Immunity, Vol. 13, 1–3, July, 2000, Copyright ©2000 by Cell Press

Xid-like Phenotypes: A B Cell Signalosome Takes Shape

Minireview

David A. Fruman,* Anne B. Satterthwaite,† and Owen N. Witte‡§
*Department of Molecular Biology and Biochemistry
University of California, Irvine
Irvine, California 92697
†Department of Internal Medicine and
Harold C. Simmons Arthritis Research Center
University of Texas Southwestern Medical Center
Dallas, Texas 75390
‡Howard Hughes Medical Institute and
Department of Microbiology, Immunology,
and Molecular Genetics
University of California, Los Angeles
Los Angeles, California 90095

A major goal in immunology research has been to delineate early signal transduction events after antigen receptor ligation. This field was propelled forward by the discovery in 1993 that mutations in Bruton's tyrosine kinase (Btk), a member of the Tec family of nonreceptor tyrosine kinases, were the cause of inherited immunodeficiency diseases in both mice (*Xid*, for X-linked immunodeficiency) and humans (XLA, for X-linked agammaglobulinemia). The finding that impaired function of a tyrosine kinase could compromise B cell development and proliferation reinforced the concept that regulated tyrosine phosphorylation plays a central role in lymphocyte activation. Many laboratories have used gene targeting technology to evaluate the role of other putative signaling components in activation of B cells and other leukocytes. A great surprise of these studies is the number of genes whose disruption causes a phenotype highly similar to *Xid*. Gene deletions resulting in close copies of *Xid* include knockouts of the p85α subunit of phosphoinositide 3-kinase (PI3K) (Fruman et al., 1999; Suzuki et al., 1999), the adaptor protein BLNK/SLP-65/BASH (Jumaa et al., 1999; Pappu et al., 1999; Hayashi et al., 2000; Xu et al., 2000), and protein kinase C beta (PKCβ) (Leitges et al., 1996). A similar but more severe phenotype is observed in mice lacking B cell receptor (BCR) signaling chains or the tyrosine kinase Syk (reviewed in Pillai, 1999). A common property of all these proteins is their regulation of, or by, calcium flux. This correlation is strengthened by the finding of *Xid*-like defects in mice lacking the γ2 isoform of phospholipase C (PLCγ2), as reported in this issue of *Immunity* (Wang et al., 2000).

One could envision different models to explain how deletion of distinct genes could yield similar phenotypes. The genes could encode proteins that function in a linear pathway with either serial or parallel connections, with each link absolutely required for the cellular response. Alternatively, the genes could encode proteins that act in concert via an interconnected complex to produce the response. Removal of one component, as from a house of cards, would destabilize the whole complex. The concept of a molecular scaffold organizing a signaling cassette has been validated genetically in yeast MAP kinase signaling (reviewed in Burack and Shaw, 2000). Deletion of any of the kinases or their scaffolding protein results in a common mating defect. This minireview will focus on biochemical and genetic evidence for such a scaffold or "signalosome" model of BCR signaling.

Xid refers to the immunodeficiency in CBA/N mice, a naturally arising variant of the CBA/J strain. Over the years, these mice have been studied at many levels, resulting in a clear picture of their immunological, cellular, and molecular defects (reviewed extensively; see Satterthwaite et al., 1998). *Xid* mice show poor antibody responses to T-independent type II (TI-II) antigens, such as NP-Ficoll, but normal responses to T-dependent (TD) antigens. Naïve animals have greatly reduced serum titers of certain Ig isotypes, particularly IgM and IgG3. This correlates with a nearly complete absence of the $CD5^+$ (B-1) subset of B cells that is thought to produce much of the serum antibodies of these isotypes. The number of mature splenic B cells (i.e., B-2 subset) is moderately reduced, especially the $IgM^{lo}IgD^{hi}$ population that represents mature recirculating cells. The remaining B cells fail to proliferate in response to BCR stimulation in vitro and show greater spontaneous apoptosis. However, the cells do respond to anti-CD40 plus IL-4, consistent with normal TD antibody responses.

A major signaling defect in *Xid* B cells is an attenuated Ca^{2+} flux following BCR stimulation. In both B cells and T cells of wild-type mice, antigen receptor stimulation causes an initial spike in intracellular Ca^{2+} concentration followed by a sustained plateau of intermediate Ca^{2+} concentration that slowly decays to basal levels (reviewed in Scharenberg and Kinet, 1998). The initial rise is known to require the hydrolysis of PIP_2 by PLCγ1 or PLCγ2 to produce DAG and IP_3. This results in release of Ca^{2+} stores from the ER lumen via IP_3-gated channels. Emptying of these stores then causes the opening of plasma membrane Ca^{2+} channels ("store-operated channels," or SOC) allowing ions from outside the cell to maintain an elevated intracellular Ca^{2+} concentration. This process is known as capacitative calcium entry. A relatively high level of IP_3 needs to be produced to empty internal stores sufficiently to open the SOC and generate a sustained flux. Lower levels of IP_3 produce a smaller release phase that generally fails to sustain a plateau. Thus, quantitative differences in the output of PLC enzymes can have clear qualitative effects on the Ca^{2+} signal and subsequent activation of downstream signaling events. The sustained phase of Ca^{2+} flux is blunted in Btk-deficient B cells and augmented in cells overexpressing Btk (reviewed in Scharenberg and Kinet, 1998).

