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Syk Mediates BCR- and CD40-Signaling Intergration during B Cell Activation

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Abstract

CD40 is essential for optimal B cell activation. It has been shown that CD40 stimulation can augment BCR-induced B cell responses, but the molecular mechanism(s) by which CD40 regulates BCR signaling is poorly understood. In this report, we attempted to characterize the signaling synergy between BCR- and CD40-mediated pathways during B cell activation. We found that spleen tyrosine kinase (Syk) is involved in CD40 signaling, and is synergistically activated in B cells in response to BCR/CD40 costimulation. CD40 stimulation alone also activates B cell linker (BLNK), Bruton tyrosine kinase (Btk), and Vav-2 downstream of Syk, and significantly enhances BCR-induced formation of complex consisting of, Vav-2, Btk, BLNK, and phospholipase C-gamma2 (PLC-γ2) leading to activation of extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase, Akt, and NF-κB required for optimal B cell activation. Therefore, our data suggest that CD40 can strengthen BCR-signaling pathway and quantitatively modify BCR signaling during B cell activation.

Keywords

B Lymphocytes; Costimulation; Cell activation; Signal transduction

Introduction

B cell activation is the combined outcome of signals generated through the coreceptors (CD19, CD21, CD22, FcγRIIB) which act to quantitatively modify BCR signaling (Hasler & Zouali, 2001), and signals generated through costimulatory receptors that represent the physical manifestation of T cell help which qualitatively modify BCR signaling (Hasler & Zouali, 2001; Bishop & Hostager, 2001b). CD40 is expressed on bone marrow (BM) B cells, mature B cells, and certain accessory cells, including monocytes and BM-derived and follicular dendritic cells. Activated CD4⁺ T cells express CD40 ligand (CD40L) which interacts with CD40 on B cells to initiate antibody (Ab) responses to T-dependent antigens (Ags) (Bishop & Hostager, 2003).

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BCR or CD40 stimulation alone is weakly mitogenic, but in combination synergistically initiate the program of B cell activation characterized by proliferation, isotype switching, up-regulation of costimulatory receptors, germinal center formation, and memory generation (Bishop & Hostager, 2001a; Schonbeck & Libby, 2001; Haxhinasto et al., 2002). In the absence of CD40, B cells are tolerant in the periphery (Buhlmann et al., 1995), suggesting a role of CD40 in induction of B cell tolerance. Engagement of CD40 may lower the threshold for BCR-mediated B cell activation (Klaus et al., 1999; Salmena et al., 2003). Previously, it has been reported that pre-ligation of B cells with CD40L can modify BCR signaling by eliminating Bruton tyrosine kinase (Btk) activation, and synergistically activating extracellular signal regulated kinase (ERK) (Mizuno & Rothstein, 2005; Mizuno & Rothstein, 2003). Activation of CD40-dependent signaling pathway is mediated primarily by several members of TNF receptor (TNFR)-associated factor (TRAF) protein family (Bishop & Hostager, 2001b). TRAFs serve as adaptor proteins that connect the cytoplasmic domain of CD40 to downstream effectors, such as c-Jun-NH₂-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK). CD40 stimulation also induces activation of PI3-K, phospholipase C- γ 2 (PLC- γ 2), and NF- κ B (Bishop & Hostager, 2001a; Craxton et al., 1998).

In this regard, TRAF-2 may be required for the synergy between BCR and CD40 (Haxhinasto, Hostager & Bishop, 2002). Phosphorylation of TRAF-2 at tyrosine 484 (Tyr484) is crucial for BCR and CD40 synergy which involves Btk activation leading to enhancement of the CD40 response by TRAF-2 in a protein kinase D (PKD)-dependent manner (Haxhinasto & Bishop, 2004). However, it is unknown whether CD40 could quantitatively modify BCR signaling pathway, and if could, at which level these two signaling pathways converge?

