A Filarial Nematode-Secreted Phosphorylcholine-Containing Glycoprotein Uncouples the B Cell Antigen Receptor from Extracellular Signal-Regulated Kinase-Mitogen-Activated Protein Kinase by Promoting the Surface Ig-Mediated Recruitment of Src Homology 2 Domain-Containing Tyrosine Phosphatase-1 and Pac-1 Mitogen-Activated Kinase-Phosphatase

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A Filarial Nematode-Secreter Phosphorylcholine-Containing Glycoprotein Uncouples the B Cell Antigen Receptor from Extracellular Signal-Regulated Kinase-Mitogen-Activated Protein Kinase by Promoting the Surface Ig-Mediated Recruitment of Src Homology 2 Domain-Containing Tyrosine Phosphatase-1 and Pac-1 Mitogen-Activated Kinase-Phosphatase

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Unraveling the molecular mechanisms by which filarial nematodes, major human pathogens in the tropics, evade the host immune system remains an elusive goal. We have previously shown that excretory-secretory product-62 (ES-62), a homologue of phosphorylcholine-containing molecules that are secreted by human parasites and which is active in rodent models of filarial infection, is able to polyclonally activate certain protein tyrosine kinase and mitogen-activating protein kinase signal transduction elements in B lymphocytes. Such activation mediates desensitization of subsequent B cell Ag receptor (BCR) ligation-induced activation of extracellular signal-regulated kinase-mitogen-activated protein (ErkMAP) kinase and ultimately B cell proliferation. We now show that the desensitization is due to ES-62 targeting two major regulatory sites of B cell activation. Firstly, pre-exposure to ES-62 primes subsequent BCR-mediated recruitment of SHP-1 tyrosine phosphatase to abolish recruitment of the RasErkMAP kinase cascade via the Igαβ-Phosphatase1 recruitement of Src Homology 2 Domain-Containing Tyrosine phosphatase.

Filarial nematodes constitute a large group of parasites of vertebrates. Three of the eight species that infect humans, Wuchereria bancrofti, Brugia malayi, and Onchocerca volvulus, represent important pathogens, in that 1000 million people are at risk of elephantiasis, chronic debilitating skin lesions, or blindness as a consequence of infection (1). The success of these parasites is due to their ability to survive unscathed in their human host in excess of 5 years, and prolonged research supports the idea that such longevity reflects an ability of filarial nematodes to suppress/modulate the host immune system. The mechanisms underlying this remain unclear, but evidence has been steadily accumulating to indicate that molecules secreted by the worms (excretory-secretory products (ES)) could be involved (2, 3). Of particular interest, filarial nematodes have been shown to secrete glycoproteins containing a known immunomodulator, phosphorylcholine (PC) (2, 4). We have demonstrated that the PC moiety of one such molecule, ES-62, a glycoprotein that is a major ES of the rodent filarial parasite Acanthocheilonema viteae (5) and has homologues in human filarial nematodes, inhibits Ag receptor-driven B and T cell proliferation by selectively uncoupling the Ag receptors from key downstream proliferative signaling pathways (4, 6–10). Thus, although ES-62-mediated inhibition of lymphocyte activation is not targeted against the early Ag receptor-coupled phospholipase C (PLC-γ)-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, it selectively down-regulates certain protein kinase C isoforms and desensitizes protein tyrosine kinase-dependent coupling of the Ag receptors to the phosphatidylinositol 3-kinase and the Ras-ErkMAP kinase signaling cascades (6–9).

