}. These mice accumulate T cells producing large amounts of interleukin 4, which leads to eosinophilia, and an accumulation of plasma cells secreting IgE. Biochemical analysis indicates that the Y136F mutation abrogates TCR-induced PLC- γ 1 activation and calcium flux, in agreement with the observation that PLC- γ 1 binds to p-Tyr136. The more severe T cell development phenotype seen in mice bearing mutations in the three distal tyrosine residues is likely due to the role of these residues in recruiting Gads and/or Grb2. Thus, different LAT tyrosine residues influence distinct biochemical pathways and thereby play different roles in positive and negative regulation of T cell activation. Tyr136, for example, appears to have a more prominent role in the negative regulation of T cell activation. A knockin approach was needed to assign different functions to different parts of the protein.

The SLP76 cytoplasmic adaptor protein is able to bind to a number of other signaling proteins through several different domains, including a cluster of three tyrosine residues that become phosphorylated upon TCR activation (Tyr112, Tyr128 and Tyr145), a proline-rich (PR) domain and an SH2 domain (Fig. 1). As with LAT, germline deletion of the gene encoding SLP76 results in a complete block in T cell development at the pre-TCR checkpoint^{3,4}, and knockin mutations revealed that the three tyrosine residues perform distinct functions. Whereas all three tyrosine residues are required for TCR-induced calcium flux, Erk activation and phosphorylation of PLC- γ 1, Tyr112 and Tyr128 were required for TCR-induced phosphorylation of Vav1, whereas Tyr145 was more important for the activation of Itk²². Interestingly, compound mutant mice expressing the Y112F,Y128F SLP76 mutant protein from one allele and the Y145F mutant from the other allele show near normal T cell activation, suggesting the involvement of at least two SLP76 molecules in a multimeric signaling complex. In contrast, mutations in the PR region and the SH2 domain result in different phenotypes. Both mutations perturb T cell activation, but to a lesser extent than mutations in the tyrosine residues²³. Furthermore, the PR domain was required for a normal calcium flux, whereas the SH2 mutant did not affect this pathway. Once again, subtle mutations were needed for fine dissection of SLP76 function.

Partial loss-of-function mutations

The ZAP70 protein tyrosine kinase plays an important role in transducing TCR signals. ZAP70 knockout mice show a complete block in thymocyte positive selection and contain no mature T cells^{1,2}. A spontaneously arising point mutation (R464C) in a conserved region of the kinase domain of ZAP70 (Fig. 1) completely eliminates kinase activity, and results in a phenotype identical to that of ZAP70 knockout mice, demonstrating that the kinase activity of ZAP70 is essential for normal thymocyte positive selection²⁴.

Interestingly, however, a series of chemically-induced point mutations in ZAP70 (I367F, W504R and the compound I367F,W504R mutation) affect kinase activity to different degrees and result in a graded spectrum of phenotypes²⁵. Different physiological processes showed different sensitivity to reductions in ZAP70 kinase activity. Mild reductions in activity caused a loss of CD4⁺ T cell positive selection, whereas a moderate reduction caused failure of thymic negative selection, germinal center memory antibody responses and regulatory T cell development. Finally, an even stronger reduction in activity was required to eliminate T cell help for IgE and IgG autoantibody production, positive selection of CD8⁺ T cells and maintenance of CD4⁺ and CD8⁺ memory T cells. As a consequence, a moderate reduction in ZAP70 activity resulted in autoimmunity, a phenotype that was not seen in ZAP70 knockout animals. Interestingly, an autoimmune arthritis had been previously reported in mice bearing a spontaneous point mutation in ZAP70 (W163C)²⁶. Together, these results illustrate that an allelic series of point mutants with partial loss-of-function can reveal roles for a protein in a range of physiological processes that could not be seen with a straightforward null mutant. Despite these insights, it remains unclear in the case of the ZAP70 mutants whether the observed phenotypes reflect roles for ZAP70 in TCR signaling in mature T cells, or are a consequence of altered thymic selection. A resolution of this issue will require the use of conditional stage-specific point mutations, where wild-type ZAP70 is expressed during thymic development, but then expression switches to the mutant protein in

mature T cells. The methodology required for these experiments has recently become available²⁷.

Proteins in complexes

Class IA PI3K heterodimers consist of a catalytic subunit (p110 α , p110 β , p110 δ) constitutively bound to an adaptor protein (p85 α , p55 α , p50 α , p85 β , and p55 γ) (Fig. 1)²⁸. This association is important for protein stability, as shown in a number of knockout models. Whereas disruption of the *Pik3r1* gene eliminates p85 α , p55 α and p50 α proteins (pan-p85 α knockout), it also causes an increase in the quantity of p85 β and a decrease in the quantity of p110 α , p110 β and p110 δ proteins^{29,30}. Conversely, knockout of p110 δ results in decreased quantities of p85 α , p55 α and p50 α ³¹. Thus interpretation of the phenotypes of these mutants is complicated, as they could be due to absence of the targeted protein, or to secondary changes in the quantities of associated proteins. To avoid these complications, another study knocked in a point mutation (D910A) into p110 δ , which eliminates kinase activity but leaves the protein otherwise intact. In this case, the quantities of p85 α , p55 α , p50 α , p110 α , p110 β and p110 δ remained unchanged, and thus any observed phenotypic changes could be ascribed to loss of p110 δ kinase activity³². Although the B cell phenotypes in the p110 δ knockout and knockin mutants appear to be similar, it is only because of the knockin mutant that we can be sure that they are caused by a lack of p110 δ kinase activity and not effects on other proteins.