In this study, we show that CD40 ligation activates Syk-dependent pathway in B cells. CD40 costimulation promotes BCR-induced phosphorylation of Btk, B cell linker (BLNK), protein kinase C- β (PKC- β), ERK, p38 MAPK, and Akt, but has no effect on phosphorylation of JNK. In addition, CD40 stimulation enhances BCR-induced NF- κ B activation. Therefore, CD40 ligation selectively enhances BCR-mediated Syk activity leading to activation of ERK, p38 MAPK, Akt, and NF- κ B, and these two pathways may converge at Syk.

Materials and Methods

Mice

Female BALB/c mice were purchased from National Cancer Institute (Frederick, MD, USA), and were used for experiments at age of 6 to 10 weeks.

Reagents

F(ab')₂ anti-mouse IgM fragments were purchased from Zymed (San Francisco, CA, USA). Anti-mouse CD40 (clone 3/23) was purchased from BD PharMingen (San Diego, CA, USA). Abs against Lyn, Syk, Vav-2, PLC- γ 2, BLNK, Btk, and PI3-K (p85) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-tyrosine Ab (4G10) and anti-phospho-Akt (Ser473) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Phospho-Abs against Lyn (Tyr507), Syk (Tyr519/520), Btk (Tyr223), BLNK (Tyr96), PLC- γ 2 (Tyr1217) was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). A murine B cell isolation kit was obtained from Miltenyi Biotec (Auburn, CA, USA). HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG were purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA). Syk inhibitor BAY61-3606 were purchased from Fisher Scientific (Hampton, NH, USA).

B cell isolation and activation

Splenic B cells from BALB/c mice were isolated (purity > 95% as determined by FACS analysis of B220 cell surface expression) using B cell isolation kit (Miltenyi Biotec., Auburn, CA, USA). Contaminating T cells were less than 1% as determined by CD3 staining. For in vitro activation, B cells (10^7 /ml) were stimulated for various time periods indicated with F(ab')₂ anti-IgM (2 µg/ml), anti-CD40 (2 µg/ml), or both. The cells were lysed in 0.5% NP-40 lysis buffer or in radioimmunoprecipitation assay (RIPA) buffer where indicated.

B cell proliferation assay

Splenic B cells (1×10^6 /ml) from BALB/c mice were cultured for 56 h at 37 °C in round-bottomed 96-well plates with anti-CD40 mAb or isotype control (R&D systems) as indicated. The cells were pulsed with 1 µCi [³H]-thymidine, and harvested 16 h later. The radioactivity was quantitated using a Wallac 1205 BetaplateTM β-liquid scintillation counter (Perkin Elmer-Wallac, Gaithersburg, MD, USA).

Immunoprecipitation and Western blotting

The conditions for immunoprecipitation and immunoblotting were described previously (Zhang et al., 2002; Li et al., 2004; Qiao et al., 2007; Qiao et al., 2008).

Results

CD40 stimulation induces Syk activation, and additively modifies BCR-induced activation/phosphorylation of Syk-mediated signaling pathway

TRAF-2 may be required for the synergy between BCR and CD40 in B cell lines (Bishop, 2004; Haxhinasto & Bishop, 2004; Haxhinasto, Hostager & Bishop, 2002). Furthermore, CD40 pre-ligation enhances BCR-induced ERK activation (Mizuno & Rothstein, 2005). However, it remains unknown whether CD40-derived signal can integrate with the proximal BCR signal. To address this, we first examined whether CD40 ligation activates well-characterized BCR signaling pathway. To this end, BALB/c splenic B cells were stimulated with F(ab')₂ anti-IgM, anti-CD40, or both, and blotted with phospho-Abs against Lyn, Syk, and BLNK. As shown in Figure 1A, CD40 stimulation induced dephosphorylation of Lyn at Tyr 507 and phosphorylation of Syk and BLNK, whereas BCR and CD40 costimulation resulted in a synergistic activation of Syk but not Lyn. These results suggest that CD40 indeed shares the components of BCR signaling pathway, and CD40 may quantitatively enhance BCR-mediated signaling derived from Syk. Vav-2 and BLNK are substrates of Syk in B cells, and are essential in activating PLC-γ2 (Schedel et al., 2002; Fu et al., 1998; Johmura et al., 2003). To detect Vav-2 phosphorylation, B cells were stimulated with F(ab')₂ anti-IgM, anti-CD40, or both, and lysed. The cell lysates were immunoprecipitated with anti-Vav-2, and blotted with anti-phospho-tyrosine. Vav-2 phosphorylation kinetics correlated with that of Syk phosphorylation. Consistent with hyper-activation of Syk, sustained phosphorylation of BLNK was observed in response to BCR and CD40 stimulation (Fig. 1A). Therefore, BCR and CD40 cooperate in B cell activation at the level of Syk in B cells.