In B cells, coupling of the Ag receptors (slg/BCR) to ErkMAP kinase is protein tyrosine kinase (PTK) dependent. Following ligation of the BCR the PTK, Lyn, tyrosine phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs) on the accessory transducing molecules Ig-α (CD79a) and Ig-β (CD79b), leading to the recruitment and activation of additional PTKs (such as Syk, Lyn, Blk, and Fyn) and signaling molecules (PLC-γ and RasGAP) and adaptors (Shc and Grb2) in an Src homology (SH)2- and SH3-domain-dependent manner (11). Thus, Shc binds to the phosphorylated ITAMs (12–14) and, in turn, is phosphorylated by Syk, permitting recruitment of the Grb2Sos complexes required for activation of Ras at the plasma membrane. Following Sos-driven guanine nucleotide exchange and generation of the GTP-bound form of Ras, Ras binds and derepresses Raf Ser/Thr kinase, triggering stimulation of MAP kinase cascade and consequent phosphorylation of a number of downstream signaling molecules.
activation of ErkMAP kinase (11). Although we have found that ES-62 profoundly suppresses BCR-stimulated tyrosine phosphorylation events in general, it does not appear to uncouple the BCR from MAP kinase activation by disrupting activation of the BCR-associated PTKs such as Lyn, Syk, Blk, and Fyn (9). Indeed, ES-62 actually induces some activation of these PTKs and, in addition, ErkMAP kinase, although these activation events are insufficient to promote B cell proliferation (9). We now provide a mechanism to explain ES-62-mediated desensitization of BCR coupling to ErkMAP kinase. Specifically, we show that ES-62 targets two major negative regulators in the control of BCR coupling to the RasErkMAP kinase cascade, SH-2 domain-containing tyrosine phosphatase (SHP)-1 tyrosine phosphatase and the MAP kinase phosphatase, Pac-1.

Materials and Methods

Antibodies

Anti-phosphotyrosine (4G10), agarose-conjugated anti-phosphotyrosine (4G10), and tyrosine phosphatase assay kits were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Shc, anti-Sos, anti-Grb2, and anti-Lyn Abs were obtained from Transduction Laboratories (Lexington, KY), and anti-SHP-1, anti-SH-2-containing inositol phosphatase, anti-PP2A, anti-Btk, anti-Syk, and anti-Fyn Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). F(ab')2 of anti-Ig Abs were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and Stratech Scientific (Luton, U.K.). In addition, anti-mouse Ig-HRP, anti-rabbit Ig-HRP, and ECL reagents were obtained from Amersham International (Little Chalfont, U.K.). Protein A- and protein G-Sepharose were obtained from Sigma (Poole, U.K.). MACS CD43 (Ly-48) microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

B cell purification

B cells (>98% s Ig+) were purified from murine BALB/c spleen by negative selection using anti-CD43-coated magnetic beads according to the manufacturer’s instructions (Miltenyi Biotec, Surrey, U.K.). Briefly, single-cell suspensions of splenocytes were centrifuged (400 g) through Ficoll cushions, and the enriched lymphocyte fraction was harvested from the Ficoll-aqueous interface. The cells were washed and resuspended (2 × 106/ml) in PBS containing 5% BSA and 2 mM EDTA and incubated with anti-CD43-coated beads (100 µg/106 cells) for 20 min at 4°C. The cells were then loaded onto a MACS negative selection column, and the B cells were eluted, washed, and resuspended in RPMI 1640 medium containing 5% FCS.

Preparation of cell lysates

ES-62 from A. viteae was prepared as described previously (6). B cells (5 × 106) were preincubated with either ES-62 (2 µg/ml in supplemented RPMI 1640 medium) or medium alone for 4 h, and then incubated with anti-Ig (50 µg/ml) or medium alone for the indicated time at 37°C. Where indicated, an alternative stimulation protocol that involved incubating small resting B cells (5 × 107) with anti-Ig (50 µg/ml), ES-62 (2 µg/ml), or medium alone for the indicated time at 37°C was employed. Following cell incubations, reactions were terminated by addition of lysis buffer (50 mM Tris (pH 7.4), 150 mM sodium chloride, 2% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium orthovanadate, 0.5 mM PMSF, chymostatin (10 µg/ml), leupeptin (10 µg/ml), antipain (10 µg/ml), and pepstatin A (10 µg/ml)), and the samples were incubated on ice for 20 min before microcentrifugation at 20,000 × g for 30 min at 4°C. The supernatants were transferred to fresh tubes and either used immediately or stored at −20°C until required.

