Brief Definitive Report

Antigen-induced B Lymphocyte Activation Involves the p21^{ras} and ras.GAP Signaling Pathway

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Summary

Ligation of a B lymphocyte surface immunoglobulin (sIg) antigen receptor (AgR) by its specific Ag ligand initiates a signaling pathway that culminates in B cell activation. However, many events of this pathway have not been elucidated. Here we present three novel findings that demonstrate directly that AgR-mediated signaling in B cells functions by the p21ras/ras.GAP-dependent pathway. First, stimulation of TA3 7.9 Ag-specific murine B lymphoma cells for 2 min with either Ag or F(ab')$_2$ anti-IgM induces p21^{ras} activation as measured by an increase in the GTP/GDP ratio of its bound nucleotides. This activation of p21^{ras} does not occur via a change in its guanine nucleotide exchange rate. Second, Ag stimulation results in the inhibition of activity of p120 ras.GAP, a protein that regulates p21^{ras} activation. Tyrosine phosphorylation of ras.GAP occurs within 1 min after Ag stimulation but is no longer detectable at 20 min after stimulation, at which time ras.GAP activity remains inhibited. Thus, tyrosine phosphorylation of ras.GAP is not required for the inhibition of its activity. Third, despite the role proposed for a ras.GAP-associated p190 protein in the control of ras.GAP activity in B cells, p190 was not detectable either in anti-ras.GAP immunoprecipitates of [^{35}S]methionine labeled lysates of Ag-stimulated or -unstimulated 7.9 cells or as a tyrosine phosphoprotein in Western blots of anti-ras.GAP immunoprecipitates of Ag-stimulated 7.9 cell lysates. Inasmuch as the TA3 7.9 B lymphoma is representative of a mature, sIgM-bearing B cell, our observations raise the intriguing possibility that the capacity of p190 to associate with ras.GAP and regulate the activities of ras.GAP and p21^{ras} in a B cell is dependent on the stage of differentiation of the B cell.

B cell transmembrane signaling induced by the crosslinking of a surface immunoglobulin (sIg) AgR involves convergent pathways of activation. Whereas rapid tyrosine phosphorylation of several components of these pathways, e.g., phospholipase Cγ, contribute to B cell activation, and a role for GTP-binding proteins (G proteins) in B cell signaling has been reported (1–3), the precise nature of these G proteins remains to be identified. Two such candidate types of proteins are the classical heterotrimeric (α, β, γ) (1–4) and the low molecular weight p21^{ras} (5) families of G proteins. The activation of a member of the p21^{ras} proto-oncogene family has been demonstrated in a variety of cell types including T lymphocytes (6). Notwithstanding that the function of p21^{ras} in transmembrane signaling has yet to be elucidated, it appears that receptors can regulate p21^{ras} activity in cells destined for proliferation. For example, p21^{ras} may be associated with the AgR complex in activated B cells, particularly since p21^{ras} cocaps with crosslinked sIgM (7). p21^{ras} can exist in either an "active" (GTP-associated) or "inactive" (GDP-associated) state. The activation state of p21^{ras} can be controlled by the activity of a GTPase activating protein, designated as p120 ras.GAP. ras.GAP potentiates an increase in the intrinsic GTPase activity of p21^{ras} causing p21^{ras} to hydrolyze its associated GTP to GDP. Gold et al. (8) recently reported that mouse and human Ag-nonspecific B cells polyclonally activated by anti-Ig undergo an increase in the tyrosine phosphorylation of ras.GAP and its associated p190 and p62 proteins that regulate p21^{ras} activation and ras.GAP-mediated signaling. This observation provided only indirect evidence that the ras.GAP/p21^{ras} pathway of activation is involved in sIg-mediated transmembrane signaling, since neither the relationship between tyrosine phosphorylation of ras.GAP and ras.GAP activity nor the activation of p21^{ras} was examined.