To confirm Syk is indeed involved in CD40-mediated signaling, splenic B cells purified from BALB/c mice were stimulated for 72 h at 37°C with anti-CD40 mAb or isotype control (R&D systems), and B cell proliferation was then determined by [³H]thymidine incorporation. Pretreating B cells with a specific Syk inhibitor, BAY 61-3606, completely diminished CD40-induced B cell proliferation (Fig. 1B), suggesting that Syk is a mediator in CD40 signaling pathway in B cells.

Both PI3-K and Btk are involved in CD40-mediated B cell costimulation

It has been shown that CD40 ligation activates phosphoinositide-3 kinase (PI3-K) (Bishop & Hostager, 2001a; Craxton, Shu, Graves, Saklatvala, Krebs & Clark, 1998), whereas PI3-K is responsible for the activation of Btk by bringing Btk to the plasma membrane through interactions between the pleckstrin homology domain of Btk and phosphatidylinositol(3,4,5)-trisphosphate (Salim et al., 1996). We were then interested in determining the activity of PI3-K and Btk in response to stimulation of BCR, CD40, or both. To this end, purified splenic B cells were stimulated and lysed. The cell lysates were blotted with anti-phospho-Akt (Ser473) and anti-phospho-Btk (Tyr223). Akt is a major target of PI3-K, and the phosphorylation state of Akt has been used as a surrogate indicator of PI3-K activation (Kane et al., 2001; Okkenhaug et al., 2001; Hirsch et al., 2000). Tyrosine 223 is a major autophosphorylation site of Btk, its phosphorylation can thus be used as an indicator of Btk activity (Clayton et al., 2002). As shown in Figure 2, CD40 stimulation had a stimulatory effect on Akt phosphorylation. In contrast, BCR/CD40 stimulation resulted in synergistic phosphorylation of Btk at Tyr223 required for full activation of Btk, indicating that CD40 ligation potentiates BCR-induced Btk activation. Tyrosine residues 753, 759, 1197 and 1217 of PLC- γ 2 have been identified as important Btk phosphorylation sites (Sangster et al., 2003). To examine whether PLC γ 2 was phosphorylated by Btk on tyrosine 1217, anti-phospho-PLC- γ 2 (Tyr1217) Ab was employed. BCR or CD40 stimulation of BALB/c B cells induced phosphorylation of PLC- γ 2 on tyrosine 1217, and this phosphorylation was strongly enhanced by BCR/CD40 costimulation (Fig. 2).

CD40 stimulation promotes BCR-induced formation of complex consisting of Syk/Vav-2/Btk/BLNK/PLC- γ 2

Engagement of the BCR initiates receptor aggregation, resulting in activation of receptor-associated Src-family kinases as well as Syk and Btk/Tec family kinases. These initial events facilitate the recruitment and activation of additional kinases and adaptor proteins within membrane raft microdomains leading to the formation of a mature BCR signalosome and promoting the full activation of several downstream signaling cascades (Guo et al., 2004). The prominent substrates of Syk are Vav-2 and BLNK. Syk efficiently phosphorylates Vav-2 and BLNK only upon BCR stimulation (Kurosaki et al., 2000; Reth & Brummer, 2004). The phosphorylated BLNK recruits Btk and PLC- γ 2, an event that results in the phosphorylation and activation of PLC- γ 2, generation of inositol 1,4,5-trisphosphate (IP₃), and the opening of intracellular Ca²⁺ stores (Kurosaki, Maeda, Ishiai, Hashimoto, Inabe & Takata, 2000; Deane & Fruman, 2004; Reth & Brummer, 2004). To address whether CD40 stimulation promoted BCR-induced formation of signalosome, BALB/c B cells were stimulated for 5 and 15 min with F(ab')₂ anti-IgM, anti-CD40, or both, and lysed in 0.5% NP-40 lysis buffer. The cell lysates were immunoprecipitated with anti-Syk, and blotted with anti-Vav-2, anti-Btk, anti-BLNK, anti-PLC- γ 2, and anti-Syk, respectively. CD40 stimulation alone induced complex formation consisting of Vav-2, Btk, BLNK and PLC- γ 2, and significantly promoted this complex formation induced by BCR stimulation (Fig. 3A). The formation of this complex potentiated by CD40 costimulation was inhibited by the presence of the Syk inhibitor (Fig. 3B), further supporting a role of Syk activity induced by CD40 in synergistic activation of BCR signaling pathway.

