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MAPkinase: a second site of G-protein regulation of B-cell activation via the antigen receptors

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SUMMARY

Ligation of the antigen receptors on B cells transduces transmembrane signals leading to the induction of DNA synthesis. We now show that a pertussis toxin-sensitive heterotrimeric G-protein(s) of the G_i class plays a key role in the regulation of surface immunoglobulin (sIg)-mediated DNA synthesis in B cells. This site of G-protein regulation is distinct from that we have previously reported to govern the coupling of the antigen receptors on B cells to the phospholipase C-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate. We have, moreover, identified a candidate target for this new G-protein regulation by showing that mitogen-activating protein kinase (MAPkinase) activity, which plays a key role in the transduction of sIg-mediated proliferative signals in B cells, is abrogated by pre-exposure to pertussis toxin that covalently modifies and inactivates heterotrimeric G-proteins of the G_i class. Furthermore, our data suggest that this pertussis toxin-sensitive G-protein couples the antigen receptors to MAPkinase activation, at least in part, by regulating sIg-coupling to Lyn, Syk and perhaps Blk and Fyn activity, results consistent with studies in other systems which show that classical G-protein-coupled receptors recruit such protein tyrosine kinases to tranduce MAPkinase activation. Interestingly, however, this G-protein plays no apparent role in the control of up-regulation of major histocompatibility complex class II expression on B cells, suggesting that such G-protein-regulated-tyrosine kinase and MAPkinase activation is not required for the induction of this biological response following antigen receptor ligation.

INTRODUCTION

B cells respond to antigen via cell surface clonatypic receptors (surface immunoglobulins; sIg). Ligation of sIg by antigen or mitogenic anti-immunoglobulin antibodies ultimately leads to B-cell proliferation and differentiation. Although the events leading to B-cell proliferation have not been fully elucidated, it is clear that ligation of the B-cell antigen receptor complex (BCR), which comprises sIg and its accessory ITAM (Immunoreceptor Tyrosine-based Activation Motif)-containing transducing molecules, lg-α (CD79a) and lg-β (CD79b), induces protein tyrosine kinase (PTKs) activity.[1] The resulting tyrosine phosphorylation induces recruitment of Syk kinase and the reorientation, enhanced binding and activation of ITAM-associated *src*-family PTK, Blk, Fyn, Lck and Lyn, leading to BCR-association of a number of key signal transducers, implicated in cellular activation and proliferation, such as phospholipase C (PLC)-γ, phosphoinositide-3-kinase (PI-3-K) and the components of the Ras/MAPkinase (mitogen-activating protein kinase) signalling cascades.[1]

We[2,3] and others[4–8] however, have also provided evidence that BCR signalling may be additionally controlled by one or more G-proteins, regulatory signal transduction elements that bind and hydrolyse GTP, which have been implicated in the regulation of key cellular events, such as transcription, transmembrane signalling, protein trafficking, proliferation and differentiation.[9] Most of this evidence focuses on the role of G-proteins in the coupling of the BCR to early signalling events such as phosphatidylinositol-4,5-biphosphate ($PtdInsP_2$) hydrolysis: for example disruption and reconstitution experiments in permeabilized B cells showed that both classes of antigen receptor (sIgM and sIgD), exhibit the classical kinetics of a G-protein-coupled inositol phosphate signalling pathway; namely rapid agonist-accelerated kinetics and reduced calcium dependence of GTPγS-driven $PtdInsP_2$ hydrolysis.[2–8] Moreover, such signalling via the sIg receptors could be blocked by introduction of the G-protein antagonist, GDPβS or by pretreatment of intact cells with the bacterial product, pertussis toxin (PTX) which specifically targets and inactivates G-proteins of the G_i family by inducing ADP-ribosylation of their Gα subunits.[5–8] Importantly, these studies

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also provided a mechanism for the G-protein regulation of BCR-coupling to $PtdInsP_2$ hydrolysis in that they demonstrated that PTX-mediated inactivation of G_i-like G-proteins abrogated all BCR-stimulated tyrosine phosphorylation and in particular, tyrosine phosphorylation, and hence presumably activation, of PLC-γ.[7] This mechanism was supported by corroborating studies which showed that whilst GTPγS or aluminium fluoride could stimulate tyrosine phosphorylation events in permeabilized B cells,[3,7,10] the essentially identical responses observed in response to anti-immunoglobulin were blocked by introduction of GDPβS.[7] Although the BCR is not structurally homologous to the classically defined, seven transmembrane spanning G-protein-coupled receptors, taken together, these results suggested that one or more G-proteins associated with sIg, act to regulate coupling of the BCR to PTK activation and downstream signalling events such as $PtdInsP_2$ hydrolysis. A precedent has already been set for such non-classical G-protein-regulation, by studies on the intrinsic tyrosine kinase receptors for insulin and epidermal growth factor (EGF), which supported similar roles for the PTX-sensitive G-proteins of the G_i class in the regulation of autophosphorylation of the insulin receptor,[11] coupling of the insulin-like growth factor-II (IGF-II)/Mannose 6-phosphate receptor to G_{i2} signalling[12] and in the EGF-Receptor-coupled activation of PLC-γ1 in normal rat hepatocytes.[13]

Although most of the above studies focused on the G-protein regulation of BCR-coupling to $PtdInsP_2$ hydrolysis, some of them also provided evidence of additional sites of G-protein regulation of B-cell activation including receptor patching and capping[5] and up-regulation of early response genes such as c-*fos*, and also DNA synthesis.[3,7,14,15] We now report that coupling of the BCR to MAPkinase, a key proliferative signal transducer in B-cell activation, is regulated, at least in part, by a PTX-sensitive G_i-like protein: since MAPkinase activation appears to play an important role in the initiation of transcriptional events, this may prove to be a major site of G-protein regulation of sIg-mediated B-cell proliferation.

