The membrane immunoglobulin receptor utilizes a Shc/Grb2/hSOS complex for activation of the mitogen-activated protein kinase cascade in a B-cell line

Gita KUMAR, Shirley WANG, Shalini GUPTA and André NEL*

Division of Clinical Immunology and Allergy, Department of Medicine, UCLA School of Medicine, 10833 LeConte Avenue, Los Angeles, CA 90024, U.S.A.

Ligation of membrane immunoglobulin M (mIgM) receptor in the Ramos B-cell line induced tyrosine phosphorylation of several intracellular substrates, including the adaptor protein Shc. Phosphorylated Shc could be seen to associate with Grb2 in a complex which included hSOS. Inasmuch as hSOS is involved in p21^{ras} activation, we also demonstrated that mIgM ligation activated a Ras-dependent kinase cascade in which sequential activation of Raf-1 and MEK-1 culminates in the activation of p42 mitogen-activated protein (MAP) kinase (ERK-2). The

tumour promoter and protein kinase C agonist, phorbol 12-myristate 13-acetate (PMA), also activated Raf-1, MEK-1, and MAP kinase in Ramos cells, but did not induce tyrosine phosphorylation of Shc or Shc/Grb2 association. Okadaic acid, another tumour promoter and serine/threonine phosphatase inhibitor, activated p42 MAP kinase without activating Raf-1 or MEK-1, suggesting the existence of a serine/threonine phosphatase which directly regulates MAP kinase activity.

INTRODUCTION

The B-cell antigen receptor (BCR), membrane immunoglobulin (mIg), plays an important role in regulation of B-cell responses [1]. While ligand binding is mediated via the immunoglobulin (mIg) domains of the BCR, signal transduction proceeds via mIg-associated complexes which are comprised of Ig α /Ig β and Ig α /Ig γ dimers [2]. Ligation of mIg triggers activation of several protein tyrosine kinases (PTKs) which results in the phosphorylation of diverse substrates, including phospholipase C- γ 2 (PLC γ 2) [3]. Activated PLC γ 2 generates the second messengers, diacylglycerol and inositol 1,4,5-trisphosphate, which, in turn, results in intracellular Ca²⁺ concentration ([Ca²⁺]_i) flux and activation of protein kinase C (PKC) [1,4]. PKC and PTKs represent two principal signalling pathways which are used by the BCR to initiate B-cell growth and differentiation [1]. While there is still uncertainty about the role of the various PTKs, including p72^{syk}, p59^{lyn}, p53/56^{lyn}, and p55^{blk}, in B-cell responses, the identities of several phosphoprotein substrates are already known [5–8]. We described induced tyrosine phosphorylation of a 42 kDa protein by the BCR which was characterized as p42 MAP kinase, also known as Extracellular Regulated Kinase-2 (ERK-2) [9]. ERK-2 is a member of the mitogen-activated protein (MAP) kinase family, and is thought to play an important role in cellular growth regulation [10,11].

Though several receptor tyrosine kinases (RTKs) activate MAP kinase without a contribution from PKC [12–15], PKC is an integral part of BCR signalling and probably acts in concert with BCR-associated PTKs to activate MAP kinase [9]. PMA, a direct PKC agonist, is a potent activator of MAP kinase in B-lymphocytes [9,16,17], which suggests that PKC contributes independently to MAP kinase activation. The MAP kinase cascade

is quite complex and has been shown to integrate signals from RTKs, receptor-associated PTKs, PKC and receptor-associated GTP-binding proteins [10,12,13,18–22]. The best-characterized pathway is the activation of p21^{ras} by RTKs, which ultimately leads to MAP kinase activation. Linkage between RTKs and Ras is provided by adaptor proteins. The critical adaptor protein is Grb2, which uses an SH2 domain to bind tyrosine phosphate groups either on the RTK or on Shc proteins associated with the receptor [23,24]. The SH3 domains of Grb2 shuttle a guanine nucleotide exchange factor (GNEF), human son-of-sevenless (hSOS), to the surface membrane where it activates Ras by exchanging GDP for GTP [23,25]. Although BCR ligation has been shown to lead to increased GTP uptake by p21^{ras} [26,27], it is unclear whether Grb2, Shc, or hSOS is involved in this pathway. Another GNEF, p95^{nav}, which is activated by a BCR-associated PTK, may also be involved in Ras activation [7,28]. Activated Ras leads to MAP kinase activation by way of an intermediary MAP kinase kinase kinase (MAPKKK) and a MAP kinase kinase (MAPKK) [14]. While mammalian cells contain several candidates at each of these steps, Raf-1 is the best-characterized MAPKKK [29–31], while MEK-1 is the best-characterized MAPKK [14]. MEK-1, a member of the MAP kinase or ERK kinase (MEK) family, acts as a dual-specificity kinase which activates MAP kinase by phosphorylating both Thr¹⁸³ and Tyr¹⁸⁵ residues [32,33]. Not all cell types conform to the above-mentioned cascade, and in certain cell types MAP kinase activation occurs independently of Raf-1 [14]. To this end, other MAPKKKs have been characterized, including the yeast Ste11 homologue, MEKK, and c-mos [13,34].

In addition to the contribution of afferent kinases, MAP kinase activity is regulated by phosphatases, including the tyrosine phosphatase PAC-1, which inactivates MAP kinase by

Abbreviations used: BCR, B-cell antigen receptor; [Ca²⁺]_i, intracellular Ca²⁺ concentration; DTT, dithiothreitol; GNEF, guanine nucleotide exchange factor; hSOS, human son of sevenless; GAP, GTPase-activating protein; GST, glutathione S-transferase; mAb, monoclonal antibody; MAP, mitogen-activated protein; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase; MBP, maltose-binding protein; mIgM, membrane immunoglobulin M; NP-40, Nonidet P-40; PKC, protein kinase C; PLC γ 2, phospholipase C- γ 2; PMA, phorbol 12-myristate 13-acetate; PTK, protein tyrosine kinase; RTK, receptor tyrosine kinase; TIU, trypsin inhibitory units.

* To whom correspondence should be addressed.

removing the Tyr¹⁸⁵ in the nucleus [35]. We have previously shown that a serine/threonine phosphatase inhibitor, okadaic acid, activates MAP kinase in B lymphocytes [16] and hypothesized that regulation occurs at the level of MAP kinase or an afferent kinase in the MAP kinase cascade.

