Brief Definitive Report

Activation of the p21^{ras} Pathway Couples Antigen Receptor Stimulation to Induction of the Primary Response Gene *egr*-1 in B Lymphocytes

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Summary

The primary response gene *egr*-1 encodes a sequence-specific transcription factor whose expression is necessary for antigen receptor–stimulated activation of B lymphocytes. The molecular processes involved in linking *egr*-1 induction to antigen receptor signaling have not been defined. The present study demonstrates that expression of an activated form of p21^{ras} results in *egr*-1 induction similar to that previously shown after antigen receptor cross-linking. In addition, both antigen receptor cross-linking and p21^{ras} use the same element in the *egr*-1 promoter to exert their effects. Using dominant-negative mutants of p21^{ras} and raf-1, we demonstrate that induction of *egr*-1 after antigen receptor cross-linking is mediated by activation of the p21^{ras}/mitogen-activated protein kinase signaling pathway. While regulation of the p21^{ras} pathway during B cell activation has been intensively studied, this report represents the first description of a biologically relevant event associated with its activation.

Activation and differentiation of B lymphocytes is initiated by an interaction between a multivalent antigen and the antigen receptor (BCR) on the cell surface. As the activation signal enters the cytoplasm, a variety of biochemical changes can be detected. These changes include phosphotidylinositol hydrolysis with subsequent Ca^{2+} mobilization and protein kinase C (PKC) activation, and increased tyrosine kinase activity (for review see reference 1). In addition, the low molecular weight G protein p21^{ras} is activated after antigen receptor cross-linking on B cells (2–4). These studies were prompted by an early report that found that p21^{ras} colocalized with the antigen receptor after cross-linking and aggregation (5).

The p21^{ras} signaling pathway has been characterized in a variety of cell types. As a G protein, p21^{ras} is active only in the GTP-bound state (6). Two mechanisms for regulating the activation state of p21^{ras} have been described. Guanine nucleotide exchange factors (GEFs) augment the exchange of GDP for GTP and are positive activators, while GTPase activating proteins (GAPs) result in inactivation of p21^{ras} (6). Directly downstream of p21^{ras} in this signaling pathway is the activation of the serine/threonine kinase raf-1 (7). The mechanism by which p21^{ras} activates raf-1 is not entirely understood, but recent reports suggest that it results from recruitment of raf-1 to the cell membrane (8, 9). Activated raf-1 phosphorylates members of the MAPK/ERK kinase (MEK) family of dual specificity kinases (10). After phosphorylation, MEKs activate the mitogen activated protein kinases (MAPKs) by threonine and tyrosine phosphorylation (11). MAPK has been directly linked to the control of transcription due to phosphorylation of certain transcription factors, including c-JUN (12) and a group of ETS family members collectively known as p62TCF (13). While the linearity of the p21^{ras}/raf-1/MEK/MAPK pathway has been confirmed in many receptor systems, points of divergence have also been described (14).

In B lymphocytes, activation of the p21^{ras} pathway after BCR cross-linking has been extensively studied (1). The GEF *vav* is activated (15, 16) and the GAP factors p120 (3, 17) and neurofibromin (18) inactivated after BCR cross-linking. In addition, recent studies (18a) have shown recruitment of the GEF SOS-1 to the BCR following receptor cross-linking. Activation of raf-1, MEK, and MAPK by BCR cross-linking have all been demonstrated (2). As yet, however, no downstream effectors of the p21^{ras}/MAPK pathway have been described in B lymphocytes. This is in contrast to T lymphocytes, where induction of the IL-2 gene, an event critical for antigen receptor–initiated proliferation, appears to be mediated by activation of the p21^{ras}/MAPK pathway (19, 20).

Among the earliest genetic events detected after BCR cross-linking is induction of the primary response gene *egr*-1 (1). Antisense studies have demonstrated that induction of *egr*-1 is necessary for antigen receptor–mediated activation of both B and T lymphocytes (21, 22). This is presumably accomplished by induction of a second wave of function-related genes whose regulation is controlled by the transcription factor encoded by *egr*-1.