PLCγ2 is the major isoform of PLCγ expressed in B cells (reviewed in DeFranco, 1997; Kurosaki and Tsukada, 2000). Wang and colleagues (2000) now have shown that PLCγ2 deficiency results in a pattern of developmental and functional abnormalities of the B cell lineage nearly identical to those observed in Btk-deficient mice, likely due to impaired BCR-induced Ca^{2+}

§To whom correspondence should be addressed (e-mail: owenw@microbio.ucla.edu).

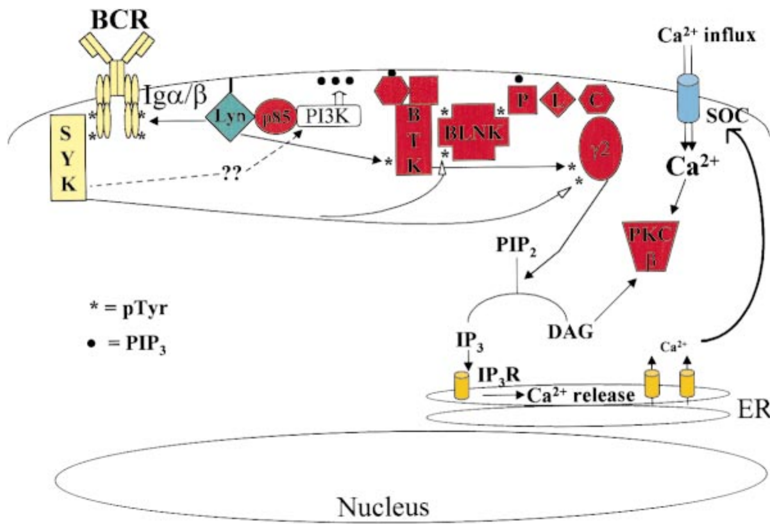

Figure 1. Schematic Diagram of the Signalosome Linking BCR Stimulation with Sustained Ca^{2+} Flux

BCR cross-linking leads to the activation of three tyrosine kinases (Lyn, Syk, and Btk) and a lipid kinase (PI3K). Production of PIP_3 and tyrosine phosphorylation of the BLNK adapter protein nucleates a complex that coordinates the activation of PLCγ2. Maximal activation of PLCγ2 is required to generate sufficient IP_3 to empty internal Ca^{2+} stores, which in turn is necessary for opening of SOC. Ca^{2+} and DAG are necessary cofactors for activation of PKCβ. Different colors represent proteins whose gene deletion results in a block at the pro-B to pre-B transition (yellow), *Xid*-like defects in development and function (red), or B cell hyperresponsiveness (green). The store-operated calcium channel mechanism is shown in blue. Arrows pointing to asterisks indicate phosphorylation events.

flux. Btk, PLCγ2, and other proteins whose deletion results in *Xid*-like defects are shown in red in Figure 1. There is some variation among the mice in the number of IgM^+ splenic B cells. For example, there are fewer mature B cells in mice lacking BLNK or p85α compared to *Xid*. On the other hand, PKCβ-deficient mice have nearly normal numbers of IgM^+ and IgD^+ cells. Some of these differences could be the result of distinct genetic backgrounds. The penetrance of the reduced B cell phenotype in *Xid* varies in different inbred strains (Khan et al., 1995). All of these genetically altered mice have a nearly complete absence of B-1 cells, reduced serum IgM and IgG3, and impaired TI-II antibody responses. Interestingly, LPS responses are also defective in B cells from several of these mouse strains.