In order to elucidate the potential role of adaptor proteins, MAPKKK, MAPKK, and the involvement of serine/threonine phosphatases in the MAP kinase cascade associated with the BCR, we used the human B-cell line, Ramos, which responds to anti-IgM treatment with increased tyrosine protein phosphorylation, PKC activation, and $[Ca^{2+}]_i$ flux [9,17]. Our results show that ligation of mIgM was associated with tyrosine phosphorylation of p52^{shc} and subsequent binding of Shc to Grb2. Direct evidence that adaptor proteins are involved in the activation of Ras by the BCR was provided by co-precipitation of hSOS with p52^{shc} following BCR stimulation. By contrast, direct PKC activation with PMA did not induce tyrosine phosphorylation of Shc or the association of Shc with Grb2. Raf-1 and MEK-1 acted as MAPKKKs and MAPKKs respectively, in the cascade induced by anti-IgM and PMA. Okadaic acid, while a potent MAP kinase activator, did not stimulate MEK-1 or Raf-1 activity in Ramos cells.

MATERIALS AND METHODS

Antibodies and reagents

Biotinylated goat anti-(human IgM) used for cellular stimulation was obtained from Southern Biotechnology Associates (Birmingham, AL, U.S.A.). Monoclonal antibodies (mAbs) included anti-ERK-2 from Biodesign (Kennebunkport, ME, U.S.A.), anti-Grb2, anti-Shc and anti-hSOS from Transduction Laboratories (Lexington, KY, U.S.A.), anti-MEK-1, clone 2E9, from Dr. Ray Erikson (Department of Cell and Developmental Biology, Harvard University, Boston, MA, U.S.A.), and anti-phosphotyrosine, clone 4G10, from UBI (Lake Placid, NY, U.S.A.). Polyclonal antibodies were anti-Shc from UBI, anti-Vav and anti-Raf-1 from Santa Cruz Laboratories (Santa Cruz, CA, U.S.A.), and anti-MEKK from Dr. Gary Johnson (Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, U.S.A.). Avidin, Protein A-Sepharose, PMA and glutathione-agarose were from Sigma (St. Louis, MO, U.S.A.). $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from ICN and ¹²⁵I-labelled Protein A and ECL reagents were obtained from Amersham (Arlington Heights, IL, U.S.A.). Myelin basic protein (MBP) peptide (APRTTDGRR), corresponding to amino acids 90–98 of MBP was purchased from UBI. Okadaic acid was obtained from LC Laboratories (Woburn, MA, U.S.A.) and tissue culture reagents were from Irvine Scientific (Santa Ana, CA, U.S.A.).

Recombinant fusion proteins

Bacteria expressing 6-histidine-tagged proteins were: kinase-inactive ERK-1 (K71, K72–RR), a gift from Dr. Thomas Geppert (Department of Medicine, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) and kinase-inactive MEK-1 from Dr. Gary Johnson. These fusion proteins were purified over nickel agarose resin from Qiagen (Chatsworth, CA, U.S.A.). Bacteria expressing the glutathione S-transferase (GST)–Grb2myc fusion protein were kindly provided by Dr. Robert Weinberg (Department of Biology, Whitehead Institute for Biomedical Research, MIT, Cambridge, MA, U.S.A.) and was purified using glutathione-agarose resin from Sigma.

Cell culture and stimulation, and preparation of lysates

The human Burkitt's lymphoma cell line (mIgM⁺), Ramos, was a gift from Dr. J. A. Ledbetter (Bristol Meyers Squibb, Seattle, WA, U.S.A.) and was maintained in RPMI 1640 with L-glutamine and supplemented with 10% (v/v) fetal calf serum and 1% penicillin/streptomycin. For stimulation, 5×10^6 cells were treated with 2 $\mu\text{g}/\text{ml}$ of biotinylated goat anti-(human Ig) for 2 min and then crosslinked with 50 $\mu\text{g}/\text{ml}$ avidin for the indicated time periods. Alternatively, cells were treated with 100 nM PMA for 5 min or 1 μM okadaic acid for 30 and 60 min at 37 °C. Cells were pelleted and lysed by sonication in 200 μl of the appropriate lysis buffer (see below). Lysates were centrifuged at 14000 *g* for 5 min at 4 °C to remove particulates and the resulting supernatants were used as described below.

MAP kinase assay

Cells (5×10^6) were lysed in Buffer A (20 mM Tris, pH 7.4, 1 mM EGTA, 10 mM PNPP, 100 μM sodium vanadate, 10 mM β -glycerophosphate, 10 mM NaF, 10 $\mu\text{g}/\text{ml}$ leupeptin and 0.004 trypsin inhibitory units (TIU)/ml aprotinin). The lysates were clarified by centrifugation. p42^{MAPK} was purified from the supernatant and reconstituted with MBP peptide for conducting *in vitro* phosphorylation assays as previously described [19].

MEK-1 immunocomplex kinase assay

Duplicate aliquots of 5×10^6 cells were lysed in Buffer B [20 mM Tris, pH 8.0, 10 mM EGTA, 5 mM MgCl₂, 20 mM β -glycerophosphate, 1 mM sodium vanadate, 1 mM dithiothreitol (DTT), 10 $\mu\text{g}/\text{ml}$ leupeptin and 0.004 TIU/ml aprotinin]. Immune complex kinase assays were conducted by incubating 80–100 μg of lysate protein with 5 μg of anti-MEK-1(2E9) mAb for 1 h at 4 °C. To these we added Protein A-Sepharose beads, previously coupled to 3 μg of rabbit anti-(mouse IgG) antibodies (Jackson ImmunoResearch, Westgrove, PA, U.S.A.). The immune precipitates were washed twice with Buffer B and once with Buffer C (50 mM Tris, pH 8.0, 1 mM DTT, 10 mM MgCl₂, 1 mM sodium vanadate, 1 mM NaF, 1 $\mu\text{g}/\text{ml}$ leupeptin and 0.004 TIU/ml aprotinin). Kinase reactions were conducted in a total volume of 50 μl containing Buffer C, 3 μg of kinase-inactive ERK-1, 50 μM ATP and 10 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 20 min at 30 °C. Reactions were stopped by boiling with 25 μl of 3 × SDS sample buffer, and proteins were resolved by SDS/10%-PAGE. Substrate phosphorylation was visualized by autoradiography [36].