MATERIALS AND METHODS

Antibodies

Anti-protein tyrosine kinase antibodies for immunoprecipitation were obtained from Transduction Products, Inc. (Lexington, KY; Anti-Lyn) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA; anti-Blk, anti-Syk and anti-Fyn). In addition, anti-mouse immunoglobulin–horseradish peroxidase (HRP), anti-rabbit immunoglobulin–HRP and enhanced chemiluminence (ECL) reagents were obtained from Amersham International, (Amersham, Bucks, UK). $F(ab'_2)$ fragments of rabbit anti-mouse immunoglobulin antibodies were obtained from Jackson Immunoresearch Laboratories, Inc., distributed by Stratech Scientific Ltd, Bedfordshire, UK. In addition, a monoclonal antibody (mAb) to Erk2 (mouse anti-peptide EETARFQPGYRS) was generated in association with Dr A. M. Campbell (Department of Biochemistry, University of Glasgow). Protein A– and protein G–Sepharose were obtained from Sigma (Poole, Dorset, UK).

B-cell culture

Tissue culture materials were from Gibco (Paisley, UK); Ficoll–Hypaque and Percoll were purchased from Sigma and radiochemicals were from Amersham International. All other reagents were of the highest grade available.

Small, dense B (>90% sIg^+) cells were prepared from the spleens of 12-week-old male CBA/Ca, (CBA × C57BL/6) F_1 or BALB/c mice as described previously.[16] Briefly, B cells were prepared by depleting T cells with anti-Thy-1 and complement, followed by Percoll density fractionation. For measurement of DNA synthesis, murine B cells (2×10^5 cells/200 μl) were cultured in triplicate in flat bottom microtitre wells in RPMI-1640 medium supplemented with glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids, 2-mercaptoethanol (50 μM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 5% fetal calf serum (FCS). [^{3}H]Thymidine, at 0·5 μCi/well was generally added at 68 hr, and cultures were harvested 4 hr later with an automated cell harvester (Skatron) and incorporated label was estimated by liquid scintillation counting.[16]

For analyses of Ia antigen expression, B cells from CBA/Ca mice were cultured at 10^6/ml with the indicated stimuli for 20–24 hr, harvested, and stained with fluorescein isothiocyanate (FITC)–anti-I-A^k (11.52.19) prior to analysis on the fluorescence-activated cell sorter as described previously (FACS; Coulter Electronics Ltd, Luton, UK).[17] Alternatively, B cells from BALB/c mice were stained with biotinylated-anti-I-A^d (34.5-3) and streptavidin–phycoerythrin and analysed in a Becton-Dickinson Facscan (Sunnyvale, CA). The 34.5-3 hybridoma was a kind gift from Dr E. C. Snow (Department of Microbiology and Immunology, University of Kentucky College of Medicine, Lexington, KY).

Reversible permeabilization of murine B lymphocytes using ATP

A wide range of cells and cell lines, including lymphocytes, can be permeabilized by incubation with ATP^{4-} in the absence of divalent cations and resealed following addition of excess magnesium ions.[18,19] Small, dense B (>90% sIg^+) cells were prepared from the spleens of 12-week-old male CBA/Ca, (CBA × C57BL/6) F_1 or BALB/c mice as described above and washed three times in a divalent cation-free salt solution (pH 7·7 comprising 137 mM NaCl, 2·7 mM KCl, 20 mM HEPES, 5·6 mM glucose and 1 mg/ml bovine serum albumin). The cells were then resuspended in this buffer at 2×10^6 cells/ml and permeabilized with 500 μM ATP for 10 min: at pH 7·7, and in the absence of divalent cations, approximately all the ATP will be fully ionized in the ATP^{4-} form. Following the permeabilization period, the cells were resealed by addition of 2 mM $MgCl_2$ and 1 mM $CaCl_2$. After a further 10 min culture, the cells were then washed three times and resuspended in supplemented RPMI medium and processed, for example, for [^{3}H]thymidine uptake experiments, as normal intact cells. Permeabilization (97·6% cells using 500 μM ATP; 67% cells using 50 μM) was monitored by the uptake of the normally impermeant fluorescent nuclear stain, ethidium bromide (25 μM) and cell viability following permeabilization/resealing was assessed by trypan blue exclusion. The permeabilization/resealing cycle had no significant effect on the anti-immunoglobulin- ($95 \pm 7\%$, $n=25$) or lipopolysaccharide-(LPS; $104 \pm 3\%$, $n=25$) stimulated DNA synthesis responses obtained relative to those of control cells which had not undergone the permeabilization/resealing cycle.

Permeabilization/resealing of the large numbers of cells required for cell signalling experiments proved cumbersome

and inefficient (for example, a 10-fold increase in cell concentration led to a 50–70% decrease in the percentage of cells permeabilized) and thus as labelling studies using [^{32}P-α]GTP and [^{35}S-γ]GTPγS showed that these compounds were readily taken up by B cells during a 4–24-hr culture period, where indicated, some of the 3-day DNA synthesis experiments were performed on intact cells simply cultured with GDPβS.

Measurement of inositol phospholipid hydrolysis

Inositol phospholipid hydrolysis in murine B cells was analysed by previously published methods.[2,16] In brief, lymphocytes were labelled with [^{3}H]myo-inositol (specific activity 110 Ci/mmol; Amersham International). Labelled cells were then stimulated either as intact cells, or following permeabilization with ATP[18] or streptolysin-O,[2] with anti-antigen receptor antibodies, with or without GTPγS and/or GDPβS. Total ^{3}H-labelled inositol phospholipids, or the individual species, were determined following ion-exchange chromatography of the water-soluble extracts obtained from the aqueous phase of the Bligh-Dyer phospholipid extraction.

Preparation of cell lysates

B cells (5×10^6) were preincubated with either dithiothreitol (DTT)-activated PTX (1 μg/ml final concentration)[7,20] from *Bordetella pertussis* (Porton Products, Porton Down, UK) or medium alone for 4 hr and then incubated with anti-immunoglobulin (50 μg/ml) or medium alone for the indicated time at 37°. Following cell incubations, reactions were terminated by addition of lysis buffer [50 mM Tris buffer, pH 7·4 containing 150 mM sodium chloride, 2% (v/v) nonidet P-40 (NP-40); 0·25% (w/v) sodium deoxycholate, 1 mM EGTA, 10 mM sodium orthovanadate, 0·5 mM phenylmethylsulphonylfluoride, 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°. The supernatants were transferred to fresh tubes and precleared for immunoprecipitations with protein G–Sepharose.