To further investigate the role of p21^{ras} and ras.GAP activities in sIg AgR-mediated signaling in B cells, we have used an Ag-specific murine B cell, reasoning that Ag is the natural ligand for the B cell AgR. Ag and anti-Ig may differentially effect B cell activation, e.g., anti-Ig reagents but not T cell–dependent Ags are able to induce B cell blastogenesis (9, 10). The TA3 7.9 transfected murine B lymphoma cells

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(designated as 7.9) used here express an anti-TNP–specific sIgM of the Sp6 idiotype, are surface $I^{d,k}$ positive and present TNP_6-OVA, a T cell–dependent Ag, about 600-fold more efficiently than OVA to OVA/I-A^d-specific T cells, indicating that they use this TNP-specific sIgM to internalize, process, and present Ag to appropriate T cells (11). 7.9 cells also undergo both Ag- and Ab-mediated transmembrane signaling. The signals generated by these two forms of B cell activation, although not identical, involve an elevation in $[Ca^{2+}]_i$ as well as the tyrosine phosphorylation of several specific substrates (10, 12, 13). In this study, we determined whether the signal initiated by either Ag– or anti-IgM–dependent sIg crosslinking in 7.9 cells involves the direct activation of the components of the $p21^{ras}$-dependent pathway. We demonstrate that engagement of the 7.9 B cell AgR by Ag or F(ab')$_2$ anti-IgM induces $p21^{ras}$ activation. This activation is associated with the rapid tyrosine phosphorylation and inhibition of activity of ras.GAP, and interestingly, does not involve the association of ras.GAP with a p190 protein. These results provide direct evidence that signaling through the AgR in B cell functions via a $p21^{ras}$/ras.GAP-dependent pathway, and suggest that the mechanism of regulation of ras.GAP and $p21^{ras}$ activities may differ according to the stage of differentiation of a B cell.

Materials and Methods

Cells. TA3 7.9, a murine B hybridoma cell line that expresses a TNP-specific sIg, was generated by transfection of parental TA3.14 D5.1 cells with plasmid pR-HL_{TNP} to derive the TA3.14 D5.1 3D3 7.9 subclone, designated as 7.9 (11). 7.9 B cells were maintained in RPMI-1640 medium supplemented with 5% FBS, as described (12). Rat-2 v-src transformed blasts were kindly supplied by Dr. M. Moran (C.H. Best Institute, University of Toronto) and were grown in DMEM supplemented with 10% fetal bovine serum, as described (14).

Assay of $p21^{ras}$ Activity and Its Rate of Guanine Nucleotide Exchange. $p21^{ras}$ activity in 7.9 B cells that were either unstimulated or stimulated by Ag or anti-μ was assayed by determination of the ras-bound GTP/GDP + GTP ratio in streptolysin-O (Wellcome Diagnostics, Greenville, NC) permeabilized cells, as described (15). To assay the rate of guanine nucleotide exchange by $p21^{ras}$, 7.9 B cells were permeabilized with streptolysin-O (0.5 U/ml) and α-[^{32}P]GTP (10 μCi; 3,000 Ci/mmol) in the presence or absence of TNP_6-OVA (1 μg/ml). The reaction was terminated by centrifuging the cells at 1,000 *g* for 30 s and resuspension of the cells in a lysis buffer, as described (6). $p21^{ras}$ was immunoprecipitated with the Y13–259 anti-p21v-H-ras mAb (Oncogene Science Inc., Manhasset, NY) or a control normal rat IgG (Jackson Immuno-Research Laboratories, Inc., West Grove, PA), and the ras-bound guanine nucleotides were eluted, separated by polyethyleneimine-cellulose thin layer chromatography and quantitated by liquid scintillation counting (15). The guanine nucleotide exchange rate was calculated as described (16) according to the following formula: ($p21^{ras}$ associated α-[^{32}P]GTP + α-[^{32}P]GDP in stimulated cells)/($p21^{ras}$ associated α-[^{32}P]GTP + α-[^{32}P]GDP in quiescent cells).

Immunoprecipitation. Lysates of 7.9 B cells (10^7/ml) or rat-2 cells (10^7/ml) were prepared, precleared with protein A–Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), immunoprecipitated with either a polyclonal rabbit anti-ras.GAP Ab (kindly provided by Dr. M. Moran), a rabbit anti-p190 Ab (kindly supplied by Dr. J. Settleman, Massachusetts General Hospital, Boston, MA) or a control preimmune normal rabbit serum (NRS) and protein A–Sepharose, washed, resolved by 10% SDS-PAGE, and Western blotted with the Py72 anti-phosphotyrosine mAb, as described (15).

