Surface Immunoglobulin-mediated Signal Transduction Involves Rapid Phosphorylation and Activation of the Protooncogene Product Raf-1 in Human B-Cells

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ABSTRACT

The protooncogene product, Raf-1, is a serine/threonine kinase and has been implicated as an intermediate in signal transduction mechanisms. We examined neoplastic and normal B cells for phosphorylation and activation of Raf-1 protein in response to anti-immunoglobulin antibody (anti-Ig). Anti-Ig-induced rapid phosphorylation of Raf-1 protein in both neoplastic B-cells of hairy cell leukemia and normal tonsillar B-cells which proliferated well in response to anti-Ig. The increase in phosphorylated protein was due primarily to an increase in phosphoserine. The immune complex assay using Histone V-S as an exogenous substrate also showed an increase in Raf-1-associated kinase activity. An inhibitor of protein kinase C, H7, inhibited the proliferation as well as the Raf-1 phosphorylation in response to the proliferative signal of anti-Ig. Further, downregulation of protein kinase C by the treatment with 12-phorbol 13-myristic acid significantly abrogated the induction of Raf-1 phosphorylation. These results suggest that, in human B-cells, Raf-1 protein may be involved in the signal transduction pathway mediated by surface immunoglobulin, and that it may be, at least partially, phosphorylated by activated PKC.

INTRODUCTION

Anti-Ig have been used to prepare models of activation of B-cells (1). Cross-linkage of the s-Ig under appropriate conditions results in the delivery of an activation signal to the B-cells, which induces them to enter into cell cycle. As for the signal transduction system of B-cells through s-Ig, the breakdown of inositol phospholipids and generation of inositol triphosphate and DAG have been reported (2). The inositol triphosphate causes an increase of intracellular Ca2+ concentration, and activation of Raf-1 seems to be mediated, at least partially, through PKC.

In murine fibroblasts, Raf-1 has been reported to be rapidly phosphorylated on serine and tyrosine in response to PDGF (8, 9) and, thus, has been linked to the signal transduction of several growth factor receptors (10). In hematopoietic cells, we and other authors reported activation of Raf-1 protein in response to various hematopoietic growth factors such as GM-CSF, IL-3, and colony-stimulating factor 1 (11-14). Raf-1 is a serine/threonine kinase and is known to be activated by phosphorylation. Thus, it is considered that the phosphorylation of Raf-1 may be a common intermediate step in the transduction of proliferative signals (10). However, in B-cells, it has not been reported whether the phosphorylation of Raf-1 is involved in the s-Ig-mediated signal transduction pathway.

In this study, we examined the expression and phosphorylation state of Raf-1 in normal and neoplastic B-cells in response to anti-Ig. Anti-Ig was found to induce rapid phosphorylation of Raf-1 in HCL cells and normal B-cells, and phosphorylation and activation of Raf-1 seemed to be mediated, at least partially, through PKC.

MATERIALS AND METHODS

Reagents. F(ab')2 fragments of goat anti-Ig Poly and anti-IgG (heavy chain specific) were purchased from N. L. Cappel Laboratories, Inc. (Cocharville, PA). Alkaline phosphatase from calf intestine was purchased from Boehringer Mannheim (Indianapolis, IN). Protein A-Sepharose CL4B was obtained from Pharmacia (Uppsala, Sweden). Rabbit anti-Raf-1 antiseraum was generated against a synthetic peptide as previously described (14). Anti-phosphotyrosine monoclonal antibody was generated by using phosphotyramine as the immunogen. This antibody is specific for phosphotyrosine as described previously (15, 16). H7 was purchased from Seikagaku Kogyo (Tokyo, Japan).

Cells. Two patients of 19 with B-CLL and two patients with HCL, described in the previous report (7), were included in this study. The diagnoses were confirmed as reported (7). Types of s-Ig in the two cases of B-CLL were µ, δ, and λ and µ and λ, respectively; they were γ and λ in both HCL cases. Cell suspensions from tonsils resected from patients with chronic tonsillitis were obtained by gentle teasing. Mononuclear cells were separated from the heparinized venous blood or cell suspensions by flotation in Ficoll sodium diatrizoate (LSM; Litton Bionetics, Kensington, MD) and washed 3 times in Hanks' balanced salt solution. The cells were divided into aliquots, frozen with programmable freezer, and cryopreserved in liquid nitrogen as reported previously (7). For use, the cells were thawed rapidly at 37°C, diluted slowly, and washed 3 times with Hanks' balanced salt solution. The viability of the re-
covered cells was greater than 90% as determined by the trypan blue exclusion test. In each preparation of mononuclear cells from the patients, the proportion of neoplastic cells was more than 95%. For tonsillar cells, B-cells were enriched to more than 95% by deletion of T-cells rosetting with neuraminidase-treated sheep erythrocytes.