Erk2 kinase assay

MAP kinase activity was immunoprecipitated from freshly prepared cell lysates using the Erk2-specific mAb (1 μg) followed by protein G–sepharose (20 μl). Immune complexes were washed once in phosphate-buffered saline (PBS) followed by a further two washes in 0·5 lithium chloride/20 mM Tris, pH 8 before being resuspended in MAPkinase buffer (40 mM HEPES, pH 8/2 mM DTT/0·1 mM EGTA/5 mM magnesium acetate/1 mM sodium orthovanadate). Myelin basic protein (10 μg) and 15 μCi of [^{32}P-γ] ATP were added and immune complexes were incubated at room temperature for 30 min. Reactions were stopped by the addition of 100 μl, 75 mM orthophosphoric acid. Samples were spotted onto phosphocellulose paper (Whatman Ltd, Kent, UK), left to dry, then extensively washed in 75 mM orthophosphoric acid. The papers were dried and counted by liquid scintillation.

In vitro *PTK assays*

The individual PTK were immunoprecipitated from freshly prepared cell lysates using the appropriate antibodies (1 μg) followed by protein G–Sepharose (20 μl) and washed once in PBS, pH 7·4 followed by a further wash in 0·5 lithium chloride/20 mM Tris, pH 8. Immune complexes were then washed once in kinase buffer (10 mM manganese chloride/50 mM Tris, pH 7·4/1 mM sodium orthovanadate) and then finally resuspended in a further 40 μl of the kinase buffer. Enolase (10 μg) was added along with 15 μCi of [^{32}P-γ] ATP and the samples were incubated at room temperature for 15 min. The reaction was stopped by the addition of 100 μl, 75 mM orthophosphoric acid. Samples were spotted onto phosphocellulose paper, left to dry, and then extensively washed in 75 mM orthophosphoric acid. The papers were dried and counted by liquid scintillation.

RESULTS

G-protein antagonists inhibit anti-immunoglobulin-stimulated DNA synthesis in ATP-permeabilized/resealed B cells

Stimulation of resting splenic B cells either by cross-linking the antigen receptors with F(ab)′$_2$ fragments of anti-immunoglobulin antibodies (Fig. 1a) or by culture with the polyclonal mitogen, LPS (Fig. 1b) leads to the induction of DNA synthesis within 72 hr. Pretreatment of B cells with PTX profoundly inhibits sIg-mediated DNA synthesis, suggesting that one or more G_i-like G-proteins are involved in the

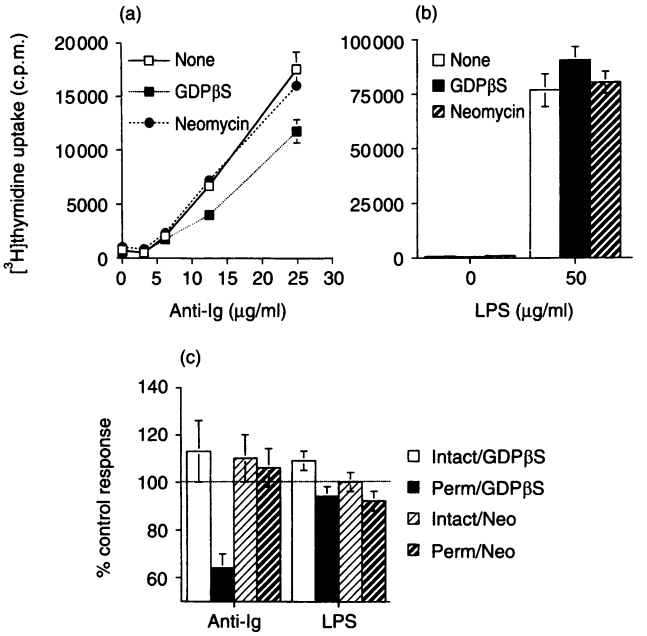

Figure 1. Introduction of GDPβS into permeabilized/resealed B cells blocks sIg-mediated DNA synthesis. Small dense resting B cells were permeabilized and resealed using ATP^{4-} in the presence of buffer, GDPβS (100 μM) or neomycin (10 mM) as indicated. Following washing, the cells were cultured in the presence of anti-immunoglobulin or LPS, at the indicated concentrations, and DNA synthesis measured after 72 hr. In (a) and (b), results are expressed as mean [^{3}H]thymidine uptake (c.p.m.) ±SD from a single representative experiment performed in triplicate. In (c), the data are normalized such that the control anti-immunoglobulin or LPS-response in mock-permeabilized (Intact) cells (no ATP) is taken as 100% and the response obtained using trapped GDPβS or neomycin is calculated as a percentage. The data presented in this panel represent the mean ±SD of normalized values taken from at least 14 independent experiments performed in triplicate.

regulation of BCR-mediated proliferation (results not shown and refs. 7,14). In order to identify key signals involved in the G-protein regulation of sIg-mediated DNA synthesis, we have exploited an *in vitro* system of transient cell permeabilization/resealing[18,19] which allows us to introduce and trap low molecular weight (<1000) antagonists of various signal transducers. Trapping of GDPβS, which is a stable antagonist of the regulatory G-proteins, substantially inhibits anti-immunoglobulin- (64±6% control response, $n=14$), but not LPS- (94±4%, $n=15$) stimulated DNA synthesis in permeabilized/resealed, but not mock permeabilized (intact), B cells (Fig. 1c). These results therefore suggest that one or more G-proteins may play a key role in the regulation of B-cell activation via the antigen receptors.

We[2] and others[4,7,8] have previously shown that coupling of the antigen receptors to $PtdInsP_2$-PLC activation occurs in a G-protein-dependent manner. To determine if trapped GDPβS blocks sIg-DNA synthesis by targetting coupling to this early signalling event, we have also compared the effects of trapping GDPβS or neomycin, a reagent which chelates $PtdInsP_2$ and blocks PLC hydrolysis,[18] on anti-immunoglobulin-and LPS-stimulated DNA synthesis and $PtdInsP_2$ hydrolysis (Fig. 1). The results obtained show that whilst GDPβS typically inhibits the DNA synthesis responses to anti-immunoglobulin by some 40%, neomycin had no significant effect on any of the anti-immunoglobulin (106±8, $n=14$) or LPS (92±4, $n=15$) DNA responses tested despite being at least as effective as GDPβS in blocking sIg-coupling to $PtdInsP_2$ hydrolysis in permeabilized/resealed cells (Fig. 2a). Moreover, we have also found that these reagents are equally effective in blocking either direct G-protein activation of $PtdInsP_2$ hydrolysis by the G-protein agonist, GTPγS or the G-protein-dependent activation via sIg in streptolysin-O-permeabilized B cells (Fig. 2b). Taken together, these results suggests that GDPβS is targetting a G-protein regulatory site distinct from that involved in $PtdInsP_2$ hydrolysis.