Assay of ras.GAP Activity. The activity of ras.GAP in Ag-stimulated and -unstimulated 7.9 B cells was assayed after immunoprecipitation of lysates with either rabbit anti-ras.GAP Ab or NRS, and protein A-Sepharose, as described (15). GAP activity in unstimulated B cells (calculated as $p21^{ras}$-bound basal cpm) – ($p21^{ras}$-bound cpm in nonactivated cells) was taken to represent maximal (100%) GAP activity. The relative GAP activity in stimulated cells was calculated as follows: 100% × [($p21^{ras}$-bound basal cpm) – ($p21^{ras}$-bound cpm in stimulated cells)/($p21^{ras}$-bound basal cpm) – ($p21^{ras}$-bound cpm in unstimulated cells)].

Western Blots. For detection of ras.GAP, aliquots of lysates (10^7 cells/sample) were first immunoprecipitated with anti-ras.GAP and then heated to 85°C for 10 min in the presence of SDS-sample buffer (1% SDS, 2.5% 2-ME, 10% glycerol, and 62.5 mM Tris-HCl, pH 6.8), centrifuged at 12,000 *g* for 10 min, analyzed by SDS-PAGE (4–20% Tris-glycine gradient gels; Helixx Technologies, Scarborough, Ontario, Canada) and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). Membranes were immunoblotted with a 1/500 dilution of either polyclonal rabbit anti-ras.GAP antiserum or NRS for 4 h followed by ^{125}I-labeled protein A (1 μCi/ml; Amersham Corp., Arlington Heights, IL) for another 4 h, and were then dried and autoradiographed for 24 h (15).

Results and Discussion

The presence of $p21^{ras}$ in Ag-specific 7.9 B cells was investigated by Western blot analysis using the Y13.259 mAb that recognizes all forms of $p21^{ras}$ (17). These B cells were found to express $p21^{ras}$ (data not shown). This anti-ras mAb also specifically immunoprecipitated $p21^{ras}$-bound guanine nucleotides after permeabilization of the B cells with streptolysin-O in the presence of α-[^{32}P]GTP. The predominant nucleotide bound to $p21^{ras}$ in unstimulated cells was GDP (Fig. 1 *A*). Stimulation of 7.9 cells for 2 min with TNP_6-OVA as Ag resulted in the accumulation of 30% (± 8%, n = 5) GTP, as compared with 6% GTP (± 4%, n = 5) in unstimulated cells. Similarly, stimulation of 7.9 B cells for 2 min with a polyclonal goat F(ab')$_2$ anti-IgM Ab also induced an accumulation of GTP significantly above basal levels. Cells treated with the control OVA carrier protein did not accumulate GTP above basal levels (data not shown).

The ability of $p21^{ras}$ to accumulate GTP may be regulated in part by its ability to increase its rate of exchange of bound guanine nucleotides (16, 18). Therefore, we examined whether $p21^{ras}$ increases its guanine nucleotide exchange rate in response to Ag. 7.9 B cells were stimulated with TNP_6-OVA (1 μg/ml) for various times in the presence of streptolysin-O and α-[^{32}P]GTP. After stopping the reactions by immunoprecipitation of $p21^{ras}$, total ^{32}P-labeled guanine nucleotide radioactivity associated with $p21^{ras}$ was determined. The rate of guanine nucleotide exchange increased only marginally, i.e., 1.3-fold (± 0.3, n = 4), in stimulated B cells (Fig. 1 *B*). Since other cell types that utilize guanine nucleotide exchange to regulate $p21^{ras}$ activity generally undergo a two-