Generation of immune complexes

Fresh cell lysates were prepared and then centrifuged at 19,800 × g for 30 min at 4°C. The samples were precleared using 10 µl protein A- or protein G-Sepharose and then incubated with the appropriate Ab at 4°C on a rotating shaker. The samples were further incubated with 10 µl protein G-Sepharose or protein A-Sepharose at 4°C for 2 h and finally centrifuged at 19,800 × g for 5 min. The supernatant was discarded. Samples required for Western blotting were resuspended in lysis buffer and stored at −20°C whereas samples required for phosphatase activity were resuspended in lysis buffer and used immediately or stored at 4°C.

Western blot analysis

Immunoprecipitation samples were thawed and centrifuged at 19,800 × g for 5 min, and the pellet was solubilized in reducing buffer containing 10% SDS and then boiled for 15 min. The sample was further centrifuged at 19,800 × g for 5 min, the supernatant was removed, and equal protein loadings of samples were electrophoresed on a 10% acrylamide resolving gel under reducing conditions. The proteins were transferred onto polyvinylidene difluoride (Millipore, Watford, U.K.) and blocked at room temperature with 10% nonfat dried milk in PBS/Tween 20 (0.1%, v/v) under constant agitation. The blots were incubated with the appropriate Ab (see figure legends for details) for at least 1 h at room temperature and then washed in PBS/Tween (0.1%, v/v) before incubating with the appropriate HRP-conjugated secondary Ab in 5% nonfat dried milk for 1 h with constant agitation. The blots were thoroughly washed, then incubated with ECL substrate (Amersham Life Sciences) and exposed to film.

Erk2 kinase assay

Erk2 kinase activity was measured in vitro as described previously (9). Briefly, MAP kinase was immunoprecipitated from freshly prepared cell lysates using an Erk2-specific mAb (1 µg), followed by protein G-Sepharose (20 µl). Immune complexes were washed once in PBS followed by a further two washes in 0.5 M lithium chloride/20 mM Tris, pH 8, before being resuspended in (MAP kinase buffer (40 mM HEPES (pH 8), 5 mM DTT, 0.1 mM EGTA, 5 mM magnesium acetate, and 1 mM sodium orthovanadate). Myelins basic protein (10 µg) and 15 µCi [γ32P]ATP were added, and immune complexes were incubated at room temperature for 30 min. Reactions were stopped by the addition of 100 µl of 75 mM orthophosphoric acid. Samples were spotted onto phosphocellulose paper (Whatman, Maidstone, U.K.), left to dry, then extensively washed in 75 mM orthophosphoric acid. The papers were dried and counted by liquid scintillation.

Phosphatase assays

SHP-1 tyrosine phosphatase activity was measured by an assay that has been previously described by Harder et al. (15) using a commercial (Upstate Biotechnology) kit specific for SHP-1 activity. Briefly, anti-Lyn and anti-SHP-1 immunoprecipitate samples were washed four times in 10 mM Tris-HCl, pH 7.4, and finally 25 µl of each sample was pipetted into wells of a microtiter plate. SHP-1-specific phosphopeptide (RRLIEDAPVYARG; 350 µM) and 100 µl of malachite green solution were added to each well, and the reaction was allowed to proceed at room temperature for 15 min. The A630 was measured for each sample, and the phosphate concentration was assessed with reference to a standard curve (16). Pac-1 activity was also assessed in anti-Lyn and anti-Erk2 immune complexes using a UBI kit by following the hydrolysis of p-nitrophenyl phosphate as described by the manufacturers. All assays were performed in triplicate, and the results are presented as the mean ± SD. The data presented are representative of at least three independent experiments.

Results

Pre-exposure to ES-62 primes BCR-mediated association of the effector PTK Lyn with the tyrosine phosphatase, SHP-1

We have previously shown that pretreatment of B cells (4 h) with ES-62 (2 µg/ml) desensitizes BCR coupling to ErkMAP kinase activity. Moreover, although such coupling is dependent on activation of BCR-associated PTKs, we have demonstrated that ES-62-induced desensitization does not involve the abrogation of BCR-mediated activation of the PTKs, Lyn, Syk, Blk, or Fyn (9). However, treatment with ES-62 profoundly suppresses BCR-mediated tyrosine phosphorylation events resulting from activation of such PTKs (9) within 10 min of ligation of sIg, and thus we investigated whether ES-62 mediates desensitization of BCR-coupled ErkMAP kinase by promoting the recruitment of the tyrosine phosphatase, SHP-1. Such dephosphorylation would result not only in the abortive termination of BCR signaling, but also, via dephosphorylating the ITAMs on the BCR-associated accessory transducing molecules, Ig-a (CD79a) and Ig-β (CD79b) (11, 17), prevent reinitiation of BCR-signaling.