Raf-1 immunocomplex kinase assay

Aliquots (1×10^7 cells) were lysed in Buffer D [10 mM sodium phosphate, pH 7.0, 150 mM NaCl, 2 mM EGTA, 50 mM NaF, 200 μM sodium vanadate, 1% deoxycholate, 1% Nonidet P-40 (NP-40), 0.1% SDS, 0.1% 2-mercaptoethanol, 0.008 TIU/ml aprotinin and 1 mM PMSF]. After centrifugation at 14000 *g* for 5 min, soluble supernatants were incubated with 1 μg of anti-Raf-1 sera for 1 h at 4 °C and subsequently with Protein A-Sepharose beads for 1 h at 4 °C. The immune complexes were layered on Buffer D containing 10% sucrose and centrifuged at 600 *g* for 10 min in an Eppendorf centrifuge. Beads were washed twice with Buffer D, twice with wash buffer (10 mM Pipes, pH 7.0, 0.1 M NaCl, 0.5% NP-40), twice with wash buffer without NP-40, and once with Buffer E (10 mM Pipes, pH 7.0, 0.008 TIU/ml aprotinin). Complexes were resuspended in 25 μl

of Buffer E. Kinase assays were performed in Buffer E with 2 μ g of kinase-inactive MEK-1, 10 mM MgCl_2 and 20 μCi of [γ - ^{32}P]ATP in a total volume of 50 μl . Reactions were terminated after 40 min at 30 °C by boiling with 25 μl of 3 $\times$ SDS sample buffer. Proteins were resolved by SDS/10%-PAGE and visualized by autoradiography [36].

Anti-Shc Immunoprecipitation

Cells (1×10^7) were lysed in 200 μl of Buffer F (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EGTA, 1.5 mM MgCl_2 , 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 10 $\mu\text{g}/\text{ml}$ leupeptin and 0.004 TIU/ml aprotinin). After clearing at 14000 g , lysates were incubated with 5 μg of polyclonal anti-Shc antibody for 1 h at 4 °C and subsequently bound to Protein A-Sepharose. Immunoprecipitates were washed twice with Buffer F and then boiled in 75 μl of SDS sample buffer. Proteins were resolved by SDS/12%-PAGE and subsequently transferred to Immobilon-P [36].

GST-Grb2 affinity association

Aliquots (1×10^7 cells) were lysed in Buffer G (20 mM Tris, pH 7.6, 1% Triton X-100, 50 mM NaCl, 100 μM sodium vanadate, 10 mM sodium pyrophosphate, 5 mM EGTA, 0.1% BSA, 10 $\mu\text{g}/\text{ml}$ leupeptin and 0.004 TIU/ml aprotinin), and the clarified supernatants were incubated with 10 μg of GST-Grb2myc or recombinant GST bound to glutathione-agarose (30 μl swollen bead volume) for 1 h at 4 °C. The beads were extensively washed with Buffer G without BSA, and the beads were boiled with 75 μl of 1 $\times$ SDS sample buffer. Proteins were resolved by SDS/9%-PAGE and transferred to Immobilon-P membrane.

Anti-Vav Immunoprecipitation

Control and stimulated cells (1×10^7) were lysed in Buffer H (10 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.009 TIU/ml aprotinin, 0.25 mM PMSF, 1 mM NaF, 1 mM sodium vanadate) according to the method of Bustelo and Barbacid [37]. The resulting lysates were cleared by centrifugation and the supernatants were incubated for 1 h at 4 °C with 1 μg of polyclonal anti-Vav sera. Immunocomplexes were allowed to bind to Protein A-Sepharose for 1 h at 4 °C and were washed twice with 1 ml of Buffer H and resuspended in 75 μl of 1 $\times$ SDS sample buffer. The samples were boiled for 5 min, resolved by SDS/9%-PAGE and transferred to Immobilon-P [36].

Anti-Grb2 and anti-hSOS Immunoprecipitation

Control and stimulated cells (2×10^7) were lysed in 300 μl of Buffer I (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 10 mM NaF, 2 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin and 20 $\mu\text{g}/\text{ml}$ aprotinin). Lysates were cleared by centrifugation and the supernatants were incubated with 10 μl of the appropriate antiserum for 1 h at 4 °C, and subsequently with Protein A-Sepharose for 1 h. Immune complexes were washed three times with 1 ml of Buffer J (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% NP-40, 1 mM sodium orthovanadate, 5 mM NaF, 2 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin and 20 $\mu\text{g}/\text{ml}$ aprotinin) and then boiled with SDS sample buffer. Proteins were resolved on SDS/10%-PAGE and transferred to Immobilon-P.

Western blotting

Proteins were resolved by SDS/PAGE and transferred to

Immobilon-P in a Bio-Rad transblot cell for 16 h at 0.35 mA. The membrane was blocked for 2 h at room temperature with 6% (w/v) BSA in Tris-buffered saline and incubated with the desired antibody for 1 h at room temperature. Detection was performed either by using the enhanced chemiluminescence (ECL) method or by overlaying the blot with ^{125}I -Protein A at 1 $\mu\text{Ci}/\text{ml}$ in blocking solution for 1 h followed by four 10 min washes in Tris-buffered saline containing 0.1% Tween and 0.1% BSA. The blots were visualized by autoradiography [36].

RESULTS

Anti-IgM ligation induces tyrosine kinase protein phosphorylation, including phosphorylation of p42 MAP kinase in Ramos cells

Antibody-mediated ligation of the mIgM receptor in the human B-lymphocyte cell line, Ramos, induced tyrosine phosphorylation of several proteins, including p160, p120, p95, p75, p72 and p42 (Figure 1a, lanes 3–5). We have previously identified p42 as p42 MAP kinase/ERK-2 and showed that this kinase is activated by the mIgM receptor [9,16]. MAP kinase activation requires tyrosine and threonine phosphorylation [33] and is accompanied by electrophoretic mobility shift, as is shown by anti-ERK-2 overlay of the blot shown in Figure 1(b) (lanes 3–5). PMA, which is a direct PKC agonist, also induced tyrosine phosphorylation of p42 along with electrophoretic mobility shift of ERK-2 (Figure 1a, lane 2; Figure 1b, lane 2). Inasmuch as the BCR utilizes PTK and PKC signalling pathways, either one or both pathways may be involved in the MAP kinase cascade in Ramos cells.