Our recent results indicate that *egr*-1 induction after BCR cross-linking requires a serum response element (SRE) and adjacent ETS motifs in the *egr*-1 promoter (McMahon, S.B.,

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and J.G. Monroe, manuscript submitted for publication). A similar combination of SRE/ETS motifs mediates c-*fos* induction in fibroblasts in response to serum growth factors (for review see reference 13). The c-*fos* SRE is occupied by a ternary complex composed of a homodimer of serum response factor (SRF) and a single molecule of p62TCF, which binds the adjacent ETS motif (13). Transcription of c-*fos* results from phosphorylation of p62TCF by MAPK (13). Because of the similarity in expression pattern and regulatory elements between c-*fos* and *egr*-1, this study was undertaken to determine whether the $p21^{ras}$/MAPK pathway was responsible for *egr*-1 induction in B cells. Using dominant-negative mutants of $p21^{ras}$ and raf-1, we demonstrate that in B lymphocytes, induction of *egr*-1 depends on activation of the $p21^{ras}$ pathway. This study represents the first demonstration of any functional consequence of $p21^{ras}$ activation after BCR cross-linking.

Materials and Methods

B Lymphocyte Isolation and Culture. The spleens of 8- to 12-wk BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were removed aseptically from animals killed by cervical dislocation. Spleens were minced and T lymphocytes removed by treatment with anti-Thy-1.2 (HO13.4) antiserum and rabbit complement (Pel-Freeze Biologicals, Rogers, AZ). Red blood cells were removed by hypotonic lysis and the remaining cells spun over a 75% Percoll cushion. Cells were placed in culture for 72 h in B cell assay media (RPMI-1640 with 10% FCS, 2 mM L-glutamine, nonessential amino acids, 100 μg/ml each of penicillin and streptomycin, and 5×10^{-5} M 2-ME) with LPS (*Salmonella Typhosa*; Sigma Chemical Co., St. Louis, MO) at 50 μg/ml. After 72 h of culture, B cell blasts were transiently transfected with vectors, as described below.

Plasmid Construction. The reporter plasmid pBL395 contains *egr*-1 promoter sequences from −395 to +65 linked to the gene encoding chloramphenicol acetyltransferase (CAT). The plasmid containing point mutations in SRE #4 was generated by the PCR method with Vent (Exo-) polymerase (New England Biolabs, Beverly, MA). The expression vector pEJ encodes a constitutively active form of cHa-*ras* (25) and was provided by Dr. R. Muschel (University of Pennsylvania, Philadelphia, PA). The expression vector pASN17 encodes a dominant-negative form of $p21^{ras}$ (26) and was provided by Dr. G. Cooper (Harvard University, Cambridge, MA). The expression vector pmt-NΔraf encodes a dominant-negative form of raf-1 (27) and was provided by Dr. D. Schaap (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Plasmid DNA was isolated by the anion-exchange method (QIAGEN). DNA from multiple preparations was used in these studies, and results were uniformly consistent.

Transient Transfection of B Lymphocytes. B cell blasts were transiently transfected essentially as described (28, 28a). Briefly, cells were washed once in transfection buffer (25 mM Tris-Cl [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$) prewarmed to 37°C and suspended in a sterile solution containing DEAE-dextran (Pharmacia Fine Chemicals, Piscataway, NJ) at 500 μg/ml in STBS and the indicated amounts of plasmid DNA. Generally, 5×10^7 cells were transfected in 5 ml of this solution for 30 min at 37°C. Cells were then washed once with STBS and suspended at 10^6/ml in B cell assay media (without LPS). Stimuli were added 24 h after transfection for PMA (Sigma Chemical Co.) (10 ng/ml), or 40 h after transfection for BCR cross-linking. BCR cross-linking was accomplished by the addition of goat anti-μ heavy chain (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) antibodies at 20 μg/ml.

CAT Assay. To assay transfected B cell blasts for CAT activity, cells were collected 48 h after transfection by centrifugation. After lysis, CAT activity was assessed as described previously (28, 28a). Results were quantitated on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). CAT activity is presented as either percent conversion or fold induction. Fold induction is defined by the ratio of the percentage of chloramphenicol converted to acetylated forms in stimulated versus unstimulated cells.