We found, however, that incubation of B cells with ES-62 did not significantly stimulate SHP-1 activity over a 30-min period (Fig. 1a). Indeed, ES-62 did not stimulate SHP-1 activity at any
point over the normal 4-h preincubation period (results not shown).

In contrast, anti-Ig strongly and transiently stimulated SHP-1 activity within 30 min of BCR ligation, kinetics consistent with the gradual return toward basal levels of tyrosine phosphorylation observed within this time period following BCR ligation (9). Importantly, however, we found that pretreatment of the cells with ES-62 followed by restimulation via the BCR modulated the kinetics of SHP-1 activation, resulting in the rapid activation of SHP-1 (Fig. 1b) with kinetics commensurate (within 10 min) with the ES-62-induced abrogation of BCR-mediated tyrosine phosphorylation (9). As the amplitude of the anti-Ig-induced response seen in ES-62-treated cells is comparable to that observed in control cells, these data suggest that ES-62 priming acts to ensure a rapid negative regulatory signal to prevent initiation of BCR signaling.

In addition, although the levels of Lyn activity resulting from ligation of the BCR were essentially the same in control and ES-62-pretreated B cells, as determined by measurement of the in vitro kinase activities of anti-Lyn immune complexes (Fig. 2a) (9), we found that pre-exposure to ES-62 greatly enhanced the recruitment of SHP-1 within 30 min of BCR stimulation to these anti-Lyn immune complexes relative to that in control cells (Fig. 2b). In contrast, Lyn expression in these immune complexes remained constant (Fig. 2b). Moreover, in vitro SHP-1 phosphatase assays on these anti-Lyn immune complexes showed enhanced SHP-1 activity following 30 min of anti-Ig treatment in cells pretreated with ES-62 relative to that observed in control cells (Fig. 2c).

SHP-1 was found to coimmunoprecipitate with Lyn in control unstimulated cells; since Lyn is the PTK that initiates BCR activation by tyrosine phosphorylating the ITAMs on Ig-α/β, this finding is likely to support the proposal that SHP-1 acts to keep the BCR in its resting state. Consistent with this, following stimulation via the BCR, SHP-1 dissociates from Lyn-based complexes, only reassociating and being stimulated (Fig. 2) with kinetics consistent with BCR-mediated activation of SHP-1 activity (Figs. 1 and 2) and termination of early (within 30 min) tyrosine phosphorylation signals (9). The virtually undetectable levels of Lyn-SHP-1 association in cells treated with ES-62 alone (Fig. 2) correlates not only with the ES-62-mediated induction of Lyn activity and subsequent tyrosine phosphorylation events we have reported previously (9), but also with the lack of significant SHP-1 activity observed in anti-SHP-1 immune complexes derived from B cells exposed to ES-62 for up to 4 h (Fig. 1 and results not shown). This targeting of Lyn-SHP-1 complexes was selective, as pre-exposure to ES-62 did not result in modulation of BCR-mediated SHP-1 association with the PTKs, Syk, or Blk (results not shown).

**FIGURE 1.** Modulation of BCR-coupled SHP-1 activity by pre-exposure to ES-62. a, B cells were stimulated for the time indicated with medium (□), anti-Ig (50 μg/ml; ○), or ES-62 (2 μg/ml; ■) for up to 60 min as indicated. b, Cells were incubated with medium (□) or ES-62 (2 μg/ml; ■) for 4 h before challenge with anti-Ig (50 μg/ml) for the times indicated. Cell lysates and anti-SHP-1 immune complexes were prepared, and SHP-1 activity was measured immediately using the Upstate Biotechnology kit as described in Materials and Methods.