Cell Proliferation Assay. The mononuclear cells from HCL Case 1 were cultured at 1 x 10^6/ml in 200 µl of RPMI 1640 supplemented with 10% fetal bovine serum in 96-well plates in the presence or absence of anti-IgG or anti-Ig Poly (100 µg/ml). In some experiments, various concentrations of PKC inhibitor, H7, were used to examine the effects of PKC on anti-Ig-induced proliferation and phosphorylation of Raf-1. Cells were cultured for 72 h, and proliferation was assessed by[^H] thymidine incorporation as described previously (7, 16).

Stimulation with Anti-Ig and Cell Lysis. The mononuclear cells from patients and the B-cell-enriched fraction of tonsillar cells were washed free of serum and incubated in serum-free RPMI 1640 containing 0.5% BSA for 2 h. The cells were then exposed to anti-IgG or anti-Ig Poly (100 µg/ml) at 37°C for 0 to 120 min. For downregulation of PKC, HCL cells (Case 1) were cultured in RPMI 1640 supplemented with 2% fetal calf serum for 24 h in the presence of PMA (10 ng/ml; Sigma) or control medium containing 0.01% DMSO, washed free of serum, and then stimulated with anti-IgG for 15 min. After stimulation, cells were washed with cold phosphate-buffered saline and lysed in 20 mM Tris-HCl (pH 8.0):137 mM NaCl:0.5% sodium deoxycholate:1% Nonidet P-40 containing protease and phosphatase inhibitors [1 mM PMSF (Sigma, St. Louis, MO), 0.15 unit/ml of aprotinin (Sigma), 10 mM EDTA, 10 µg/ml of leupeptin (Sigma), 100 µM sodium fluoride, and 2 mM sodium orthovanadate] at 4°C for 20 min. Insoluble materials were removed by centrifugation at 4°C for 15 min at 10,000 x g. Total protein content of the lysate was determined by the Bio-Rad protein assay.

Cet Electrophoresis and Immunoblotting. Lysates (120 µg) were mixed 1:1 with 2X SDS sample buffer with 2-mercaptoethanol and heated at 100°C for 5 min prior to SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred from the gel onto a 0.2-mm nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) and immunoblotted with anti-Raf-1 antibody as described previously (14). Specificity of the anti-Raf-1 antibody was evaluated by blocking with the immunizing peptide (CTLTTSPRLPVF) as described previously (14). Raf-1 protein was performed as described previously (14). Briefly, HCL cells were treated with anti-IgG for 15 min, and the lysates from stimulated or unstimulated cells (10^6/cells) were used for Raf-1 immunoprecipitation. For phosphatase treatment, the Raf-1 immunoprecipitates were washed 5 times with 100 mM Tris (pH 9.5):100 mM NaCl:5 mM MgCl2 (alkaline phosphate reaction buffer) containing protease inhibitors (1 mM PMFSF, 10 µg/ml of leupeptin, and 0.15 unit/ml of aprotinin) and then incubated with calf intestine alkaline phosphatase (600 units/ml) for 1 h at 37°C. After washing, treated and untreated immunoprecipitates were electrophoresed on SDS-PAGE and transferred to nitrocellulose filters. Raf-1 proteins were detected by probing the blot with anti-Raf-1 antibody.

Labeling of the Cells with [32P]Orthophosphate and Phosphoamino Acid Analysis. HCL cells were washed 3 times with phosphate-free RPMI 1640 containing 0.5% BSA and cultured for 2 h in the phosphate-free medium. After 2 h of incubation, the cells (10^6) were washed once with the phosphate-free medium and then incubated for a further 2 h in this medium containing 1 mCi (37 MBq) of carrier-free [32P]orthophosphate (Amersham, Arlington Heights, IL). Labeled cells were cultured with or without anti-IgG (100 µg/ml) for 15 min and lysed in Nonidet P-40 buffer with protease and phosphatase inhibitors. Raf-1 proteins were immunoprecipitated, separated by 7.5% SDS-PAGE, and visualized by autoradiography. The phosphoamino acid composition of [32P]-labeled bands excised from the SDS-PAGE gel was determined as described previously (14). Phosphoserine, phosphothreonine, and phospho-tyrosine controls (Sigma) were detected by reaction with ninhydrin, and the radioactive amino acids were detected by autoradiography.