Results

Expression of a Constitutively Active Form of $p21^{ras}$ Activates the egr-1 Promoter in B Lymphocytes. Previous studies have demonstrated that promoter elements including an SRE and two adjacent ETS motifs mediate *egr*-1 induction in response to BCR cross-linking (McMahon, S.B., and J.G. Monroe, manuscript submitted for publication). Because of recent studies demonstrating that activation of the $p21^{ras}$/MAPK pathway is responsible for SRE-dependent expression of c-*fos* in fibroblasts (13), the participation of this pathway in induction of *egr*-1 in B lymphocytes was evaluated. B cell blasts were transiently transfected with a combination of a construct (pBL395) containing the *egr*-1 promoter upstream of the CAT reporter gene and the $p21^{ras}$ expression vector pEJ (25). pBL395 contains *egr*-1 promoter sequences from −395 to +65 which includes four SREs, a cAMP response element, and a binding site for the EGR-1 protein, as well as potential binding sites for other transcription factors (23). The pEJ vector encodes a $p21^{ras}$ molecule, which is constitutively active due to a substitution at amino acid 12 (glycine → valine), resulting in decreased GTP hydrolysis and accumulation of $p21^{ras}$ molecules in the active, GTP-bound form (25). As is evident in Fig. 1, cotransfection of pEJ resulted in a dose-dependent increase in transcription from the *egr*-1 promoter as assessed by CAT activity. Cotransfection of the empty vector (pBR322) had no detectable effect on *egr*-1 promoter activity.

The Promoter Element Mediating egr-1 Induction by Activated $p21^{ras}$ Colocalizes with the Element Mediating Induction by BCR Cross-linking. While the results presented in Fig. 1 indicate that activated $p21^{ras}$ causes an increase in the activity of a construct containing a large region of the *egr*-1 promoter, these data fail to address whether $p21^{ras}$ and BCR cross-linking use the same *cis*-regulatory elements. Our previous studies demonstrated that induction of *egr*-1 after BCR cross-linking in B lymphocytes was dependent on the presence of an SRE at −360 (SRE #4) (McMahon, S. B., and J. G. Monroe, manuscript submitted for publication). To determine whether expression of activated $p21^{ras}$ mimics BCR cross-linking in this regard, an *egr*-1 promoter containing a mutated version of SRE #4 was tested for activity in the presence of activated $p21^{ras}$. Like pBL395, this CAT reporter construct contains *egr*-1 promoter sequences from −395 to +65. However, four point mutations were introduced into the core of SRE #4. These mutations have previously been shown to inhibit SRF binding to the c-*fos* SRE and consequently abolish transcription mediated by this element (29).

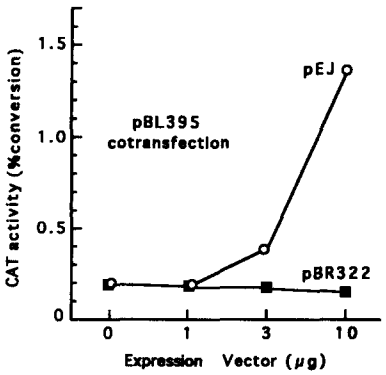

Figure 1. Cotransfection of an activated form of p21[ras] results in a dose-dependent increase in *egr*-1 promoter activity. LPS-induced B cell blasts were transiently transfected with 20 μg of the *egr*-1 promoter/CAT vector pBL395 along with the indicated amounts of either pEJ (*open circles*) or the empty cassette pBR322 (*solid squares*). pEJ encodes a constitutively active form of cHa-*ras* (25). Cells were harvested 48 h after transfection and lysates assayed for CAT activity. The results are expressed as the percentage of chloramphenicol converted to acetylated forms.