ES-62 uncoupling of the BCR from the MAP kinase cascade correlates with modulation of tyrosine phosphorylation of Ig-β and Shc adaptor complexes

Pretreatment with ES-62 appears to prime B cells for the induction of total and Lyn-associated SHP-1 activation following subsequent

**FIGURE 2.** ES-62 promotes BCR-mediated recruitment of SHP-1 to anti-Lyn immune complexes and primes anti-Ig-stimulated SHP-1 activity. B cells were incubated with either ES-62 (2 μg/ml) or medium for 4 h. Following the ES-62 treatment the cells (5 × 10⁵) were incubated with anti-Ig (50 μg/ml) or medium for up to 30 min and assessed for ES-62-mediated desensitization of sIg-mediated activation of Lyn in in vitro kinase assays of anti-Lyn immune complexes (a). b, These anti-Lyn immune complexes were probed by Western blotting for expression of Lyn and SHP-1. c, B cells were stimulated with medium (□) or ES-62 (2 μg/ml; ■) for 4 h before challenge with anti-Ig (50 μg/ml) for the times indicated. Cell lysates and anti-Lyn immune complexes were prepared, and SHP-1 activity was measured immediately using the Upstate Biotechnology kit as described in Materials and Methods.
stimulation of the Ag receptors (Figs. 1 and 2). To determine whether this induction of SHP-1 activity correlated with the desensitization of BCR-MAP kinase activity, we decided to investigate whether potential targets of BCR-associated SHP-1 activity involved in the coupling of the BCR to MAP kinase were dephosphorylated in ES-62-pretreated cells. As a first approach, we determined whether BCR-associated ITAMs were dephosphorylated; we found in control cells that anti-Ig induced a strong enhancement of tyrosine phosphorylation of Ig-β, whereas this response was strongly suppressed in ES-62-pretreated cells (Fig. 3), results consistent with ES-62 acting to block initiation of BCR signaling. Interestingly, ES-62 alone did not induce tyrosine phosphorylation of Ig-β and, indeed, appeared to slightly suppress its phosphorylation, providing further evidence that ES-62 does not abortively activate B cells via the BCR transduction machinery (6).

The abrogation of BCR-stimulated tyrosine phosphorylation of Ig-β in cells pre-exposed to ES-62 was reflected by a decrease in the level of BCR-driven tyrosine phosphorylation of Shc, an adaptor molecule that has been shown to recruit the RasMAP kinase regulatory complex Grb2-Sos to the plasma membrane (11); ES-62 modulates the tyrosine phosphorylation of Shc (both p52 and p46 isoforms of Shc) such that the response to stimulation via sIg is significantly reduced compared with that observed in control cells (Fig. 3). Interestingly, as we have seen for other signal transduction molecules, e.g., PTKs such as Lyn and ErkMAP kinase (9), exposure to ES-62 per se is able to cause significant activation of this adaptor molecule. Our data examining the effect of ES-62 on Ig-β (Fig. 3), however, indicates that such phosphorylation of Shc is unlikely to follow complexing with Ig-β, but, rather, probably occurs in a BCR-independent manner.

Recent data have suggested that Blnk, a central linker protein in B cell activation via Syk kinase activation, also associates with Grb2 (18) to transduce ErkMAP kinase signals (19). However, we have found that ES-62 pretreatment does not modulate BCR-mediated Syk-Blnk association (results not shown), suggesting that the effects of ES-62 do not target this route of Erk activation, but are confined to the Shc pathway.

We find that PC has essentially identical effects as ES-62 on the tyrosine phosphorylation of Ig-β and Shc (Fig. 3) and, likewise, in preventing subsequent phosphorylation and activation of these molecules normally induced by BCR ligation. Similarly, while exposure to ES-62 or PC results in the tyrosine phosphorylation of Erk1 and Erk2 MAP kinases, such treatment abrogates the tyrosine phosphorylation of these kinases normally resulting from ligation of the BCR (Fig. 3) (9). These results therefore further support our previous studies, which suggested that PC could mimic ES-62 in the induction of many of the signaling lesions that we have described in B and T cells (4, 6, 7, 9, 10). Moreover, our data are consistent with the proposal that PC-containing ES uncouple the BCR from ErkMAP kinase activation at least in part by disrupting early tyrosine phosphorylation-dependent signals, including the Shc-mediated recruitment of the MAP kinase cascade. Consistent with this, we found that while anti-Ig stimulates association of Shc and Sos, the guanine nucleotide exchange factor for Ras, pretreatment with ES-62 completely abrogates complex formation between Shc and Sos (Fig. 3e).