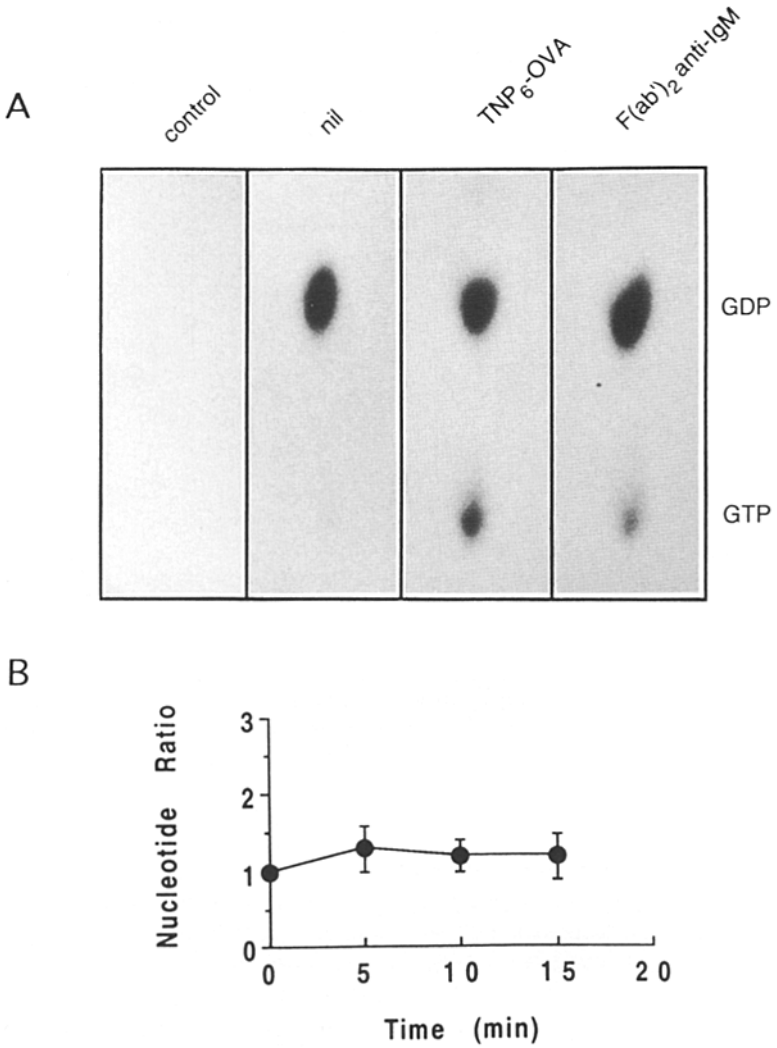

Figure 1. (*A*) $p21^{ras}$ is activated in 7.9 B cells stimulated by either Ag or $F(ab')_2$ anti-IgM. TNP-specific 7.9 B cells (5×10^7 cells/ml) were incubated for 2 min at 37°C in the absence (lanes *1* and *2*) or presence (lane *3*) of either TNP_6-OVA (1 μg/ml) or $F(ab')_2$ anti-IgM Ab (15 μg/ml) (lane *4*). The cells were then permeabilized and labeled for 10 min at 37°C with α-[^{32}P]GTP. $p21^{ras}$ was immunoprecipitated with either the Y13-259 mAb (lanes *2–4*) or normal rat IgG (lane *1*), and $p21^{ras}$-bound guanine nucleotides were then eluted and autoradiographed. (*B*) Rate of guanine nucleotide exchange is not increased significantly during $p21^{ras}$ activation in Ag stimulated 7.9 B cells. 7.9 B cells were permeabilized with streptolysin-O and α-[^{32}P]GTP in the presence or absence of TNP_6-OVA (1 μg/ml). The ratio of ^{32}P-labeled GDP- plus GTP-bound $p21^{ras}$ in stimulated cells to that in unstimulated cells was quantitated at various times after Ag stimulation, as indicated. A representative experiment of three separate experiments is shown in *A* and *B*.

to sixfold increase in the exchange rate (15, 18), our results indicate that Ag-stimulated $p21^{ras}$ activation in 7.9 B cells is not controlled by an increase in the exchange rate of guanine nucleotides on $p21^{ras}$. A similar finding was previously reported for activated T cells (6).