The class IB PI3K is a heterodimer consisting of a p110 γ catalytic subunit and a p101 adaptor protein. Both a knockout and a knockin mutant of p110 γ have been generated. The latter mutation, K833R, eliminates kinase activity. Remarkably, whereas both mutants show a similar reduction in chemokine receptor function in hematopoietic cells, they show clear differences in signaling in cardiomyocytes^{33–35}. In p110 γ knockout cardiomyocytes, contractility is increased, but this effect is not seen in the knockin mutant. This phenotypic difference revealed an unexpected kinase-independent role of p110 γ as an adaptor protein. In its capacity as an adaptor protein, p110 γ binds to phosphodiesterase 3B (PDE3B), an enzyme necessary to degrade cAMP, a second messenger that regulates cardiac contractility. Once again the knockin mutation was able to provide mechanistic insight that could not be obtained from the knockout.

Compensation by family members

The Cbl E3 ubiquitin ligase negatively regulates TCR signaling by causing ubiquitination and subsequent degradation of key signaling proteins³⁶. Cbl contains a number of domains, including a tyrosine kinase binding (TKB) domain that binds to substrates, a RING finger domain that binds to an E2 ubiquitin ligase, and a proline-rich domain (Fig. 1). Unlike the Cbl knockout, a point mutation (C379A) that inactivated the RING finger domain of Cbl resulted in embryonic lethality³⁷. The most likely explanation for this discrepancy is that in the knockout, the related Cbl-b protein is able to take the place of Cbl, whereas in the C379A knockin mutant, access is blocked by the mutant Cbl protein. This interpretation is supported by the Cbl, Cbl-b double knockout, which also dies *in utero*. Thus, in this instance, a knockin mutation was able to reveal a fuller picture of the physiological role of

the protein than could be seen with a conventional knockout mutation. Nonetheless, it is clear from this example, and others, that the knockout and knockin mutants provide complementary information, emphasizing the utility of studying both genetic alterations in parallel.

Interestingly, a knockin mutation into the TKB domain of Cbl (G304E) illustrates another important reason for taking the time to use knockin gene targeting strategies to study protein function. Studies in the Jurkat T cell line had shown that this mutation eliminated negative regulation of the ZAP70 kinase, leading to its hyperphosphorylation³⁶. However this phenotype was not observed in the knockin mice, in which phosphorylation of ZAP70 was unaffected as were downstream ZAP70-dependent pathways, such as TCR-induced activation of Erk and Akt. These findings underscore the dangers associated with extrapolation of conclusions from transformed cell lines to signaling processes in primary lymphocytes³⁸.

Concluding remarks

The ability to use gene targeting to introduce essentially any mutation into the mouse genome has allowed the study of mutated signaling proteins in primary lymphocytes. Such knockin mutations are shedding light on mechanistic aspects of signaling processes that could not have been deciphered using knockout mutations. Clearly, in the study of any new signaling protein it will usually be advisable to make the null mutation by knocking out gene function fully. However, where insights into protein structure allow, these should be combined with point mutants to enable accurate analysis of protein function in distinct signaling pathways.

In this regard, knowledge of protein structure is a prerequisite for the rational design of such mutations. To understand the role of any given domain it is important to mutate one or more residues critical for function, but not for domain folding. Such unfolded domains could destabilize the rest of the protein leading to its degradation, could interfere with the function of other domains, or, by exposure of hydrophobic surfaces, could lead to protein insolubility. All of the above phenomena would simply result in replication of the knockout phenotype. Similarly, domain deletions can also affect the remainder of the protein, as domains often interact with each other in a native structure, and removal of one domain can affect the folding, solubility or function of another. In the case of some domains, such as kinase or SH2 domains, many atomic resolution structures are available, and so it is fairly straightforward to design mutations that eliminate function of the domain without unfolding it. However, in other cases, less structural insight may make it difficult to identify suitable residues to mutate. In such cases it is advisable to first carefully examine the effects of planned point mutations *in vitro*, looking at domain function and stability, before committing effort to the generation of a knockin mouse mutant.

The analysis of signal transduction in lymphocytes has changed over the last 25 years, driven primarily by the availability of genomic sequences and by technical improvements in genetic manipulation. Access to genomic sequences has made possible whole genome analysis, thereby allowing simultaneous analysis of multiple signaling pathways. The

genetic analysis of signaling has become easier with the advent of gene targeting, allowing the generation of constitutive knockouts, conditional knockouts, and point mutations of any gene in the mouse genome. Most recently, a combination of these approaches has allowed the generation of conditional knockin mutations²⁷. Thus it is now possible to generate specific point mutations in a tissue-specific or inducible manner, allowing an unprecedented level of genetic control during the analysis of signaling pathways.

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Figure 1.

Domain structure of signaling proteins discussed in the text. Location of amino acids mutated in knockin mice is shown. Abbreviations of domain labels: CH, calponin homology; Ac, acidic; DH, Dbl homology; PH, pleckstrin homology; C1, protein kinase C homology 1; SH3, Src homology 3; SH2, Src homology 2; TM, transmembrane; PR, proline-rich; C2, Protein kinase C homology 2; PI3/4-K, phosphatidylinositol-3 and -4 kinase; Rho-GAP, Rho GTPase activating protein homology; TKB, tyrosine kinase binding; RING, RING finger.