Simply culturing B cells with GDPβS during the 3-day period required for optimal anti-immunoglobulin-induced DNA synthesis allowed enhanced uptake of GDPβS (see the Materials and Methods) and provided results which corroborated and extended those obtained using the permeabilization/resealing system: for example, specificity studies using a range of nucleotide diphosphates showed that whilst GDPβS and GDP inhibit anti-immunoglobulin-mediated, but not LPS-mediated, DNA synthesis, ADP, CDP, IDP and UDP have no significant effect on either anti-immunoglobulin- (Fig. 3a) or LPS (Fig. 3b)-mediated DNA synthesis, results confirming that we are specifically targetting a G-protein regulatory site.[21] Moreover, in addition to confirming our previous findings that whilst GDPβS had no significant effect on LPS-stimulated DNA synthesis, this new approach allowed us to completely abrogate the anti-immunoglobulin-mediated response and partially block the DNA synthesis response induced by costimulation of B cells with the phorbol ester, phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore, ionomycin (Fig. 3c). Costimulation with PMA and ionomycin activates protein kinase C isoforms and calcium/calmodulin-dependent kinases, and thus mimics signalling events downstream of $PtdInsP_2$-PLC. Furthermore, time-course experiments showed that anti-immunoglobulin-mediated DNA synthesis is still substantially inhibited (60–70%) even when introduction of

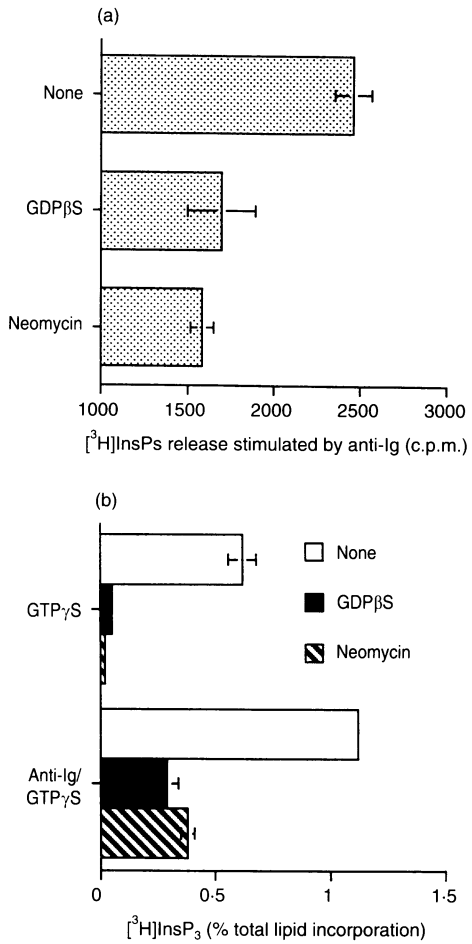

Figure 2. GDPβS and neomycin are equally effective at inhibiting sIg-coupled InsP release. In (a), small dense resting B cells prelabelled with [^{3}H]inositol were permeabilized and resealed using ATP^{4-} in the presence of buffer, GDPβS (100 μM) or neomycin (10 mM). Following washing, the cells were cultured with anti-immunoglobulin or medium and total stimulated [^{3}H]InsP release measured after 30 min. In (b), [^{3}H]inositol-labelled B cells were permeabilized with streptolysin-O in the presence of pCa7 buffer, GDPβS (100 μM) or neomycin (10 mM) as indicated and the release of stimulated [^{3}H]$InsP_3$ in response to GTPγS (100 μM) or GTPγS (100 μM) plus anti-immunoglobulin (50 μg/ml) measured after 10 min. Results are expressed as mean stimulated [^{3}H]InsP release (c.p.m.)±SD from single experiments performed in triplicate representative of at least two other independent experiments.

GDPβS to the B-cell cultures is delayed for 24 hr, but not 48 hr, following initiation of BCR cross-linking (Fig. 3d). Similarly, the DNA synthesis stimulated by PMA plus ionomycin is inhibited by some 40–50% when addition of GDPβS is delayed for 24 hr (results not shown).

Taken together these results suggest that GDPβS is targetting one or more G-proteins which play a key role(s) in the regulation of sIg-coupled DNA synthesis downstream of $PtdInsP_2$ hydrolysis. Evidence that this G-protein is specific for the regulation of BCR-coupled DNA synthesis was provided by parallel studies investigating the role of G-proteins in the regulation of major histocompatibility complex (MHC) Class II up-regulation which showed that neither GDPβS nor neomycin had any significant effect on I-A up-regulation in