Early B cell signaling involves the tyrosine phosphorylation of several substrates including p120 ras.GAP (7), whose activity controls the activation state of $p21^{ras}$. To investigate the role of ras.GAP activity in Ag-mediated B cell transmembrane signaling, we determined whether Ag-induced B cell activation elicits a change in ras.GAP activity. ras.GAP was immunoprecipitated from unstimulated and Ag-stimulated 7.9 cells and was then tested for its enzymatic activity. Ag stimulation induced a strong inhibition of ras.GAP activity in these B cells, with maximum inhibition being detectable between 1 and 5 min after Ag stimulation (Fig. 2 *A*). This inhibition of ras.GAP activity persisted for at least 20 min. For all ras.GAP activity determinations, assay of each sample by immunoblotting with an anti-ras.GAP Ab demonstrated that the amount of immunoprecipitated ras.GAP did not vary significantly between samples (Fig. 2 *B*), and that changes in activity did not result from a decrease in the relative amount of immunoprecipitated ras.GAP. We also found that maximal tyrosine phosphorylation of immunoprecipitated ras.GAP occurred at 1 min after Ag stimulation and, interestingly, at 20 min after stimulation, tyrosine phosphorylation of ras.GAP was no longer detectable despite the fact that ras.GAP activity was strongly inhibited at this time. The latter results are consistent with the observations of Gold et al. (8) who demonstrated that tyrosine phosphorylation of ras.GAP occurs rapidly and transiently in anti-Ig stimulated B cells. The independence of tyrosine phosphorylation and ras.GAP activity has also been observed in other cell types (14). Thus, whereas tyrosine phosphorylation of ras.GAP does not appear to be correlated with its activity in B cells, the in vivo inactivation of ras.GAP may require an initial tyrosine phosphorylation event. Early phosphorylation of ras.GAP would also likely facilitate its interaction with other src-homology region 2 (SH_2) containing signaling proteins (8, 19). ras.GAP possesses two SH_2 domains, which allow it to bind a 62-kD protein that is thought to be involved in mRNA processing (20). ras.GAP also interacts with another protein, p190, that possesses sequence homology with a transcriptional repressor (21). Thus, the inhibition of ras.GAP activity in Ag-stimulated

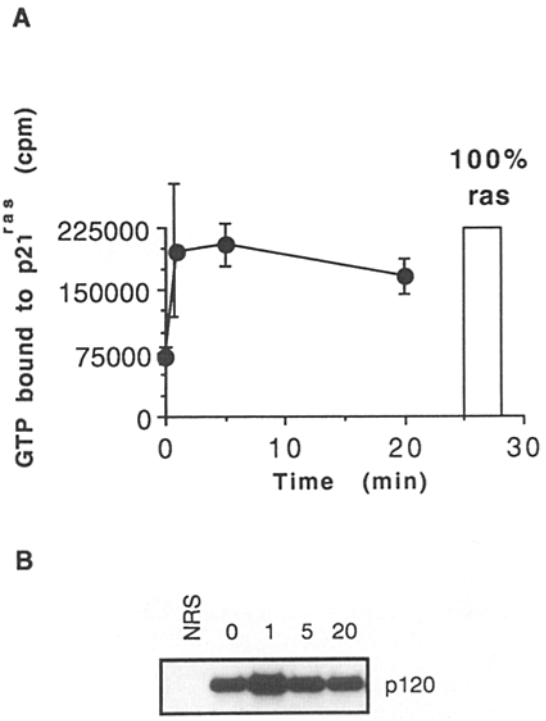

Figure 2. (*A*) Ag stimulation of 7.9 B cells induces inhibition of ras.GAP activity. 7.9 B cells were activated by TNP_6-OVA (1 μg/ml) for either 0, 1, 5, or 20 min. At each time after activation, cell lysates were immunoprecipitated with rabbit anti-ras.GAP or NRS. Washed immunoprecipitates were resuspended for 10 min at 25°C in the presence of γ-[^{32}P]GTP-labeled p21^{ras} (1 μg), and p21^{ras}-bound radioactivity was monitored by β-scintillation counting. The open bar indicates the cpm of GTP bound to p21^{ras} in the absence of ras.GAP. A representative experiment of three experiments is shown. (*B*) Ag-induced changes in ras.GAP activity do not result from changes in relative amounts of ras.GAP. Aliquots of each immunoprecipitated sample in (*A*) were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-ras.GAP. NRS denotes a control immunoprecipitation performed with NRS on a lysate prepared from unstimulated 7.9 cells. Samples denoted 0, 1, 5, and 20 were prepared by immunoprecipitation of 7.9 cell lysates after Ag stimulation for 0, 1, 5, and 20 min, respectively. One representative experiment of three is shown.