ERK, p38 MAPK, Akt, and NF- κ B are synergistically activated by BCR and CD40 stimulation

It has been well established that CD40 extends a unique signal for B cell activation and proliferation. Both BCR and CD40 have been reported to stimulate kinases such as ERK, JNK, p38 MAPK and ultimately several transcription factors such as NF- κ B, NF-AT, and AP-1 (Hsing & Bishop, 1999; Mathur et al., 2004). We have shown that CD40 ligation activates JNK, p38 MAPK, ERK, and Akt. However, it is unknown whether BCR and CD40

have a synergistic role in activating these kinases. To this end, purified splenic B cells were stimulated for 5 and 15 min at 37°C with F(ab')₂ anti-IgM, anti-CD40, or both and lysed. The cell lysates were blotted with anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38 MAPK, and anti-IκBα, respectively. Figure 4A showed that although both BCR and CD40 ligation activated ERK, JNK, p38 MAPK, and Akt, BCR/CD40 costimulation resulted in a synergistic phosphorylation of ERK and p38 MAPK and Akt, but had no effect on JNK activation.

Proteasomal degradation of IκBα, an inhibitor of NF-κB, allows NF-κB to translocate to the nucleus and induce transcription (Zhang, 2004). In the presence of CD40 stimulation, BCR-induced IκBα degradation was significantly enhanced (Fig. 4B), suggesting that BCR and CD40 stimulation synergistically activates NF-κB in B cells. It has been reported that PKC-β is specifically required for BCR-mediated NF-κB activation (Saijo et al., 2002; Su et al., 2002). Although it appears that CD40-mediated NF-κB activation does not require PKC-β (Saijo, Mecklenbrauker, Santana, Leitger, Schmedt & Tarakhovsky, 2002; Su, Guo, Kawakami, Sommer, Chae, Humphries, Kato, Kang, Patrone, Wall, Teitell, Leitges, Kawakami & Rawlings, 2002), it is unknown whether CD40 stimulation promotes BCR-induced PKC-β activation. To test this, BALB/c splenic B cells were stimulated as described in Figure 4B, and lysed. The cell lysates were immunoprecipitated with anti-PKC-β, and blotted with anti-phospho-pan-PKC. Consistent with increased degradation of IκBα, CD40 stimulation synergistically induced PKC-β phosphorylation. These results suggest that although CD40-mediated NF-κB activation may not require PKC-β, the synergistic activation of NF-κB by BCR and CD40 stimulation may be mediated by PKC-β. In support of this notion, B cells lacking CARMA1, a downstream target of PKC-β, do not proliferate in response to BCR, CD40, or BCR and CD40 stimulation, suggesting that CARMA1 regulates both BCR- and CD40-mediated NF-κB activation (Egawa et al., 2003).

Discussion

Previous studies have shown that optimal B cell activation depends signals generated upon recognition of Ag by the BCR as well as additional signals provided by cognate interaction with T helper cells, including the CD40-CD40L interaction (Haxhinasto & Bishop, 2004; Haxhinasto, Hostager & Bishop, 2002). However, it is poorly defined whether and how CD40 signaling quantitatively affects BCR signaling. In this study, we first demonstrate that CD40 ligation modifies BCR-induced B cell activation by promoting activation of Syk and formation of signaling complex consisting of Syk, Vav-2, Btk, BLNK, and PLC-γ2, thus leading to activation of p38 MAPK, ERK, Akt, and NF-κB. These results suggest that Syk is involved in signal integration during BCR and CD40 costimulation of primary murine B cells and may also play an important role in the induction of B cell anergy.