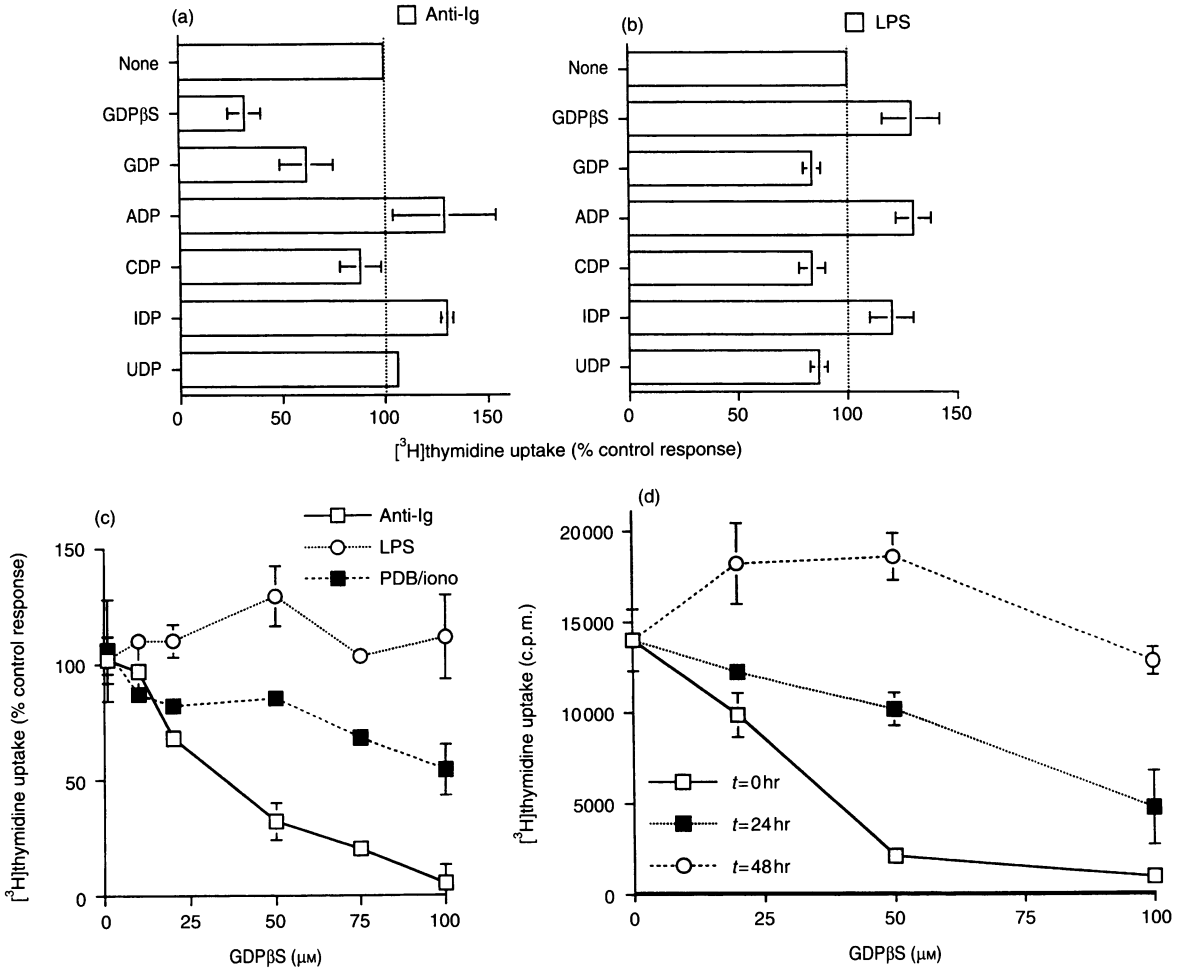

Figure 3. Specificity of GDPβS-mediated inhibition of sIg-mediated DNA synthesis in murine B cells. Small dense B cells were cultured with anti-immunoglobulin (a; 50 μg/ml) or LPS (b; 50 μg/ml) in the absence or presence of the indicated nucleotide diphosphates (100 μM) and DNA synthesis was measured after 72 hr. The data are normalized such that the control anti-immunoglobulin reponse or LPS response is taken as 100% and the response obtained with the various NDP was calculated as a percentage. The data presented in these panels represent the mean ± SD of normalized values of a single representative experiment performed in triplicate. In (c), small dense B cells were cultured with anti-immunoglobulin (50 μg/ml), PMA (100 ng/ml) plus ionomycin (1 μg/ml), or LPS (50 μg/ml) in the presence of the indicated concentration of GDPβS and DNA synthesis measured after 72 hr. The data presented in this panel represent the mean ± SD of normalized values taken from at least three independent experiments performed in triplicate. In (d), small dense B cells were cultured with anti-immunoglobulin (50 μg/ml) in the presence of the indicated concentration of GDPβS added at $t=0$, 24 or 48 hr and DNA synthesis was measured 72 hr after addition of anti-immunoglobulin. The results are expressed as mean stimulated [^{3}H]thymidine uptake (c.p.m.) ± SD from a single representative experiment performed in triplicate.

response to stimulation of B cells with anti-immunoglobulin, LPS or interleukin-4 (IL-4) (Table 1). Moreover, pretreatment with PTX, which targets and inactivates G-proteins of the G_i family, had no effect on sIg-mediated up-regulation of MHC Class II molecules (Table 2).

Antigen receptor-coupling to MAPkinase activation in B cells is regulated by a PTX-sensitive G-protein

It has recently emerged that many classical G-protein-coupled receptors are coupled to the key mitogenic signalling pathway, the RasMAP kinase cascade by recruitment of a variety of intermediate PTK-dependent signals.[22–25] Since the antigen receptors on B cells are regulated by one or more pertussis toxin-sensitive G-proteins and are coupled to PTK activation,[3,7] we decided to determine if the second site of G-protein regulation of sIg-activation outlined above was MAPkinase by investigating whether pretreatment with pertussis toxin blocked sIg-coupling to MAPkinase activation. As the data presented in Fig. 4 clearly show, pretreatment with PTX substantially blocks sIg-mediated activation of the MAPkinase, Erk2. These results therefore suggest that sIg-coupling to Erk2 activation constitutes a site of G-protein regulation during B-cell activation via the antigen receptors.

PTX differentially inhibits sIg-associated PTKs

Given that previous studies had shown that pretreatment with PTX profoundly inhibits sIg-stimulated tyrosine phosphorylation[3,7] and that classical G-protein-coupled receptors can

Table 1. GDPβS does not inhibit sIg-mediated up-regulation of I-A antigen levels on B cells

Stimulus	Median fluorescence (channels)		
	None	GDPβS	Neomycin
None	69	66	69
Anti-Ig (50 μg/ml)	96	92	99
IL-4 (10 U/ml)	122	118	118
LPS (50 μg/ml)	91	91	92

B cells (CBA/Ca mice) were permeabilized/resealed in the presence of buffer, GDPβS (100 μM) or neomycin (10 mM) and then were cultured with the indicated stimuli for 22 hr and then stained with FITC-anti-I-A^k and analysed by FACs (Coulter EPICS). Median fluorescence intensities are expressed in numbers of channels, on a log scale. The data presented are from a single representative of at least four independent experiments.