**Pre-exposure to ES-62 primes the BCR-mediated recruitment and activation of the MAP kinase phosphatase, Pac-1**

The above data provide mechanisms to explain how ES-62 acts to prevent activation of the MAP kinase cascade following restimulation via the BCR. However, treatment with ES-62 alone stimulates sustained ErkMAP kinase activity (9), although such Erk activity is also rapidly terminated following restimulation via the BCR (9). This might suggest that cross-talk between signals emanating via ES-62 and the BCR induces a negative regulatory switch acting directly at the level of ErkMAP kinase activity. A family of dual specificity (Thr/Tyr) MAP kinase phosphatases has recently been identified that act to terminate ongoing MAP kinase activity (20), and one such phosphatase, Pac-1, has been shown to be involved in the regulation of Ag receptor-directed Erk activation in lymphocytes (21). We therefore determined whether B cells, pre-exposed to ES-62 and subsequently challenged via the BCR, demonstrated enhanced recruitment and activation of Pac-1 phosphatase.

To do this we used a combination of techniques, including assessment of MAP kinase activity by Western blot analysis of the dual phosphorylation (Thr183/Tyr185) status of Erk1 and Erk2 (22, 23) and measurement of the in vitro kinase activities of anti-Erk2 immune complexes (9). We also probed the Erk2-containing immune complexes for expression of associated MAP kinase phosphatases such as Pac-1 and, in addition, the Ser/Thr phosphatase PP2A, which has also been reported to modulate ErkMAP kinase activity (20, 24). This experimental approach not only allowed us to assess the effects of pre-exposure to ES-62 on the kinetics of BCR-mediated Erk1 and Erk2 activation, but also to determine whether any loss of activity reflected abrogation of the dual phosphorylation that is required for full activation of Erk and is the substrate for Pac-1 MAP kinase phosphatase.

Cross-linking of the BCR with anti-Ig (50 μg/ml) induces the rapid activation of MAP kinase, as determined by analysis of dual phosphorylation of Erk1 and Erk2 (Fig. 4a). Similarly, cells pre-treated with ES-62 alone for 4 h exhibit significant dual phosphorylation of both Erk isoforms. This dual phosphorylation, however,
is rapidly terminated following restimulation of the ES-62-treated cells via the BCR, such that the normal response observed following BCR ligation is essentially ablated within 10 min (Fig. 4a). These results were corroborated by parallel in vitro kinase assays of anti-Erk2 immune complexes that similarly showed that the Erk2 activity normally resulting from stimulation via the BCR was virtually abrogated by pre-exposure to ES-62 (Fig. 4b).

Western blot analysis of these Erk2 immune complexes showed that pretreatment with ES-62 for 4 h led to a substantial increase in the Erk2 activity normally resulting from stimulation via the BCR. In contrast, levels of Erk2 in these immune complexes remained constant (Fig. 4c). This increase in Erk2 activity normally resulting from stimulation via the BCR was virtually abrogated by pre-exposure to ES-62 (Fig. 4b).