Evidence for the involvement of p52^{shc}, Grb2, and hSOS in signalling via the mIgM receptor

MAP kinase activation by RTKs is dependent on the activation of p21^{ras} [21,38] via the interposition of adaptor proteins and a GNEF, hSOS [23,24]. Since BCR has been shown to activate p21^{ras} [26,27], we looked at the possible involvement of the adaptor proteins, Shc and Grb2, in Ramos cells. We immunoprecipitated Shc from Ramos cells which were either untreated or treated with anti-IgM antibodies. Anti-Shc Western blotting of the immunoprecipitates clearly showed precipitation of the 52 kDa Shc species in Ramos cells (Figure 2a, lanes 2–5). Anti-phosphotyrosine overlay of the same blot demonstrated tyrosine phosphorylation of the 52 kDa protein as well as a co-precipitating 46 kDa protein in anti-IgM-treated cells (Figure 2a, lanes 4 and 5). p46 is probably the p46^{shc} and probably co-migrates with the immunoglobulin H-chain in the Shc blot. Tyrosine phosphorylation of Shc provides a binding site for the SH2 domain of Grb2. We therefore examined Shc immunoprecipitates for the presence of co-precipitating Grb2. The result of the Grb2 overlay is shown in Figure 2(b) and demonstrates co-precipitation of Grb2 with Shc in anti-IgM-treated (lane 3), but not untreated, Ramos cells (lane 2). Further confirmation of this finding was sought by performing the immunoprecipitation in reverse, that is precipitating Grb2 and demonstrating the presence of Shc by anti-Shc immunoblotting (Figure 2c). This confirmed the association of Shc with Grb2 in anti-IgM-treated cells (lanes 4 and 5) but not control cells (lane 2). Similar findings were made when a Grb2 fusion protein was used as an affinity matrix and incubated with extracts from Ramos cells; the 52 kDa Shc species from anti-IgM-treated cells, but not control cells, associated with GST-Grb2 (results not shown). In addition to binding to Shc, Grb2 is likely to play additional roles in BCR signalling because anti-phosphotyrosine overlay of anti-Grb2 immune precipitates showed co-precipitation of several tyrosine

Figure 1 BCR ligation induces tyrosine phosphorylation and p42 MAP kinase activation in Ramos cells

(a) Anti-phosphotyrosine immunoblot. 5×10^6 Ramos cells were unstimulated (lane 1), treated with 100 nM PMA for 5 min (lane 2) or treated with 2 μ g/ml biotinylated anti-IgM plus 50 μ g/ml avidin for 1 min (lane 3), 5 min (lane 4) or 15 min (lane 5). Whole-cell lysates were centrifuged to remove nuclei and the supernatants were boiled with SDS sample buffer and separated by SDS/10%-PAGE. Proteins were transferred to Immobilon-P membranes and probed with 1 μ g/ml anti-phosphotyrosine mAb, 4G10. (b) Anti-ERK2 overlay. The blot in (a) was stripped and reprobed with a mAb to ERK-2. The blot was developed by enhanced chemiluminescence.

phosphoproteins (p140, p100, p85 and p72) in anti-IgM-treated cells compared with untreated cells (Figure 2d, lanes 3 and 4).

In contrast to the results with anti-IgM-treated cells, PMA failed to induce tyrosine phosphorylation of Shc (Figure 2a, lane 3), co-precipitation of Grb2 with Shc (Figure 2b, lane 4) or co-precipitation of Shc with Grb2 (Figure 2c, lane 3). PMA also failed to induce the association of any tyrosine phosphoproteins with Grb2 (Figure 2d, lane 5). This result indicates that PKC engages the MAP kinase cascade without stimulating tyrosine phosphorylation of Shc and is compatible with the notion that PKC functions downstream of the BCR-associated PTKs.

In order to study the involvement of hSOS more directly, we immune-precipitated Grb2 from Ramos cells and probed for hSOS on a Western blot (Figure 3). While hSOS co-precipitated with Grb2 in untreated (Figure 3, lane 2) and anti-IgM-stimulated cells (lanes 4 and 5), there was a small but definitive increase in the quantity of hSOS associated with Grb2 in the stimulated cells. The difference between stimulated and unstimulated cells was accentuated, however, when we immune-precipitated hSOS and determined co-precipitation of Shc by Western blotting

(Figure 4). Compared with control cells (lane 2), anti-IgM induced an activation complex in which the 52 kDa Shc species could be clearly shown to associate with hSOS (Figure 4, lanes 3 and 4). Moreover, anti-phosphotyrosine overlay of the same blot showed that these Shc proteins were phosphorylated on tyrosine residues (Figure 4, lanes 7 and 8). The anti-phosphotyrosine blot also shows co-precipitation of a group of tyrosine phosphoproteins, around 70–75 kDa, as well as a 46 kDa protein which may represent p46^{shc} (Figure 4, lanes 7 and 8).

While these results strongly suggest the involvement of hSOS in BCR signalling, it was necessary to consider the role of another putative GNEF, p95^{vav}, which leads to Ras activation in lymphocytes [28]. Vav has also been shown to be phosphorylated on tyrosine residues and activated during BCR ligation [27,37]. We immune-precipitated Vav from Ramos cells and analysed the protein on anti-phosphotyrosine immunoblots. The results show that, while equal amounts of Vav protein were precipitated from control and stimulated cells, Vav was constitutively phosphorylated on tyrosine residues and did not undergo increased phosphorylation during anti-IgM or PMA treatment (Figure 5, lanes 6–8). In contrast, anti-CD3 treatment of the Jurkat T-cell line did show increased tyrosine phosphorylation of p95^{vav} compared with control (Figure 5, lanes 9 and 10). Interestingly, the T-cell antigen receptor in Jurkat cells does not induce tyrosine phosphorylation of Shc but does lead to MAP kinase activation [36]. These results suggest that hSOS, rather than p95^{vav}, is involved in the PTK pathway associated with the BCR in Ramos cells.