Table 2. PTX does not inhibit sIg-mediated up-regulation of I-A antigen levels on B cells

Stimulus	Median fluorescence	Mean fluorescence	% positive cells
None	148	181	82
PTX (1 μg/ml)	108	145	82
Anti-Ig (50 μg/ml)	274	352	80
PTX/Anti-Ig	274	349	81

B cells (BALB/c mice) were incubated in the presence or absence of PTX (1 μg/ml) for 4 hr and then cultured with media or anti-immunoglobulin (50 μg/ml) for 22 hr and then stained with biotinylated-anti-I-A^d and streptavidin-phycoerythrin and analysed by FACs (Becton-Dickinson, Facscan) using Lysys II version 1·1 software.

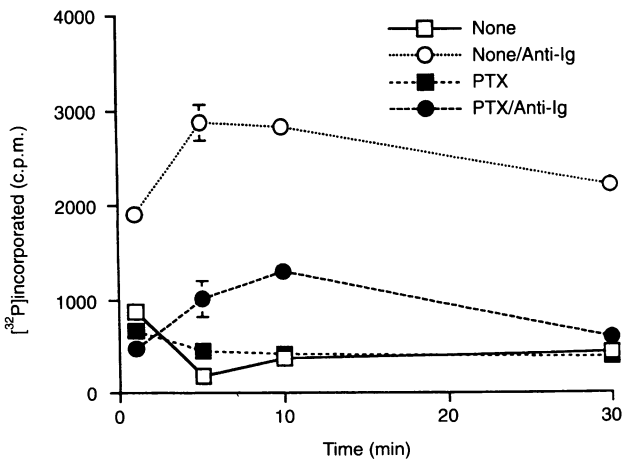

Figure 4. PTX inhibits sIg-coupled activation of Erk2. B cells were preincubated with either PTX (1 μg/ml) or media, alone for 4 hr. Samples (5×10^6 cells) were then incubated with 50 μg/ml anti-immunoglobulin or media alone for 1, 5, 10, or 30 min at 37° before assaying for Erk2 activity as described in the Materials and Methods. The results are expressed as means ± SD of a single experiment performed in triplicate representative of at least three independent experiments.

recruit MAPkinase via PTKs known to be associated with the BCR such as Syk and Lyn,[22] we investigated the effect of PTX on a number of BCR-associated PTKs in order to identify which, if any, are involved in transducing the G-protein-coupled MAPkinase signals resulting from ligation of the antigen receptors.

Firstly, we confirmed that PTX, or trapped GDPβS, blocked sIg-stimulated tyrosine phosphorylation in permeabilized/resealed B cells (Fig. 5a) and thus provided evidence that GDPβS was not simply mediating its inhibition of sIg-stimulated DNA synthesis by targetting BCR-coupling to Ras and/or other low molecular weight GTPases. Stimulation of control permeabilized/resealed B cells (Fig. 5, lanes 1 and 2) with anti-immunoglobulin for 15 min induced a strong tyrosine phosphorylation of a number of target proteins. In contrast, basal and sIg-mediated tyrosine phosphorylation was almost completely abrogated in permeabilized/resealed B cells pre-treated with PTX (lanes 3 and 4) or containing trapped GDPβS (lanes 5 and 6). The observed modulation of basal tyrosine phosphorylation probably simply reflected inhibition of serum-stimulated tyrosine phosphorylation by PTX or the trapped GDPβS during the 4-hr princubation period prior to anti-immunoglobulin challenge. The sIg-associated PTKs involved in this site of G-protein regulation, were targetted by identifying those whose subsequent activation via sIg was desensitized following pretreatment with PTX for 4 hr (Fig. 5b–e). *In vitro* kinase assays of anti-PTK immunoprecipitates showed that whilst the anti-immunoglobulin-induced stimulation of Lyn, Syk, Blk and Fyn exhibited differential activation kinetics, in each case activation was sustained for at least up to 30 min. Moreover, whilst pre-exposure to PTX had little or no effect on the basal activities on the PTK tested, it not only totally abrogated the subsequent coupling of sIg to Lyn (Fig. 5b), but also appeared to substantially inhibit sIg-stimulated Syk (Fig. 5c) activation. In contrast, the effects of PTX on sIg-mediated Blk (Fig. 5d) and Fyn (Fig. 5e) activation were rather more complex: for example, whilst the early (< 10 min) stages of Fyn or Blk activation were essentially unaffected, the later (30 min) phases of activation of Blk (Fig. 5d) and, to a lesser extent, Fyn (Fig. 5e) appeared to be somewhat desensitized (Fig. 5).

DISCUSSION

We, and others, have previously shown that the coupling of the antigen receptors on B cells to $PtdInsP_2$ is regulated by a PTX-sensitive G-protein(s) which acts at a site upstream of PTK-mediated recruitment of PLC-γ.[2–4,7,8] We now report that one or more G-proteins regulate a second early site of BCR-signalling which is distinct from that governing $PtdInsP_2$ hydrolysis and that this site of G-protein regulation plays a key role in the regulation of sIg-mediated DNA synthesis (Figs 1,2,3). Indeed, our data suggest that $PtdInsP_2$ hydrolysis is not essential for B-cell proliferation, a finding consistent with the growing body of evidence that PLC-mediated hydrolysis of $PtdInsP_2$ may simply play a role in the transduction of early activation signals such as Na^+/H^+ exchange and induction of c-*fos*, c-*myc* and c-*jun*, but is not sufficient, or even necessary, to stimulate or maintain cell proliferation.[26] We have, moreover, identified a candidate site for the second site of G-protein regulation by showing that MAPkinase activity,