Discussion

We have previously shown that ES-62-mediated inhibition of sIg-coupled B cell proliferation in vitro is associated with the selective modulation of BCR-mediated tyrosine phosphorylation and uncoupling from key proliferative pathways, such as the RasMAP kinase cascade (4, 6, 8–10). We now report that ES-62 uncouples the BCR from ErkMAP kinase signaling by selectively priming B cells for the BCR-mediated induction of two major negative regulatory elements in this pathway. Hence, pre-exposure to ES-62 promotes the BCR-mediated recruitment of 1) the tyrosine phosphatase SHP-1 to return the active BCR to its resting state and prevent re-initiation of PTK-dependent signals leading to recruitment of She-containing complexes and, hence, the downstream components of the MAP kinase cascade (Figs. 1 and 2); and 2) the dual specificity phosphatase Pac-1 to terminate any ongoing ErkMAP kinase signals. Thus, we propose that the targeting and priming of such key negative regulatory elements by filarial nematode PC-containing molecules such as ES-62 (Fig. 5) allow both a rapid and profound desensitization of BCR-coupled ErkMAP kinase signaling. An intriguing feature of this immunomodulatory system is that stimulation of B cells with ES-62 alone induces many of the signals associated with ligation of the BCR, such as activation of the PTKs Lyn, Syk, and Blk and downstream signaling elements such as ErkMAP kinase (9). This has led us to propose that pre-exposure to the parasite molecule may induce an
FIGURE 5. ES-62 uncouples the BCR from ErkMAP kinase activation by priming B cells for BCR-mediated recruitment of the negative regulatory elements SHP-1 and Pac-1. 1, ES-62 uncouples PTK signaling by promoting the BCR-mediated recruitment of Lyn-SHP-1 protein tyrosine phosphatase complexes. 2, Dephosphorylation of Ig-β promotes recruitment of Shc-Sos complexes and, hence, RasMAPK recruitment and activation. 3, ES-62/BCR cross-talk induces Erk-Pac-1 complex association and stimulates Pac-1 activity to terminate ongoing ErkMAP kinase activity.

abortive activation of B cells, such that cross-talk with signals consequently emanating from the BCR results in the coordinate induction of negative regulatory elements such as SHP-1 and Pac-1 and thereby induces a state of B cell anergy.

At present, the cross-talk signals responsible for enhanced Lyn-SHP-1 and Erk2-Pac-1 complex formation and resultant phosphatase activity observed in response to signaling via the BCR in cells pre-exposed to ES-62 are not clear. Although we have yet to elucidate how pre-exposure to ES-62 primes SHP-1 to interact with the BCR and dephosphorylate Ig-β, it is well established that this phosphatase interacts directly with the BCR in unstimulated cells to maintain the BCR in its resting state. Moreover, it has been proposed that SHP-1 may mediate its effects on stimulated cells via Lyn- and CD22-driven negative feedback mechanisms. Indeed, a recent paper has also shown that Lyn and SHP-1 associate, and these authors propose that such SHP-1/Lyn association mediates down-regulation of Lyn effector functions such as CD19 phosphorylation and, hence, desensitization of B cells. Interestingly, our preliminary data suggest that BCR-stimulated tyrosine phosphorylation of CD19 is reduced in ES-62-treated cells (results not shown). Although we have found that pretreatment with ES-62 does not significantly alter the amount of Lyn (or Syk, Fyn, or Btk) activity that we observe in response to BCR signaling alone (9), as ES-62 treatment alone induces substantial Lyn activity, these findings suggest that the increased association with SHP-1 may act to suppress the enhanced Lyn activity that might have been expected in response to stimulation with ES-62 and anti-Ig. Thus, as Lyn is likely to be a key element in the phosphatidylinositol 3-kinase pathway and recruitment of key signaling adaptors such as Shc, colocalization of Lyn and SHP-1 could maximize the efficiency of desensitization of BCR signaling by ES-62. Pertinent to this, it has previously been shown that SHP-1 can interact with Grb2 and Sos in hemopoietic cells, and so BCR signaling may drive formation of a Shc-Grb2-Sos complex that also binds Lyn and SHP-1 and, hence, allows for phosphorylation (Lyn) and dephosphorylation (SHP-1) of Shc. Indeed, as Grb2 is not a substrate for SHP-1, it has already been proposed that this interaction may localize SHP-1 near some of its substrates in lymphocytes, and, hence, this mechanism could explain our observed dephosphorylation of Shc and uncoupling from Sos-mediated activation of the RasErkMAP kinase cascade.