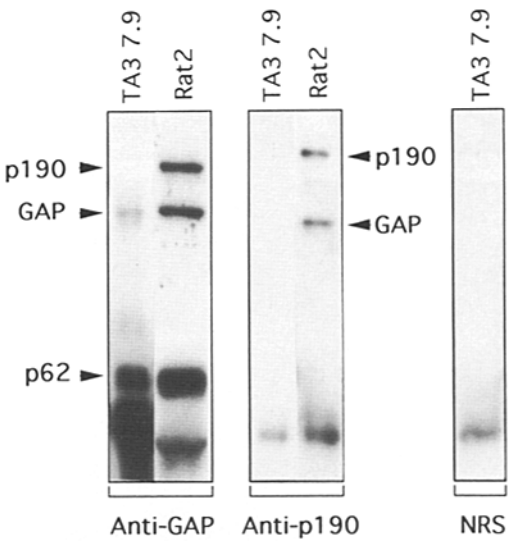

Figure 3. Tyrosine phosphorylation of ras.GAP-associated proteins. 7.9 B cells (10^7/ml) were stimulated with TNP_6-OVA (1 μg/ml), lysed, and immunoprecipitated with either rabbit anti-ras.GAP (lane *1*), rabbit anti-p190 (lane *3*), or NRS (lane *5*). In positive control experiments, rat-2 fibroblasts (10^7/ml) were lysed and immunoprecipitated with either rabbit anti-ras.GAP (lane *2*) or rabbit anti-p190 (lane *4*). Lysates were analyzed by 10% SDS-PAGE and immunoblotted with the Py-72 anti-phosphotyrosine mAb.

B cells may not only control p21^{ras} activation but may also play an important role in the activation of gene transcription by the p62 and p190 proteins.

Despite this proposed role for p190, and although we can detect p190 in anti-p190 Western blots of total SDS lysates of quiescent 7.9 B cells, p190 was either extremely faint or not detectable in anti-ras.GAP immunoprecipitates of [^{35}S]methionine labeled lysates of Ag-stimulated or -unstimulated 7.9 B cells (our unpublished observations). Moreover, we have been unable to identify p190 as a tyrosine phosphoprotein in immunoblots of anti-ras.GAP immunoprecipitates of Ag stimulated 7.9 cell lysates (Fig. 3). These results demonstrate that p190 either does not associate or associates very weakly with ras.GAP in these activated B cells. In contrast, the association of ras.GAP with tyrosine phosphorylated p190 is readily detectable in rat-2 v-src transformed fibroblasts (Fig. 3). In addition, tyrosine phosphorylated p62 coimmunoprecipitates with ras.GAP from lysates of both Ag-activated 7.9 B cells and rat-2 fibroblasts under these conditions (Fig. 3). Our unexpected findings with the lack of association between p190 and ras.GAP may arise from the fact that TA3 7.9 is a murine B cell lymphoma line representative of a mature sIgM-bearing B cell, since the association of p190 with ras.GAP appears to be considerably weaker in mature than immature B cells (8). Association of p190 with ras.GAP inactivates ras.GAP and enables the activation of p21^{ras}. In the absence of formation of a p190/ras.GAP complex, ras.GAP activity would therefore not be expected to be inhibited and this would lead to p21^{ras} activation. Since we have found that Ag stimulates the inhibition of ras.GAP activity and concomitant activation of p21^{ras} in 7.9 B cells, this raises the possibility that association of p190 with ras.GAP plays a lesser role in the inhibition of ras.GAP and activation of p21^{ras} in more highly differentiated 7.9 B cells.

In conclusion, engagement of the B cell sIgM receptor by Ag induces a signal transduction pathway that includes the activation of p21^{ras}. This activation is associated with the rapid tyrosine phosphorylation and inhibition of activity of ras.GAP, a known regulator of p21^{ras}. The efficiency of binding of p190 to ras.GAP and the role of this p190/ras.GAP complex in p21^{ras} activation may depend on the stage of differentiation of a B cell.

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