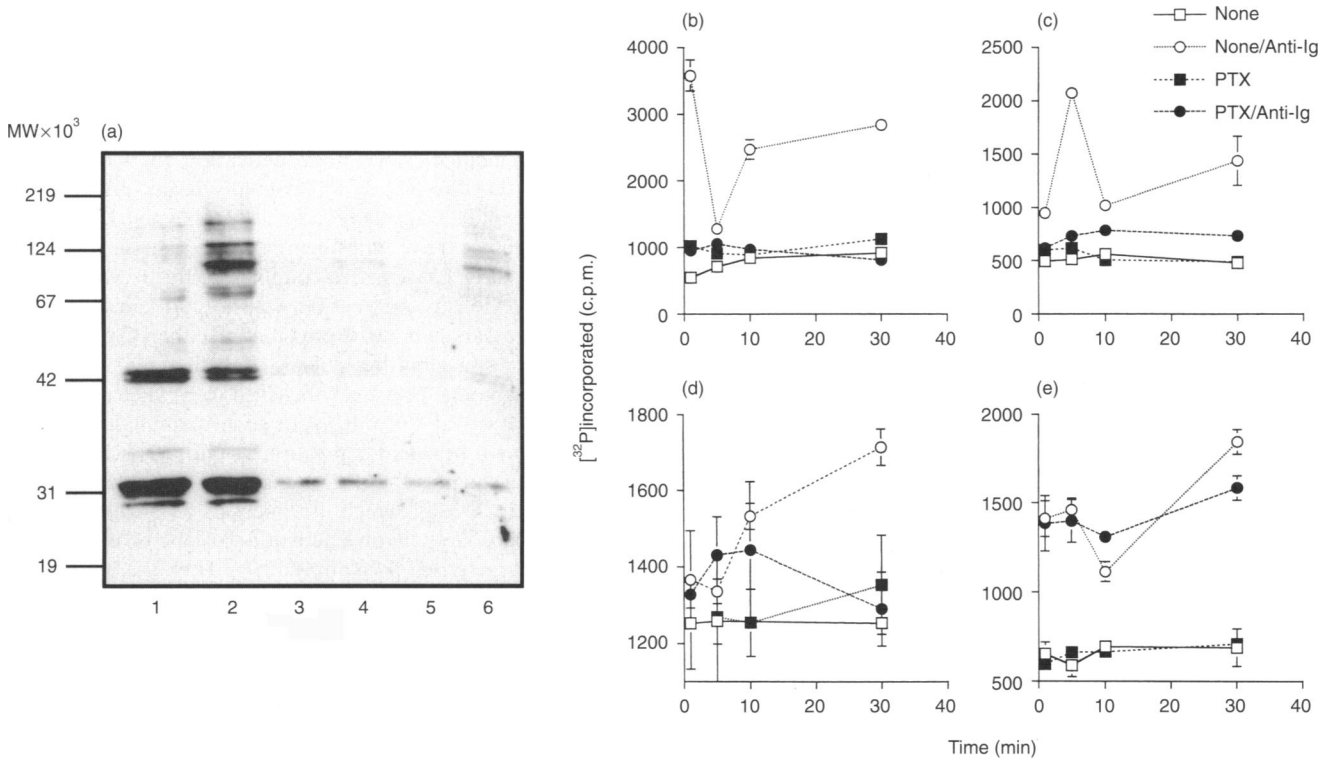

Figure 5. PTX desensitizes sIg-mediated tyrosine phosphorylation and activation of PTKs in B cells. In (a) B cells were permeabilized/resealed in the presence of buffer (lanes 1–4) or GDPβS (lanes 5 and 6) and then incubated with either PTX (1 μg/ml; lanes 3 and 4) or media (lanes 1, 2, 5, and 6) for 4 hr. Following the PTX treatment the cells (5×10^6/sample) were incubated with anti-immunoglobulin (50 μg/ml; lanes 2, 4 and 6) or media (lanes 1, 3 and 5) for 30 min. Following stimulation, cell lysates were prepared and the samples subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis/Western Blot analysis using the anti-phosphotyrosine mAb, 4G-10 as described in the Materials and Methods to allow assessment of GDPβS or PTX-mediated desensitization of sIg-mediated tyrosine phosphorylation. In (b)–(e), B cells were incubated with either PTX (1 μg/ml) or media for 4 hr. Following the PTX treatment the cells (5×10^6) were incubated with anti-immunoglobulin (50 μg/ml) or media for 30 min and assessed for PTX-mediated desensitization of sIg-mediated activation of Lyn (b), Syk (c), Blk (d), or Fyn (e) immunoprecipitates in *in vitro* kinase assays. All the results are expressed as means ± SD of a single experiment performed in triplicate representative of at least two (Blk/Fyn) or three (Lyn/Syk) independent experiments.

which plays a key role in the transduction of sIg-mediated proliferative signals in B cells, is abrogated by pre-exposure to PTX (Fig. 4). Furthermore, our data suggest that this PTX-sensitive G-protein couples the BCR to MAPkinase activation, at least in part, by regulating sIg-coupling to Lyn, Syk and perhaps Blk and Fyn activity (Fig. 5), results consistent with studies in other systems which show that classical G-protein-coupled receptors recruit such PTKs to tranduce MAPkinase activation.[22–25] Interestingly, however, this G-protein plays no apparent role in the control of up-regulation of MHC Class II expression (Tables 1 and 2), suggesting that such G-protein-regulated PTK and MAPkinase activation is not required for the induction of this biological response resulting from ligation of the BCR.

In addition, our data also support a role for G-protein regulation of later signalling events (>24 hr poststimulation) leading to sIg-mediated or PMA plus ionomycin-stimulated DNA synthesis (Fig. 3d and results not shown). To our surprise, stimulation with PMA/ionomycin only induced a very low amount of Erk activation over 30 min relative to that observed with anti-immunoglobulin and pretreatment with PTX only slightly modulated this activation (results not shown). These results, are not necessarily inconsistent with our data showing G-protein regulation of sIg-coupling to MAPkinase activation; however, as it is well established that PMA (PKC) can stimulate MAPkinase via Raf or MEK in a manner that would short-circuit the sIg-coupled PTK-dependent RasMAPkinase cascade targetted by PTX. Indeed, some 30–40% of the sIg-mediated MAPkinase activity at 5 and 10 min poststimulation is insensitive to PTX inhibition (Fig. 4) suggesting that there is a residual pool of sIg-coupled MAPkinase activity that is regulated in a G-protein-independent manner, presumably by PKC. Moreover, these results probably provide an explanation for the fact that whilst GDPβS can completely abrogate sIg-mediated DNA synthesis, it cannot induce more than a 40–50% decrease in the PMA/ionomycin response: indeed, at concentrations of GDPβS (50 μM) which inhibit the the anti-immunoglobulin response by >70%, the PMA/ionomycin response is only barely inhibited (some 15%; Fig. 3c). These results therefore either suggest that PMA/ionomycin predominantly signals via the G-protein-independent form of MAPkinase or alternatively, given the low levels of PMA/ionomycin-mediated Erk activation, indicate that PMA/ionomycin may mediate DNA synthesis via some other mitogenic pathway: thus, the inhibition resulting from GDPβS probably reflects targetting of