MAP kinase activation by BCR and PMA involves an MAPKKK and an MAPKK

Activation of MAP kinase proceeds via a divergent cascade of MAPKKKKs which converge at the level of MEK-1, an MAPKK [13]. Along one axis, Raf-1 acts as the MAPKKKK, while along the other axis, MEKK fulfills a similar role. While the activation of Raf-1 is Ras-dependent, the pathway leading to activation of MEKK is unknown, but has been tentatively linked to receptors which activate GTP-binding proteins [13]. Since BCR-mediated PTK activity is sensitive to pertussis toxin and GTP γ S induces a set of tyrosine phosphoproteins in B cells similar to those induced by BCR ligation, it is possible that MEKK does play a role in MAP kinase activation in Ramos cells [39].

In vitro kinase assays were set up with an antibody which immobilizes Raf-1 on Protein A-Sepharose beads, together with [³²P]ATP, Mg²⁺ and kinase-inactive MEK-1 [36]. The resulting autoradiogram of the phosphorylated products showed increased phosphorylation of kinase-inactive MEK-1 following anti-IgM treatment (Figure 6a, lanes 1 and 2). This was not due to different amounts of Raf-1 protein recovered from untreated or treated cells because parallel immune precipitation and Raf-1 blotting showed equal staining intensity in each lane (Figure 6b). The activation of Raf-1 was also reflected by a slight mobility shift of the kinase on SDS/PAGE (Figure 6b, lane 3). We also set up immune complex kinase assays utilizing a precipitating antibody to MEKK along with kinase-inactive MEK-1 as a substrate. The resulting autoradiogram yielded several phosphoproteins, including a protein of 70 kDa, which is in the molecular-mass range of murine MEKK (not shown). Anti-IgM did not increase the phosphorylation of this or any other phosphoproteins, which suggests that MEKK is not activated by the BCR (results not shown).

In the next series of experiments we employed kinase-inactive ERK-1, which maintains the allosteric effector TEY domain, as a substrate to identify the MAPKK utilized by the mIgM

Figure 2 mIgM ligation induces assembly of a complex of tyrosine phosphoproteins, including the adaptor proteins Shc and Grb2

(a) Anti-Shc blot and anti-phosphotyrosine blot of Shc immunoprecipitates. 1×10^7 Ramos cells were untreated (lane 2), treated with PMA for 5 min (lane 3) or treated with biotinylated anti-IgM plus avidin for 1 min (lane 4) or 5 min (lane 5). Precleared lysates were incubated with 5 μ l of anti-Shc sera (lanes 2–5) or pre-immune serum (lane 1) and subsequently with Protein A–Sepharose. Immunoprecipitates were boiled in SDS-sample buffer and analysed on SDS/10%-PAGE. The proteins were transferred to Immobilon-P and blotted with 1 μ g/ml polyclonal anti-Shc antibody followed by ^{125} I-Protein A overlay; the blot was stripped and reprobed with 4G10 mAb. (b) Grb2 immunoblot of Shc immunoprecipitates. 1×10^7 Ramos cells were unstimulated (lane 2), stimulated with anti-IgM plus avidin for 5 min (lane 3) or treated with PMA for 5 min (lane 4) and lysed by sonication. Immune precipitation was carried out as in (a) with anti-Shc (lanes 2–4) or with pre-immune serum (lane 1). Proteins were separated by SDS/12%-PAGE and the resulting blot was probed with 1 μ g/ml monoclonal Grb2 antibody. The blot was developed by ECL. (c) Shc immunoblot of Grb2 immune precipitates. 2×10^7 Ramos cells were left untreated (lane 2), treated with PMA for 5 min (lane 3), or treated with anti-IgM antibodies plus avidin for 1 min (lane 4) and 5 min (lane 5). Cleared lysates were incubated with 1 μ g of polyclonal Grb2 antibody (lanes 2–5) or with 3 μ l of pre-immune serum (lane 1) for 1 h followed by Protein A–Sepharose. Washed beads were boiled with SDS-sample buffer, and the proteins were resolved by SDS/10%-PAGE and transferred to Immobilon-P membranes. The blot was probed with 1 μ g/ml monoclonal Shc antibody and detection was performed by ECL. (d) Anti-phosphotyrosine blot of Grb2 immune precipitates. 2×10^7 Ramos cells were unstimulated (lane 2), stimulated with biotinylated anti-IgM plus avidin for 1 min (lane 3) or 5 min (lane 4), or stimulated with PMA for 5 min (lane 5). Cells were lysed and nuclei were removed by centrifugation. Proteins were immune-precipitated as in (c) with anti-Grb2 (lanes 2–5) or pre-immune serum (lane 1), resolved by SDS/10%-PAGE and transferred to Immobilon-P. The blot was overlaid with 4G10 mAb.

receptor. We used an immunoprecipitating antibody to MEK-1 to conduct an immune complex kinase assay [36], which showed increased phosphorylation of kinase-inactive ERK-1 in anti-IgM-treated Ramos cells compared with control cells (Figure 6c, lanes 3 and 4).

Although PMA differed from anti-IgM with respect to the use of adaptor proteins, PMA was a potent stimulus for the activation of Raf-1 (Figure 6a, lane 3) and MEK-1 (Figure 6c, lane 5). This finding is in agreement with the ability of PMA to activate p21^{ras} in Ramos cells [17].

Figure 3 hSOS associates with Grb2 in Ramos cells

Cells (2×10^7) were unstimulated (lane 2), stimulated with PMA for 5 min (lane 3), or treated with anti-IgM for 1 min (lane 4) or 5 min (lanes 1 and 5). Soluble cell lysates were incubated with $1 \mu\text{g}$ of anti-Grb2 for 1 h and then Protein A-Sepharose for 1 h. Immune complexes were washed well and boiled with SDS-sample buffer. Proteins were resolved on SDS/8%-PAGE and transferred to Immobilon-P membrane, and then blotted with $1 \mu\text{g}/\text{ml}$ polyclonal anti-hSOS followed with ^{125}I -linked Protein A. In an accompanying anti-Grb2 overlay of these immune precipitates analysed on SDS/12%-PAGE, we showed that equal amounts of Grb2 protein were being precipitated (not shown). CL, crude lysate.