Although Hashimoto et al. (32) showed that Shc, presumably due to functional redundancy with Blnk, was dispensable for BCR coupling to the RasMAPK cascade in chicken DT40 cells, many other groups have shown a role for Shc in such BCR signaling in mammalian B cells (reviewed in Refs. 19 and 33). Moreover, and consistent with our proposal that ES-62-mediated suppression of BCR-stimulated phosphorylation of Shc correlates with uncoupling of the BCR from Erk, we have found that ES-62 promotes the dissociation of Shc-Sos complexes (Fig. 3). In contrast, pretreatment with ES-62 did not inhibit association of Syk, Blnk, and Grb2 signaling complexes (data not shown), which have been postulated to comprise an alternative BCR-RasMAPK kinase adaptor cascade (reviewed in Refs. 19 and 33). Moreover, although the existence of Shc-dependent and independent pathways for localization of Grb2-Sos complexes suggests functional redundancy in BCR-Ras coupling, Kelly and Chan (33) argue that the Shc-Grb2-Sos pathway may represent a mechanism for modulation of Ras activity. In this model the degree of Ras activation through Grb2-Sos may be increased or decreased depending on the involvement of Shc, and hence, Shc may act as a rheostat in determining the degree of Ras activation. Taken together, these data suggest that ES-62 effectively modulates RasErkMAP kinase signaling and B cell proliferation by selectively targeting the rheostat control of BCR-RasMAPK kinase coupling.

Our data indicate that the observed activity of ES-62 is almost certainly dependent on PC, a structure previously recognized as possessing both immunomodulatory (34, 35) and signaling (36, 37) properties. The parasite product, like many filarial ES, including those obtained from human parasites (38), contains PC covalently attached to N-type glycans. Indeed, we had previously shown that the PC moiety can mimic many of the effects of ES-62 on BCR signaling (Fig. 3) (6, 9) and B cell activation (4, 6, 9, 10). Moreover, preliminary evidence from BioCore, ES-62-binding/pull-down studies to surface biotinylated B cells and Far Western studies suggests that ES-62 mediates its effects on B cells by binding with high affinity to a PC-dependent manner to B cell proteins of 82 and 135 kDa, respectively. Furthermore, preliminary Western blotting studies show that the p82-ES-62 binding chain (which is also present on T cells and macrophages) appears to be TLR2. One or more of these ES-62-binding cell surface proteins may be the PC receptor reported previously to be expressed by a subset of B cells (39). However, with respect to PC/ES-62 desensitization of BCR-coupled Erk activation, the signal is essentially fully ablated, suggesting that in our study essentially all cells are targeted. Similarly, the fact that BCR coupling to PLC is not affected by pre-exposure to ES-62 (6) argues against targeting of PC-specific BCRs. Further supporting evidence argued against recruitment of PC-specific BCRs or, indeed, weak polyclonal BCR signaling by ES-62 is provided by our findings that exposure to ES-62 alone does not stimulate tyrosine phosphorylation of Ig-β (Fig. 3) or PLC activation (6). Our proposal that ES-62 binds to a non-BCR receptor is also supported by our previous studies of the effects of pretreatment with a range of concentrations of anti-Ig on several aspects of BCR signaling. In contrast to what we observe with ES-62, pretreatment with anti-Ig not only profoundly desensitizes BCR-coupled inositol trisphosphate production and calcium mobilization (40), but also up-regulates PKC expression and activation (8, 41). Moreover, and in direct contrast to what we observed with ES-62, pretreatment with low concentrations of anti-Ig is widely established to prime and enhance BCR-stimulated DNA synthesis (42).

To summarize, pre-exposure to the parasite molecule appears to induce an abortive activation of B cells that, following cross-talk with signals consequently emanating from the BCR, results in the
coordinate induction of negative regulatory elements such as SHP-1 and P-ac to induce rapid and effective desensitization of B cells to sustained proliferative signaling via the BCR. We would argue that ES-62 abortively activates the system rather than globally exhausts it, as we found that stimulation of B cells with ES-62 and IL-4 is comitogenic (10). Moreover, the similarities we have observed when comparing the effects of ES-62/PC on B and T cells to date (4, 6–10) enable us to predict with some confidence that ES-62 may desensitize T cells by a similar mechanism. There have, in fact, been more studies described in the literature on defects in proliferative responses of the latter cell type (35, 43) than on B cells from filariasis patients, but as with B cells, elucidating mechanisms has proved frustratingly difficult (44). We hope that the data presented here are a significant step to resolving this situation.

References