some convergent downstream signal shared by independent sIg- and PMA/ionomycin-signalling cascades and which may be the late site of G-protein regulation identified by the delayed (24 hr) addition of GDPβs which resulted in a more comparable concentration-dependent inhibition of both sIg-stimulated (max. 60–70%) and PMA/Ionomycin-stimulated (max. 40–50%) DNA synthesis (Fig. 3d and results not shown). This latter suggestion is also consistent with previously published evidence indicating that the later stages of B-cell activation, namely the induction of early response genes and DNA synthesis, involve PTX-sensitive signal transduction events.[7]

The finding that many proliferative signals such as PKC, Ras and PI-3-K appear to converge at the level of the MAPKinase signalling cascade[27–29] led to the proposal that MAPkinase plays a key role in the regulation of cell growth. In B cells, Ras is reported to couple the BCR to the MAPkinase cascade via activation of the intermediate kinases, Raf and MEK (MAPkinase kinase)[30,31]. Since such phosphorylation and activation of MAPkinase has been postulated to be an important event in B-cell proliferation downstream of Ras activation, as demonstrated by the use of dominant-negative mutants of Ras and Raf-1,[32] any interference with MAPkinase activation could explain the inhibitory effects of GDPβS (Figs 1,2,3) or PTX[7,14] on sIg-mediated proliferation. In B cells, sIg-mediated Ras activation has been reported to be mediated by the regulatory elements, GAP and Shc/Grb2/Sos. However, the relative contribution of these regulatory elements to sIg-coupled Ras activation during B-cell proliferation is not clear.[1] For example, the Shc/Grb2/Sos complex, shown to be involved in coupling the classical intrinsic receptor tyrosine kinases to Ras activation, has been implicated in BCR recruitment of Ras via a SH2–phosphotyrosine interaction initiated by Lyn- and Syk-mediated phosphorylation of Shc.[1,33] Indeed, it has been proposed that an activated Lyn–Syk complex may phosphorylate Shc bound to the phosphorylated ITAM of Ig-α/β, allowing assembly of a Shc–Grb2–Sos–Ras complex in close proximity to the activated BCR. Moreover, BCR-coupled tyrosine phosphorylation and inhibition of the regulatory element, GAP (which functions as a negative regulator of Ras by promoting the conversion of Ras to the inactive GDP-bound state) correlates with sIg-mediated Ras activation[34] and in resting B cells, GAP has been shown to be associated with the N-terminal unique region of Lyn.[35] Thus, the disruption of the SH2-dependent recruitment of the RasMAPkinase cascade resulting from PTX-mediated inhibition of Lyn and Syk activity (Fig. 5), together with the lack of Lyn-mediated tyrosine phosphorylation and desensitization of GAP, may provide a two-pronged rationale for the PTX-uncoupling of sIg from MAPkinase activation and B-cell activation.

Moreover, this model would be consistent with previously published studies dissecting the manner by which classical seven transmembrane spanning G-protein-coupled receptors recruit and activate the RasMAPkinase cascade.[22–25] Although the precise details of the coupling mechanisms for any receptor in a particular cell type depends on the repertoire of G-proteins and PTK available, there appears to be a common pathway involving PTK-mediated recruitment of the RasMAPkinase cascade to a Grb2/Sos scaffold.[24,25] For example, whilst M1 muscarinic receptors, which couple to MAPkinase via pertussis toxin-sensitive G_i-like proteins, have recently been shown to require recruitment of Syk and Btk, the pertussis toxin-insensitive Gq system employed by M2 muscarinic receptors couples to MAPkinase in a Lyn-and Syk-dependent manner in avian B cells.[22] Interestingly, we find evidence for G-protein regulation of sIg-coupling to Lyn and Syk, underscoring previous studies suggesting a pivotal role for Syk in directing G-protein-regulated MAPkinase activation in B cells[22] and confirming that the precise PTKs involved are likely to vary with the receptor, G-protein and cell type involved.[22–25]

Although we have not identified the precise G-protein involved in the PTX-sensitive coupling of sIg to MAPkinase, we have previously shown that whilst $G\alpha_{i2}$ can be readily detected, we can find no expression of either $G\alpha_{i1}$ or $G\alpha_{i3}$ in mature resting murine splenic B cells.[36] Likewise, $\beta_{1/2}$ G-protein subunits, previously believed to be virtually ubiquitously expressed at the mRNA level in mammalian tissues, show little or no protein expression in murine B cells.[36] This latter finding suggests that PTX-sensitive, G_{i2}-regulated signalling events in B cells, which are likely to be mediated by βγ subunits,[25] probably involve activation of one or more of the less well characterised βγ complexes.[9,37] How the βγ subunits would recruit Syk and Lyn and hence, MAPkinase is less clear. However, it has been reported that Btk,[38] which has been implicated in the G_i-mediated activation of MAPkinase in avian B lymphoma cells,[22] can bind G-protein-βγ subunits via its' PH domain.[39,40] Thus, as Btk can associate with PTKs such as Lyn or Syk in a SH2 domain-dependent manner,[22] it is possible that Btk may act to couple the BCR-stimulated G-protein to Syk and/or Lyn and hence MAPkinase activation. However, it has been reported that BCR-coupling to MAPkinase activation is normal in Btk-deficient avian B lymphoma cells[41] suggesting either that Btk is not involved in the coupling of the BCR to MAPkinase or that another Btk-family PTK such as Tec could substitute for Btk in these cells.[42] Alternatively, the G-protein may interact with Pyk2, a FAK-like PTK which has previously been shown to play a pivotal role in G-protein-regulated MAPKinase activation[25] and indeed, has been proposed to co-operate with Src PTK to link G-protein-coupled receptors to MAPkinase.[43]

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