We have previously shown that the tumour promoter okadaic acid activated MAP kinase with potency similar to PMA, albeit with less rapid kinetics (Figure 7a). Okadaic acid is an inhibitor of the serine/threonine phosphatases PP1 and PP2A [40]. Immune complex kinase assays for Raf-1 (Figure 7c) and MEK-1 (Figure 7b) demonstrated, however, that okadaic acid did not activate the respective MAPKK (Figure 7b, lane 3) or MAPKKK (Figure 7c, lane 3). These results differed when compared with stimulation with PMA (Figure 7b, lane 5; Figure 7c, lane 4). This suggests that PP1/PP2A may regulate MAP kinase activity directly.

Figure 4 Anti-phosphotyrosine and Shc immunoblotting demonstrates Shc association with hSOS in activated Ramos cells

2×10^7 cells were unstimulated (lanes 2 and 6), or treated with biotinylated anti-IgM and avidin for 1 min (lanes 3 and 7) or 5 min (lanes 4 and 8). Cleared lysates were incubated with $1 \mu\text{g}$ of polyclonal anti-hSOS for 2 h and subsequently with Protein A-Sepharose for 1 h. Lanes 1 and 5 represent pre-immune serum precipitates. Washed beads were boiled in SDS-sample buffer; proteins were separated by SDS/10%-PAGE and transferred to Immobilon-P. The membrane was probed with $1 \mu\text{g}/\text{ml}$ monoclonal anti-Shc using ECL detection, then stripped and reprobed with anti-phosphotyrosine mAb.

Figure 5 Anti-phosphotyrosine immunoblot of Vav immune precipitates shows that p95^{vav} is constitutively phosphorylated in Ramos cells and is not altered by mIgM ligation

2×10^7 Ramos cells (lanes 1–8) were unstimulated (lanes 2, 5 and 9), stimulated with anti-IgM plus avidin for 1 min (lanes 3 and 7) or for 5 min (lanes 4 and 8). For comparison, 2×10^7 Jurkat cells were stimulated with $10 \mu\text{g}/\text{ml}$ OKT3 (anti-CD3 mAb) for 5 min (lane 10) or left unstimulated (lane 9). Soluble cell lysates were incubated with $1 \mu\text{g}$ of polyclonal anti-Vav (lanes 2–4, 6–10) or $3 \mu\text{l}$ of pre-immune serum (lanes 1 and 5) and Protein A-Sepharose. After washing, immune precipitates were boiled in SDS sample buffer, resolved by SDS/10%-PAGE, and transferred to Immobilon-P. The blot shown in lanes 1–4 was probed with $1 \mu\text{g}/\text{ml}$ polyclonal anti-Vav antibody and overlaid with ^{125}I -linked Protein A. This blot was stripped and probed with monoclonal anti-phosphotyrosine (lanes 5–8). The Jurkat cell extracts were subjected to anti-phosphotyrosine immunoblotting only (lanes 9 and 10).

DISCUSSION

In this paper we show that MAP kinase activation by the mIgM receptor in Ramos cells involves a cascade of events which commence with the assembly of a Shc/Grb2/hSOS complex at the B-cell membrane. PMA, a direct PKC agonist and Ras activator, did not induce Shc tyrosine phosphorylation or Shc/Grb2 association. Both anti-IgM antibody and PMA stimulated the MAPKKK, Raf-1, and the MAPKK, MEK-1. We also showed that the serine/threonine phosphatase inhibitor, okadaic acid, activated MAP kinase in Ramos cells independent of Raf-1 or MEK-1.

MAP kinase activation by the T-cell antigen receptor is dependent on p21^{ras} and can be abrogated by dominant-negative Ras transfection [41]. Our studies suggest that Ras activation by mIgM ligation in Ramos cells is dependent on the involvement of Shc, Grb2 and hSOS (Figures 2–4). These components probably form part of a signalling complex which may include many components, as judged by the number of tyrosine phosphoproteins which associate with Grb2 (Figure 2d, lanes 3 and 4). The exact localization of this complex in relation to the BCR is still under study but may involve the mIgM-associated signal-transducing molecules Ig α and Ig β [1]. In an analogy with the ζ chain of the TCR, which contains consensus tyrosine phosphorylation motifs and binds Shc on receptor ligation [42,43], we postulate that related motifs on Ig α and Ig β provide a binding site for the SH2 domain of Shc [2].

In addition to hSOS, there are at least two other mechanisms by which Ras may be activated in B-lymphocytes, including the use of an alternative GNEF, p95^{vav}, or the inhibition of GTPase-activating protein (GAP) [6,28,44,45]. The GNEF activity of Vav is apparently controlled by tyrosine phosphorylation by BCR- or TCR-associated PTKs [7,28]. However, our results indicate that

Figure 6 Stimulation of Ramos cells via BCR ligation or PMA treatment activates an MAPKKK (Raf-1) and an MAPKK (MEK-1)

(a) Autoradiogram of Raf-1 immune complex kinase assay. 1×10^7 Ramos cells were unstimulated (lane 1), treated with anti-IgM plus avidin for 5 min (lane 2) or with PMA for 5 min (lane 3). The soluble cell lysates were incubated with $1 \mu\text{g}$ of polyclonal anti-Raf antibody for 1 h and then with Protein A-Sepharose for 1 h. The immune complexes were then centrifuged through a 10% sucrose buffer, and were washed three times with lysis buffer and three times with kinase buffer before incubation with 10 mM MgCl_2 , $1 \mu\text{g}$ of kinase-inactive MEK-1, and

mIgM ligation did not increase tyrosine phosphorylation of Vav, which contains high basal phosphorylation in Ramos cells (Figure 5). This contrasts with the TCR signalling pathway in Jurkat cells which utilizes tyrosine phosphorylation of Vav, but not of Shc, in the MAP kinase cascade [36]. The BCR signalling pathway in Ramos cells, which represent a mature B-cell lineage, appears to differ from other B-cell lines in which BCR ligation has been shown to result in tyrosine phosphorylation of Vav [7]. In addition, ligation of the BCR in a number of B-cell lines induces tyrosine phosphorylation of p120 GAP and association of GAP with p62 and p190; these effects are thought to inhibit the GAP activity, resulting in a net increase of Ras-GTP within the cell [6,44]. However, it is not known whether this mechanism of Ras activation is employed by the mIg receptors in all B-lymphocytes. Clearly there may be differences in the mechanism(s) by which Ras is activated following BCR ligation in different B-cell lineages, and it is likely that in some systems a combination of mechanisms are engaged. These differences may also reflect differences in mode of stimulation, such as ligation by antibody or stimulation by a high-affinity antigen, or even by stimulation with an antigen which has a low affinity for the BCR.