Using B cell lines, Dr. Bishop and her colleagues have identified TRAF-2 and TRAF3 in the CD40-signaling pathway together with BCR-activated PKD as important cooperative factors in BCR and CD40 synergy (Haxhinasto & Bishop, 2004). It was shown that phosphorylation of TRAF-2 is increased upon BCR but not CD40 engagement and that of the potentially phosphorylated residues of TRAF-2 at tyrosine 484 is crucial for BCR-CD40 synergy which is prevented by xid mutated Btk (Mizuno & Rothstein, 2003). On the other hand, CD40 crosstalk has a profound effect on subsequent BCR signaling. CD40 pre-ligation enhances BCR-induced ERK activation in primary B cells (Mizuno & Rothstein, 2005). However, little is known whether and how proximal BCR signaling is altered by CD40 costimulation. Using primary B cells, we found that CD40 stimulation directly activates Syk, and significantly enhances BCR-induced activation of Syk (Fig. 1A). Our data suggest that BCR

and CD40 signaling converges at the levels of Syk. In support of this notion, specific inhibition of Syk leads to impaired B cell proliferation induced by CD40 ligation (Fig. 1B).

The “signalosome” envisions a molecular complex that is regulated both temporally and spatially and whose outputs include quantitative and qualitative effects on the amount of intracellular Ca^{2+} and subsequent activation of downstream signaling events (Deane & Fruman, 2004). Therefore, a formation of complex consisting of Syk, Vav-2, Btk, BLNK, and PLC- γ 2 is critical for B cell activation (Kurosaki, Maeda, Ishiai, Hashimoto, Inabe & Takata, 2000; Deane & Fruman, 2004; Reth & Brummer, 2004). In support of this notion, Ca^{2+} influx is reduced in Vav-1 $^{-/-}$ or Vav-2 $^{-/-}$ B cells (Doody et al., 2001; Tedford et al., 2001). Our data showed that the phosphorylation of Syk, Vav-2, Btk, BLNK, and PLC- γ 2 is not only induced by IgM but also by CD40 stimulation, and these phosphorylation events are synergistically augmented by IgM/CD40 costimulation (Fig. 2). These data suggest a role of CD40 in BCR signalosome formation. Intriguingly, CD40 ligation significantly augments the formation of BCR signalosome (Fig. 3A) which leads to optimal B cell activation. To our knowledge, this is the first piece of evidence indicates a crucial role of CD40 in BCR signalosome formation.

It has been shown that PI3-K plays a critical role in B cell development and proliferation (Clayton, Bardi, Bell, Chantry, Downes, Gray, Humphries, Rawlings, Reynolds, Vigorito & Turner, 2002; Fruman, 2004; Koyasu, 2004). Mice lacking p85 regulatory subunit of PI3-K have reduced numbers of peripheral mature B cells and decreased serum Ig (Deane & Fruman, 2004). The B cells that develop have diminished proliferative responses to BCR, CD40, and LPS stimulation (Fruman, 2004; Koyasu, 2004). This phenotype is similar to defects observed in mice lacking Btk (Saito et al., 2003). More recently, it has been shown that Btk also activates phosphatidylinositol-4-phosphate 5-kinase, thereby stimulating a positive feedback loop that generate $\text{PI}(4,5)\text{P}_2$, the substrate for both PI3-K and PLC- γ 2 (Reth & Brummer, 2004). BCR or CD40 ligation alone induces activation of Akt and Btk, whereas BCR/CD40 stimulation resulted in heightened phosphorylation of Btk at Tyr223 which is required for full activation of Btk and Akt. These data suggest that CD40 promotes BCR-induced PI3-K and Btk activation. However, it is unknown whether CD40-induced Btk activation is dependent upon PI3-K since Btk activation is intact in B cells lacking either p85 α or p110 δ (Deane & Fruman, 2004; Bishop et al., 2003).