Biochemical evidence supports the genetic link between these various gene products. One consequence of BCR cross-linking is the activation of the p85/p110 form of PI3K and the resulting generation of 3′-phosphorylated phosphoinositides. One of these lipids, PIP_3, binds selectively to the pleckstrin homology (PH) domain of Btk, facilitating membrane recruitment of the kinase, whereupon Src family kinases phosphorylate Tyr-551 in the activation loop (reviewed in Satterthwaite et al., 1998). The importance of PI3K in Btk function is illustrated by the fact that *Xid* is the result of a single amino acid substitution (R28C) in the pleckstrin homology (PH) domain, and this change eliminates selective binding to PIP_3 (reviewed in Satterthwaite et al., 1998). Although the protein is still produced and the kinase domain is intact, the R28C mutation appears to be a functional null since a nearly identical phenotype is observed in Btk knockout mice (Khan et al., 1995). PLCγ2 appears to be a substrate for Btk in intact cells based on coexpression studies in heterologous cells and increased BCR-induced PLCγ2 tyrosine phosphorylation in B cells overexpressing Btk (reviewed in Scharenberg and Kinet, 1998). This is probably facilitated by BLNK, an adapter protein that is highly tyrosine phosphorylated following BCR stimulation and binds several SH2 domain-containing proteins, including Btk and PLCγ2 (reviewed in Kurosaki and Tsukada, 2000). PKCβ is activated by diacylglycerol (DAG) and Ca^{2+} that are produced following PIP_2 hydrolysis by PLC enzymes (reviewed in DeFranco, 1997).

The interdependence of different inputs to PLCγ2 provides strong support for the signalosome model. The complex appears to be nucleated by two components: PIP_3 and BLNK. Although PI3K has not been demonstrated to associate with this complex, its lipid products appear to act as "glue" for membrane assembly of the components via phosphoinositide-binding domains in Btk and probably PLCγ2 as well. In different systems, the SH2 domains and PH domain of PLCγ2 have been shown to interact with PIP_3 (reviewed in Scharenberg and Kinet, 1998). Tyrosine-phosphorylated BLNK interacts with both Btk and PLCγ2 via SH2 domains in those proteins, facilitating tyrosine phosphorylation of PLCγ2 (reviewed in Kurosaki and Tsukada, 2000). One component that may fit the linear signaling model is PKCβ. As a Ca^{2+}- and DAG-regulated enzyme, PKCβ probably functions downstream of PLCγ2 activation (reviewed in DeFranco, 1997). It may also exert feedback inhibition of the signal by phosphorylating Btk on serine and threonine residues, but this function appears not to be required in the absence of its positive role (reviewed in Satterthwaite et al., 1998).

Several proteins may function within the signalosome even though their knockout phenotypes are distinct from *Xid*. Syk is recruited to the activated BCR and can directly tyrosine phosphorylate both BLNK and PLCγ2 (reviewed in Kurosaki and Tsukada, 2000). In addition, genetic studies have shown a requirement for Syk in activation of PI3K (Beitz et al., 1999). However, deletion of BCR components or Syk (shown in yellow in Figure 1) results in a nearly complete block in B cell development at the pro-B to pre-B transition (reviewed in Pillai, 1999). This indicates that the BCR/Syk complex has additional functions besides activating Btk and PLCγ2. These pathways may partially require BLNK and/or p85α, whose deletion results in a greater developmental block than *Xid*. Lyn, one of the major Src family tyrosine kinases expressed in B cells, is another example of a protein likely to affect signalosome function though its

knockout phenotype is distinct. Lyn can activate Btk by phosphorylating its activation loop (reviewed in Satterthwaite et al., 1998) and may also activate PI3K via an interaction with p85α (reviewed in DeFranco, 1997). However, although there is a reduction in mature B cell numbers in young Lyn-deficient mice, the remaining B cells are hyperresponsive and the mice develop an autoimmune syndrome. Indeed, Lyn plays an important role in downregulation of B cell activation via CD22/SHP-1- and FcγRIIb/SHIP-mediated pathways. In contrast, its positive function may be partially redundant with other Src family kinases (DeFranco et al., 1998).

It is not yet certain how the Ca^{2+}-regulating signalosome is brought into proximity with the BCR/Syk complex and Src family kinases. A potential explanation was provided by the recent demonstration that the BCR, Lyn, and PLCγ2 are found in lipid rafts following BCR stimulation and that disruption of the rafts attenuates BCR-mediated PLCγ2 phosphorylation and Ca^{2+} flux (Aman and Ravichandran, 2000). This suggests that the components of the B cell signalosome may be spatially organized in these membrane subdomains along with the BCR. In support of this idea, confocal microscopy with antibodies against specific phosphorylated tyrosines in Btk has revealed colocalization of activated Btk with the BCR complex (Nisitani et al., 1999).

How does the signalosome ensure optimal Ca^{2+} flux following BCR stimulation? It is likely that all activating phosphorylations of PLCγ2 must be delivered at the right time and place in order to produce sufficient levels of IP_3 to induce the sustained phase of Ca^{2+} flux. Thus, PIP_3 and BLNK concentrate PLCγ2 at the membrane, probably in lipid rafts (Aman and Ravichandran, 2000) where it receives necessary inputs from other components and obtains access to its substrates. As discussed in Wang et al. (2000), there is good evidence that similar signaling complexes work together to orchestrate Ca^{2+} flux in other antigen and F_c receptor systems.