Downstream of Ras, the mIgM receptor in Ramos cells activates the MAPKKK, Raf-1, and the MAPKK, MEK-1 (Figure 6). While we have preliminary data suggesting that MEKK is not involved as an MAPKKK in anti-IgM-treated Ramos cells, it has recently come to light that there may be multiple members of the MEKK family of kinases. Because MEKK may be activated by receptors which activate G-proteins, including the BCR [39], we cannot exclude the possibility that unidentified members of this family may play a role in the BCR-associated MAP kinase cascade. These findings are in agreement with those of Tordai et al. [17].

Insofar as the BCR signalling pathway involves PKC activation subsequent to PTK activation [46], it is likely that PKC may contribute independently to Ras and MAP kinase activation. We suggest that the PKC-activated pathway may intersect the TPK pathway at the level of Ras activation; this idea is compatible with the activation of Raf-1 and MEK-1 by PMA (Figure 6). The mechanism by which PMA activates Ras is not known yet, but it may operate via Vav, or it may inhibit GAP activity as shown in T lymphocytes by Downward et al. [44].

Inasmuch as signalling is regulated by balanced activities of kinases and phosphatases, it is interesting that the serine/threonine phosphatase inhibitor, okadaic acid, activated MAP kinase without activating any of the studied afferent kinases (Figure 7). This suggests the presence of a phosphatase which acts directly on MAP kinase or which regulates a heretofore unknown MAP kinase activator. We favour the former possibility

$20 \mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 40 min at 30°C . Kinase reactions were stopped by boiling in SDS-sample buffer, the reaction products were separated by SDS/10%-PAGE and visualized by autoradiography. (b) Anti-Raf blot of Raf-1 immune precipitates in Ramos cells. Parallel immune precipitations were run with the ICK shown in Figure 5(b). Lane 2 is unstimulated cells; lane 3, treated with anti-IgM for 5 min; and lane 4, treated with PMA for 5 min. Lane 1 represents proteins precipitated with pre-immune serum. The precipitates were separated by SDS/10%-PAGE and transferred to Immobilon-P for overlay with $1 \mu\text{g}/\text{ml}$ polyclonal Raf-1 antibody. The antibody was detected by ^{125}I -linked Protein A overlay. (c) Autoradiogram of reaction products of MEK-1 *in vitro* kinase assay. 1×10^7 cells were either not stimulated (lanes 1 and 2) or treated with anti-IgM plus avidin for 1 min (lane 3), 5 min (lane 4), or 30 min (lane 5), or with PMA for 5 min (lane 6) and lysed by sonication. Soluble extracts were incubated with $5 \mu\text{l}$ of polyclonal anti-MEK-1 antibody for 1 h (lanes 2–6) or $3 \mu\text{l}$ pre-immune serum (lane 1) and then with Protein A-Sepharose for 1 h. Immune precipitates were washed and incubated with 10 mM MgCl_2 , 50 μM ATP, $1 \mu\text{g}$ of kinase-inactive ERK-1, and $10 \mu\text{Ci}$ of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 20 min at 30°C . Reactions were stopped by boiling in SDS-sample buffer. Phosphorylated proteins were resolved by SDS/10%-PAGE and visualized by autoradiography.

Figure 7 Okadaic acid activates MAP kinase without stimulating MEK-1 or Raf-1 activity in Ramos cells

(a) MAP kinase assay showing activation by okadaic acid and PMA. 5×10^6 cells were untreated, stimulated with 100 nM PMA for 5 min, or treated with $1 \mu\text{M}$ okadaic acid for the indicated time period. MAP kinase was enriched from soluble cell lysates as previously described (19). Kinase reactions were then run with 1 mg/ml MBP peptide, 20 μM unlabelled ATP and 10 mM MgCl_2 for 8 min at 30 °C and stopped by the addition of 10% trichloroacetic acid. The peptide supernatant was spotted on Whatman P81 paper squares and washed with 75 mM phosphoric acid and ³²P incorporation was determined. (b) *In vitro* MEK-1 kinase assay. An immune complex kinase assay for MEK-1 was performed as in Figure 5(a). The reaction products were separated by SDS/10%-PAGE and visualized by autoradiography. Lane 2 represents unstimulated cells, lane 3 treatment with $1 \mu\text{M}$ okadaic acid for 30 min, lane 4 okadaic acid for 60 min, and lane 5 represents 100 nM PMA treatment for 5 min (positive control). Lane 1 is immune precipitation with pre-immune serum. (c) Raf-1 immune complex

kinase assay. 1×10^7 Ramos cells were untreated (lane 1), treated with $1 \mu\text{M}$ okadaic acid for 30 min (lane 2) or for 60 min (lane 3), or treated with 100 nM PMA for 5 min (lane 4). An *in vitro* kinase assay was performed as in Figure 5(b) and the reaction products were analysed by SDS/10%-PAGE and autoradiography.

because the kinetics of MAP kinase activation by okadaic acid are slow (Figure 7a) and are best explained by interference with a phosphatase which regulates a slow turnover of MAP kinase in the cell cycle [16].

In summary, we propose that a sequence of events commencing with BCR ligation and culminating in the translocation of activated MAP kinase to the nucleus utilizes a Shc/Grb2/hSOS complex to initially activate Ras. This is followed by the activation of Raf-1 and MEK-1 as an MAPKKK and an MAPKK respectively. The PKC-activated pathway, which is activated downstream of RTKs, intersects the BCR-activated pathway at the level of Ras, thus amplifying the PTK-mediated activation of MAP kinase.

This work was supported by the Center Grant for Asthma, Allergy and Immunologic Disease (A134567) and GM-41576. We would like to thank Dr. John Cambier for reading the manuscript and providing constructive comments.