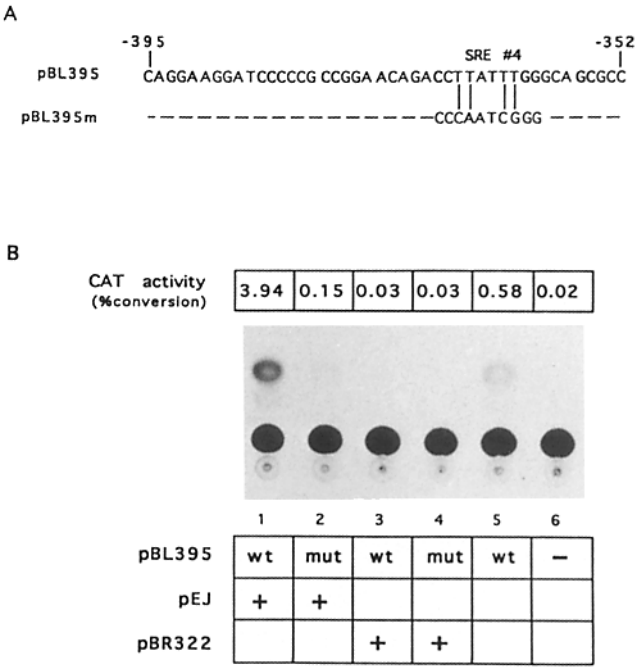

Figure 2. Activated p21[ras] induces *egr*-1 through the same promoter element necessary for antigen receptor–mediated induction. Transient transfections were carried out by use of either of two separate *egr*-1 promoter/CAT constructs. (*A*) Sequence of the *egr*-1 promoter region surrounding SRE #4. The top line indicates the wild-type sequence included in pBL395. The lower line indicates the sequence of the mutated version (*pBL395m*), with vertical lines indicating nucleotides that were altered. (*B*) The reporter constructs (5 μg of pBL395 in lanes *1* and *3* or pBL395mut in lanes *2* and *4*) described above were cotransfected with 7.5 μg of either the activated p21[ras] expression vector pEJ (lanes *1* and *2*) or the empty cassette pBR322 (lanes *3* and *4*). Cells were harvested 48 h after transfection, as described in Materials and Methods, and results of the CAT assay are displayed. Values presented represent percent conversion.

The wild-type or mutated versions of pBL395 were cotransfected into B cell blasts with the p21[ras] expression vector pEJ. As shown in Fig. 2, expression of activated p21[ras] strongly induced CAT activity from the wild-type *egr*-1 promoter (3.94% conversion). Mutation of SRE #4 resulted in a marked decrease in CAT activity (0.15% conversion). These values reflect a decrease from 131-fold induction with the wild-type promoter to 5-fold with the mutant SRE promoter (relative to levels obtained with the empty cassette pBR322), indicating that SRE #4 is necessary for the induction of *egr*-1 promoter activity by p21[ras].

Expression of a Dominant-negative Mutant of p21[ras] Inhibits BCR-mediated Induction of egr-1 in B Lymphocytes. The demonstration that *egr*-1 promoter activity is induced by a constitutively active form of p21[ras] fails to directly address the role of the endogenous p21[ras] molecule in mediating *egr*-1 induction during BCR-mediated activation of B lymphocytes. To determine whether activation of p21[ras] plays a role in this process during normal BCR signaling, a construct encoding a dominant-negative mutant of p21[ras] (pASN17) was transfected into B lymphocytes (26). The dominant-negative p21[ras] mutant contains a substitution at position 17 (serine → asparagine) (26). Position 17 is adjacent to the nucleotide binding pocket and, by analogy to other G proteins (26), may be critical for interacting with the γ phosphate of GTP. ASN17 is consequently 40-fold less efficient at binding GTP in vitro (26) and presumably inhibits the function of normal p21[ras] molecules by sequestering the cellular pool of GEF into inactive complexes (6). Cotransfection studies were performed with pASN17 (or the empty vector, pMSG) together with the *egr*-1 promoter construct pBL395. As demonstrated in Fig. 3 *A*, the induction of *egr*-1 promoter activity after BCR cross-linking is decreased by ~75% by cotransfection of pASN17. Induction of *egr*-1 was inhibited at two different ratios of the dominant-negative/reporter vectors (1:20 and 1:2). Somewhat unexpectedly, induction of *egr*-1 promoter activity in response to treatment with the phorbol ester PMA was also inhibited by the expression of ASN17 (Fig. 3 *B*). Like the inhibition of antigen receptor–mediated induction, this effect was evident at two different ratios of the dominant-negative/reporter vectors. Inhibition of PMA-mediated *egr*-1 induction by ASN17 suggests that at least some of the effects of PMA in B cells occur upstream of p21[ras] activation.