Assuming that human B cells share similar circuitry as mouse B cells, it seems likely that some patients with B cell immunodeficiency have mutations in genes encoding signaling components other than Btk. Indeed, many cases of XLA-like syndromes with normal Btk have been reported. One such patient was found to bear a homozygous mutation in BLNK causing an absence of BLNK transcripts and protein (Minegishi et al., 1999). The relative infrequency of such patients may reflect the location of BLNK and other candidate genes on autosomes, whereas Btk is on the X chromosome. It is noteworthy that the phenotype of human patients lacking either Btk or BLNK is more severe than in their murine counterparts, resembling the complete block at the pro-B to pre-B transition observed in Syk-deficient mice. This could result from differences in genetic background (Khan et al., 1995) or a stricter requirement for the Ca^{2+}-regulating signalosome at the early stages of human B lymphopoeisis.

Conclusions

The molecular events linking BCR stimulation to Ca^{2+} flux are coming into focus. Considering together the results of biochemical studies and mouse genetic analyses, strong support now exists for a "signalosome" model of B cell activation. In this view, the central response regulator (PLCγ2) is activated by multiple independent inputs that are organized by interactions with membrane phosphoinositides and a docking protein (BLNK). A further level of organization may be provided by localization in lipid rafts. It should be emphasized that the picture of the B cell signalosome is probably incomplete. It is likely that other proteins that interact with Btk may be involved in signal integration and modification. It will be important to apply genetic tests of function to these components. Another challenge for the future is to determine whether distinct signalosomes are organized by other receptors on B cells. The application of gene targeting technology, including conditional inactivation and RAG chimeric approaches, should continue to clarify the complexities of lymphocyte activation.

Selected Reading

Aman, M.J., and Ravichandran, K.S. (2000). Curr. Biol. *10*, 393–396.

Beitz, L.O., Fruman, D.A., Kurosaki, T., Cantley, L.C., and Scharenberg, A.M. (1999). J. Biol. Chem. *274*, 32662–32666.

Burack, W.R., and Shaw, A.S. (2000). Curr. Opin. Cell. Biol. *12*, 211–216.

DeFranco, A.L. (1997). Curr. Opin. Immunol. *9*, 296–308.

DeFranco, A.L., Chan, V.W., and Lowell, C.A. (1998). Semin. Immunol. *10*, 299–307.

Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W., and Cantley, L.C. (1999). Science *283*, 393–397.

Hayashi, K., Nittono, R., Okamoto, N., Tsuji, S., Hara, Y., Goitsuka, R., and Kitamura, D. (2000). Proc. Natl. Acad. Sci. USA *97*, 2755–2760.

Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M., and Nielsen, P.J. (1999). Immunity *11*, 547–554.

Khan, W.N., Alt, F.W., Gerstein, R.M., Malynn, B.A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A.B., Herzenberg, L.A., et al. (1995). Immunity *3*, 283–299.

Kurosaki, T., and Tsukada, S. (2000). Immunity *12*, 1–5.

Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S., and Tarakhovsky, A. (1996). Science *273*, 788–791.

Minegishi, Y., Rohrer, J., Coustan-Smith, E., Lederman, H.M., Pappu, R., Campana, D., Chan, A.C., and Conley, M.E. (1999). Science *286*, 1954–1957.

Nisitani, S., Kato, R.M., Rawlings, D.J., Witte, O.N., and Wahl, M.I. (1999). Proc. Natl. Acad. Sci. USA *96*, 2221–2226.

Pappu, R., Cheng, A.M., Li, B., Gong, Q., Chiu, C., Griffin, N., White, M., Sleckman, B.P., and Chan, A.C. (1999). Science *286*, 1949–1954.

Pillai, S. (1999). Immunity *10*, 493–502.

Satterthwaite, A.B., Li, Z., and Witte, O.N. (1998). Semin. Immunol. *10*, 309–316.

Scharenberg, A.M., and Kinet, J.P. (1998). Cell *94*, 5–8.

Suzuki, H., Terauchi, Y., Fujiwara, M., Aizawa, S., Yazaki, Y., Kadowaki, T., and Koyasu, S. (1999). Science *283*, 390–392.

Wang, D., Feng, J., Wen, R., Marine, J.-C., Sangster, M.Y., Parganas, E., Jackson, C.W., Cleveland, J.L., Murray, P.J., and Ihle, J.N. (2000). Immunity *13*, this issue, 25–35.

Xu, S., Tan, J.E., Wong, E.P., Manickam, A., Ponniah, S., and Lam, K.P. (2000). Int. Immunol. *12*, 397–404.