REFERENCES

- Cambier, J. C., Bedzyk, W., Campbell, K., Chien, N., Friedrich, J., Harwood, A., Jensen, W., Pleiman, C. and Clark, M. R. (1993) *Immunol. Rev.* **132**, 85–105
- Reth, M., Hombach, J., Weinands, J., Campbell, K. S., Chien, A., Justement, L. B. and Cambier, J. C. (1991) *Immunol. Today* **12**, 196–201
- Carter, R. H., Park, D. J., Rhee, S. G. and Fearon, D. T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2745–2749
- Nel, A. E., Wooten, M. W., Landreth, G. E., Goldschmidt-Clermont, P. J., Stevenson, H. C., Miller, P. J. and Galbraith, R. M. (1986) *Biochem. J.* **233**, 145–149
- Burkhardt, A. L., Brunswick, M., Bolen, J. B. and Mond, J. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7410–7414
- Gold, M. R., Crowley, M. T., Martin, G. A., McCormick, F. and DeFranco, A. L. (1993) *J. Immunol.* **150**, 377–386
- Gulbins, E., Langlet, C., Baier, G., Bonnefoy-Berard, N., Herbert, E., Altman, A. and Coggeshall, K. M. (1994) *J. Immunol.* **152**, 2123–2129
- Hutchcroft, J. E., Harrison, M. L. and Geahlen, R. (1992) *J. Biol. Chem.* **267**, 8613–8619
- Casillas, A., Hanekom, C., Williams, K., Katz, R. and Nel, A. E. (1991) *J. Biol. Chem.* **266**, 19088–19094
- Pelech, S. and Sanghera, J. S. (1992) *Trends Biochem. Sci.* **17**, 233–237
- Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J. and Cobb, M. H. (1990) *Science* **249**, 64–67
- L'Allemain, G., Pouyssegur, J. and Weber, M. J. (1991) *Cell Regul.* **2**, 675–684
- Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J. and Johnson, G. L. (1993) *Science* **260**, 315–319
- Roberts, T. M. (1993) *Nature (London)* **360**, 534–535
- Schanen-King, C., Nel, A., Williams, L. K. and Landreth, G. (1991) *Neuron* **6**, 915–922
- Casillas, A. M., Amaral, K., Chehni-Farahani, S. and Nel, A. E. (1993) *Biochem. J.* **290**, 545–550
- Tordai, A., Franklin, R. A., Patel, H., Gardner, A. M., Johnson, G. L. and Gelfand, E. W. (1994) *J. Biol. Chem.* **269**, 7538–7543
- Howe, L. R., Leever, S. J., Gomez, N., Nakiely, S., Cohen, P. and Marshall, C. J. (1992) *Cell* **71**, 335–342
- Nel, A. E., Pollack, S., Landreth, G., Ledbetter, J. A., Hultin, L., Williams, K., Katz, R. and Akerley, B. (1990) *J. Immunol.* **145**, 971–979
- Nel, A. E., Hanekom, C. and Hultin, L. (1991) *J. Immunol.* **147**, 1933–1939
- Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J. S. (1992) *Cell* **68**, 1031–1041
- Williams, N. G., Paradis, H., Agarwal, S., Charest, D. L., Pelech, S. and Roberts, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5772–5776
- Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. and Weinberg, R. A. (1993) *Nature (London)* **363**, 45–51
- Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Batzer, A., Thomas, S., Brugge, J., Schlessinger, J. and Pawson, T. (1992) *Nature (London)* **360**, 689–692

- 25 Bonfini, L., Karlovich, C. A., Dasgupta, C. and Banerjee, U. (1992) *Science* **255**, 603–606
- 26 Harwood, A. E. and Cambier, J. C. (1993) *J. Immunol.* **151**, 4513–4522
- 27 Kamauchi, K., Lazarus, A. H., Rapoport, M. J., Harwood, A., Cambier, J. C. and Delovitch, T. L. (1994) *J. Immunol.* **152**, 3306–3316
- 28 Gulbins, E., Coggeshall, K. M., Baier, G., Katzav, S., Burn, P. and Altman, A. (1993) *Science* **260**, 822–825
- 29 Dent, P., Haser, W., Haystead, T. A., Vincent, L. A., Roberts, T. M. and Sturgill, T. W. (1992) *Science* **257**, 1404–1407
- 30 Kyriakis, J. M., App, H., Zhang, X. F., Banerjee, D. L., Brautigan, D. L., Rapp, U. R. and Arruch, J. (1992) *Nature (London)* **358**, 417–421
- 31 Moodie, S. A., Willumsen, B. M., Weber, M. J. and Wolfman, A. (1993) *Science* **260**, 1658–1661
- 32 Crews, C. M., Alessandrini, A. and Erikson, R. L. (1992) *Science* **258**, 478–480
- 33 Anderson, N. G., Maller, J. L., Tonks, N. K. and Sturgill, T. (1990) *Nature (London)* **343**, 651–653
- 34 Nebreda, A. R., Hill, C., Gomez, N., Cohen, P. and Hunt, T. (1993) *FEBS Lett.* **333**, 183–187
- 35 Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R. J. and Kelly, K. (1994) *Nature (London)* **367**, 651–654
- 36 Gupta, S., Weiss, A., Kumar, G., Wang, S. and Nel, A. E. (1994) *J. Biol. Chem.* **269**, 17349–17357
- 37 Bustelo, X. R. and Barbacid, M. (1992) *Science* **256**, 1196–1199
- 38 Wood, K. W., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992) *Cell* **68**, 1041–1050
- 39 Melamed, I., Wang, G. and Roifman, C. M. (1992) *J. Immunol.* **149**, 169–174
- 40 Cohen, P. (1989) *Annu. Rev. Biochem.* **58**, 453–508
- 41 Izquierdo, M., Leesters, S. J., Marshall, C. J. and Cantrell, D. (1993) *J. Exp. Med.* **178**, 1199–1208
- 42 Ravichandran, S. R., Lee, K. K., Songyang, Z., Cantley, L. C., Burn, P. and Burakoff, S. J. (1993) *Science* **262**, 902–905
- 43 Weiss, A. (1993) *Cell* **73**, 209–212
- 44 Downward, J., Graves, J. D., Warne, P. H., Rayter, S. and Cantrell, D. A. (1990) *Nature (London)* **346**, 719–723
- 45 Lazarus, A. H., Kawauchi, K., Rapoport, M. J. and Delovitch, T. L. (1993) *J. Exp. Med.* **178**, 1765–1769
- 46 Roifman, C. M. and Wang, G. (1992) *Biochem. Biophys. Res. Commun.* **183**, 411–416