In summary, in this study we demonstrate the molecular basis of BCR and CD40 signaling integrity. CD40 stimulation strongly enhances BCR-induced activation of Syk, and subsequent assembly of signaling complex composed of Syk, Vav-2, Btk, BLNK, and PLC- γ 2 leading to optimal B cell activation.

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Abbreviations

Ab	antibody
Ag	antigen
BLNK	B cell linker
BM	bone marrow

Btk	Bruton tyrosine kinase
ERK	extracellular signal regulated kinase
IP₃	inositol 1,4,5-trisphosphate
JNK	c-Jun NH2-terminal kinase
PKC	protein kinase C
MAPK	mitogen-activated protein kinase
PI3-K	phosphoinositide Kinase-3
PLC-γ2	phospholipase C- γ 2
RIPA	radioimmunoprecipitation assay
Syk	spleen tyrosine kinase
TNFR	TNF- α receptor
TRAF	TNFR-associated factor

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Fig. 1.

CD40 stimulation modifies BCR-induced phosphorylation/activation of Syk-dependent signaling pathway. (A) Purified splenic B cells were stimulated for 5 and 15 min with F(ab')₂ anti-IgM, anti-CD40, or both, and lysed in RIPA buffer. The cell lysates were blotted with phospho-Abs against Lyn, Syk, and BLNK, respectively, or immunoprecipitated with anti-Vav-2, and blotted with anti-phospho-tyrosine mAb. The membranes were stripped and reprobed with anti-actin or anti-unphosphorylated forms of the proteins to serve as loading controls. (B) BALB/c B cells were pretreated with BAY 61–3606 (10 μM) or DMSO at 37°C for 30 min, then stimulated with anti-CD40 (2 μg/ml) or isotype control for 72 h, and B cell proliferation was determined. (C) BALB/c B cells were pretreated with BAY 61–3606 (10 μM) or DMSO at 37°C for 30 min, then stimulated with anti-CD40 (2 μg/ml) or isotype control for 5 min and lysed in RIPA buffer. The cell lysates were blotted with antibodies against phospho-Syk and actin. These data are representative of three independent experiments.

Fig. 2.

CD40 stimulation potentiates BCR-induced activation of PI3-K and Btk in B cells. Splenic B cells from BALB/c mice were stimulated for 5 and 15 min with F(ab')₂ anti-IgM, anti-CD40, or both, and lysed. The cell lysates were blotted with phospho-Abs against Akt, Btk, and PLCγ2, respectively. The membrane was stripped and reprobed with anti-Akt or anti-actin. One result representative of three experiments is shown.

A **B**

Fig. 3. CD40 promotes BCR-induced complex formation consisting of Syk, Vav-2, Btk, BLNK, and PLC-γ2. (A) Purified splenic B cells were stimulated for 5 and 15 min with F(ab')₂ anti-IgM, anti-CD40, or both, and lysed in 0.5% NP-40 lysis buffer, and immunoprecipitated with anti-Syk, blotted with anti-BLNK, anti-Vav-2, and anti-PLC-γ2, respectively. The membrane was stripped and reprobed with anti-Syk. (B) Splenic B cells were pretreated with BAY 61-3606 (10 μM) or DMSO at 37°C for 30 min, then stimulated with anti-IgM and anti-CD40 for 5 min, and lysed. The BCR signal complex was determined as in A. The data shown is representative of three independent experiments.

Fig. 4. BCR and CD40 stimulation synergistically activate ERK, p38 MAPK, Akt, and NF-κB in B cells. (A) splenic B cells were stimulated for 5 and 15 min with F(ab')₂ anti-IgM, anti-CD40, or both, and lysed. The cell lysates were blotted with anti-phospho-Abs against ERK, JNK and p38 MAPK, respectively. The membranes were stripped and reprobed with Abs against ERK, JNK and p38 MAPK, respectively. (B) Purified splenic B cells were stimulated as in A and lysed. The cell lysates were blotted with anti-phospho-κBα and actin, or immunoprecipitated with anti-PKC-β and blotted with anti-pan-phospho-PKC Ab and anti-PKC-β. The data shown is representative of two independent experiments.