Expression of a Dominant-negative Mutant of raf-1 Inhibits Antigen Receptor–mediated Induction of egr-1. Activation of p21[ras] by growth-promoting agents in all cell types that have been examined results in activation of MAPK. As discussed above, a key intermediate in this cascade is the serine/threonine kinase raf-1 (10). To determine whether p21[ras] activation after antigen receptor cross-linking was functioning through this pathway, a dominant-negative version of raf-1 (NΔraf) was used (27). NΔraf contains only the NH_2-terminal regulatory region of raf-1, lacks the COOH-terminal kinase domain, and efficiently blocks raf-1–mediated events (27). When an expression vector (pmt-NΔraf) encoding the dominant-negative raf-1 mutant was cotransfected into B cells along with pBL395, induction of *egr*-1 promoter activity was substantially decreased (Fig. 4). As with the p21[ras] dominant-negative vector, this decrease was evident whether the

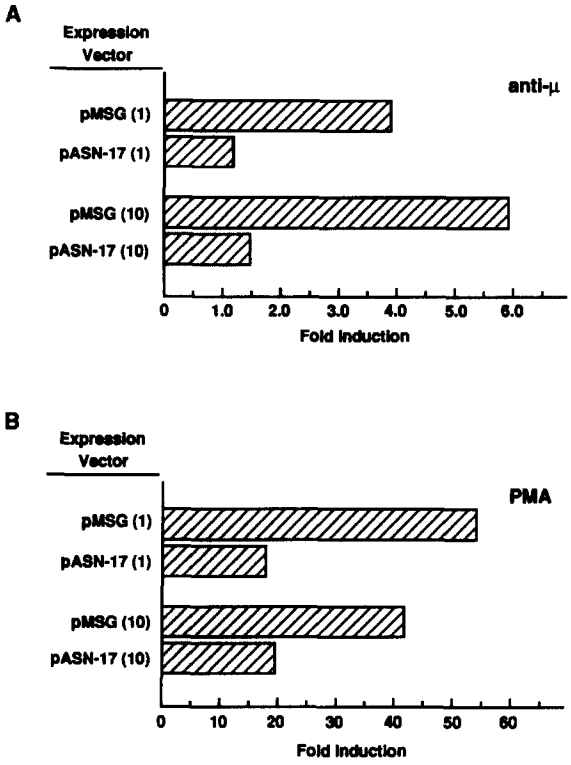

Figure 3. Expression of a dominant-negative form of $p21^{ras}$ inhibits *egr*-1 induction in B cells. LPS-induced B cell blasts were cotransfected with 20 μg of the *egr*-1 promoter/CAT construct pBL395 and 1 μg (*1*) or 10 μg (*10*) of the $p21^{ras}$ dominant-negative expression vector pASN-17 (or the empty cassette *pMSG*). Transfected cells were stimulated with either goat anti-μ antibodies at 20 μg/ml (*A*) or PMA at 10 ng/ml (*B*), as described in Materials and Methods. Results of the CAT assay are expressed as fold induction.

cells were stimulated with anti-μ (63% inhibition) or PMA (89% inhibition), suggesting that raf-1 plays a role in the induction of *egr*-1 by BCR cross-linking or PMA treatment.

Discussion

The results presented here demonstrate that activation of the $p21^{ras}$ pathway couples antigen receptor cross-linking to induction of *egr*-1 in B lymphocytes. Transfection of a constitutively active form of $p21^{ras}$ resulted in a dose-dependent increase in *egr*-1 promoter activity. Previous studies (McMahon, S. B., and J. G. Monroe, manuscript submitted for publication) demonstrated that induction of *egr*-1 after antigen receptor cross-linking requires the presence of a specific SRE, that is, SRE #4. In this study, we demonstrate that SRE #4 is also necessary for *egr*-1 induction in response to activated $p21^{ras}$. Finally, using dominant-negative mutants of $p21^{ras}$ and raf-1, we demonstrate that induction of *egr*-1 promoter activity after BCR cross-linking involves activation of the $p21^{ras}$/raf-1/MAPK pathway. By using transient transfection of primary B cells, we have been able to directly implicate this pathway in activation of the *egr*-1 promoter.

In addition to stimulation of B lymphocytes by antigen receptor cross-linking, we examined the effects of PMA treatment. PMA treatment directly activates PKC. As such, the finding that the dominant-negative $p21^{ras}$ mutant inhibited the PMA effect was somewhat unexpected and suggests that the effect of PMA on *egr*-1 transcription is mediated through the $p21^{ras}$ pathway. When these studies were initiated, PKC activation had not been shown to effect any of the early events in the $p21^{ras}$ pathway. Recent reports have suggested a mechanism for the inhibition seen in Fig. 3 by demonstrating that in addition to activating PKC, PMA can directly bind and activate the GEF *vav* (30). This potential effect of PMA on an early regulatory component in the $p21^{ras}$ pathway may explain the results obtained here, which show that inhibitory mutants of $p21^{ras}$ and raf-1 block induction of *egr*-1 by PMA.

As already noted, in B lymphocytes, *egr*-1 induction after BCR cross-linking or by expression of an activation form of $p21^{ras}$ (shown here) requires a specific SRE (SRE #4). This is noteworthy since the *egr*-1 gene contains five SREs, each of which are capable of binding SRF and regulating transcription in various cell types. In myeloid cells, for example, a variety of stimuli, including PMA, preferentially require SRE #5 for induction (31), while in PC12 cells, SREs #1 and #2 are necessary for nerve growth factor-induced expression (32). Similar to the studies presented here, expression of the v-raf oncogene in fibroblasts induces *egr*-1 promoter activity with a preference for SRE #4 (33). These results suggest a requirement for SRE #4 by receptors and stimuli that activate the $p21^{ras}$ signaling pathway.

A potential explanation for preferential utilization of SRE #4 by stimuli that activate the $p21^{ras}$ pathway may be provided by recent studies. Induction of *c-fos* by growth factors in fibroblasts results from phosphorylation of p62TCF by MAPK (13). Furthermore, p62TCF binds to a subset of ETS motifs that are adjacent to SREs (13). In the *egr*-1 promoter,

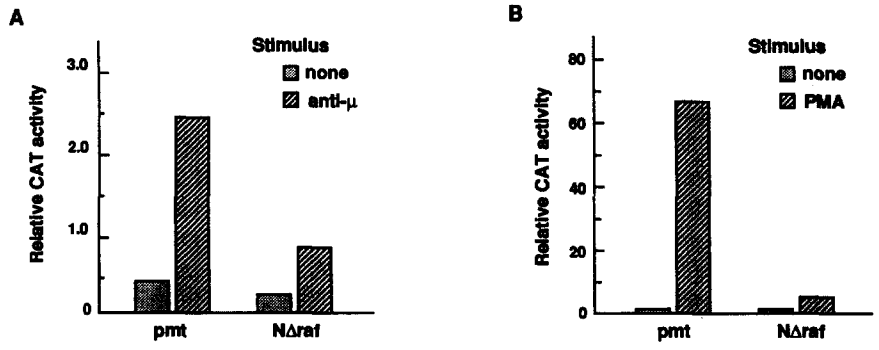

Figure 4. Expression of a dominant-negative form of raf-1 inhibits *egr*-1 induction in B cells. LPS-induced B cell blasts were cotransfected with 15 μg of the *egr*-1 promoter/CAT construct pBL395 and 30 μg of the raf-1 dominant-negative expression vector pmt-NΔraf (or the empty cassette *pmt*). Transfected cells were stimulated with either goat anti-μ antibodies at 20 μg/ml (*A*) or PMA at 10 ng/ml (*B*), as described in Materials and Methods. Relative CAT activity is expressed as percent conversion.

motifs that are adjacent to SREs (13). In the *egr*-1 promoter, high affinity p62TCF binding sites occur at only three locations, two of which are adjacent to SRE #4 (13, 23). We have recently shown that the two p62TCF binding motifs adjacent to SRE #4 are necessary for *egr*-1 promoter activity in response to BCR cross-linking or PMA treatment (McMahon, S. B., and J. G. Monroe, manuscript submitted for publication). Therefore, the presence of these motifs adjacent to SRE #4 is likely to mediate its preferential utilization in response to antigen receptor activation of the $p21^{ras}$ pathway in B lymphocytes by allowing efficient ternary complex formation by p62TCF and SRF.

Involvement of the $p21^{ras}$ pathway in BCR-generated signal transduction in B lymphocytes has been the subject of intensive study. Consequently, a significant amount of information is available regarding the proteins that regulate $p21^{ras}$ in B cells (4, 5, 15–17). In addition, several studies have identified the kinases that are active in this pathway (1, 2). However, to date, no functional consequence of $p21^{ras}$ activation in B cells has been demonstrated. In this report, we show that induction of *egr*-1, a gene whose expression is necessary for lymphocyte activation, is mediated by the $p21^{ras}$ pathway after cross-linking of the B cell antigen receptor. These findings assign a crucial role to $p21^{ras}$ in B cell activation.

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