Immune Complex Kinase Assay. Raf-1 was immunoprecipitated with anti-Raf-1 antibody from lysates of control (without anti-Ig) or anti-Ig-stimulated cells. The immune complexes were collected on Protein A-Sepharose beads, washed, and incubated in a kinase buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4):1 mM dithiothreitol:10 mM MgCl2;25 µM ATP] containing 10 µCi/ml of [γ-32P] ATP for 20 min at 37°C. The immune complexes were then again washed and separated by SDS-PAGE. In some experiments, Histone type V-S (5 µg/50-µl sample; Sigma, St. Louis, MO) was added as an exogenous kinase substrate.

RESULTS

Anti-Ig Induces Altered Migration of Raf-1 in Neoplastic B-Cells. Neoplastic B-cells from HCL Case 1 were analyzed as a monoclonal model of cells which proliferate in response to anti-IgG. The cells were treated with medium alone or anti-IgG at a concentration of 100 µg/ml, which was shown to be sufficient to induce proliferation of the HCL cells (7), and lysed; then an equal aliquot of protein was analyzed by SDS-PAGE. Raf-1 was detected by immunoblotting with a polyclonal antibody generated against a Raf-1 synthetic peptide. This antibody detected a major band at a molecular weight of approximately 74,000. After treatment with anti-Ig, more slowly migrating forms of Raf-1 were detected as early as 1 min. The change of the migration peaked at 10 to 30 min and slightly diminished at 2 h (Fig. 1). Reactivity of the anti-Raf-1 antibody with the M, 74,000 and slowly migrating forms could be completely blocked by the addition of Raf-1 synthetic peptide, indicating that those bands contained authentic Raf-1. In agreement with previous results in myeloid cells (14), the slower migrating forms of Raf-1 were eliminated by treatment with alkaline phosphatase (Fig. 2). These results suggested that the slower migration of Raf-1 is due to an increased phosphorylation.

In Vivo Labeling and Phosphoamino Acid Analysis. In order to directly demonstrate the increase in phosphorylation of Raf-1 in response to anti-Ig, HCL cells were labeled in vivo with [32P]orthophosphate and treated media alone or anti-IgG for 15 min. The cells were then lysed, and Raf-1 was detected by immunoprecipitation, SDS-PAGE, and autoradiography. The treatment with anti-IgG increased [32P] incorporation into Raf-1 (Fig. 3A). In addition, phosphoamino acid analysis was performed to determine which amino acids were phosphorylated in Raf-1 (Fig. 3B). Phosphoserine was the predominant phosphoamino acid in Raf-1 before stimulation with anti-IgG. After anti-Ig stimulation, increased phosphorylation was observed.

![Fig. 1. Altered migration of Raf-1 in HCL cells induced by anti-Ig stimulation.](image-url)

HCL cells from Case 1 were stimulated with anti-IgG (100 µg/ml) for 0 to 120 min at 37°C. The cells were then lysed, and equal aliquots of protein (120 µg) were analyzed by SDS-PAGE. Raf-1 was detected by immunoblotting with anti-Raf-1 antibody. Molecular standards were BSA (M, 84,000) and ovalbumin (M, 47,000).
Fig. 2. Effects of phosphatase treatment on electrophoretic mobility of Raf-1 immunoprecipitated from anti-IgG-stimulated HCL cells (Case 1). Raf-1 was immunoprecipitated from lysates of stimulated or unstimulated cells and then incubated with calf intestine alkaline phosphatase (600 units/ml) for 1 h at 37°C. After washing, the immunoprecipitates were electrophoresed by SDS-PAGE and transferred to nitrocellulose filters. Raf-1 proteins were detected by anti-Raf-1 antibody. HCL cells were treated with anti-IgG (100 μg/ml) for 15 min.

Anti-Ig-induced Phosphorylation of Raf-1 in Normal and Other Neoplastic B-Cells. In order to determine if Raf-1 phosphorylation is induced in normal and neoplastic B-cells, the cells were treated with anti-IgG in HCL cells or anti-Ig Poly in B-CLL cells and tonsillar B-cells for 15 min and examined for phosphorylation of Raf-1 by immunoblot as described above. The concentration of anti-Ig was sufficient to induce proliferation in HCL cells and normal B-cells as reported in previous study (7). After treatment with anti-Ig, the slower migrating forms of Raf-1 were distinctly observed in HCL cells from two patients, in tonsillar B-cells used as a source of normal B-cells, and in a case (Case 1) of two B-CLL patients, albeit to a lesser degree (Fig. 4). By contrast, the change of Raf-1 migration was barely observed in B-CLL cells (Case 2) following treatment with anti-Ig (Fig. 4). This result roughly coincides with the previous findings (7) that B-CLL cells showed lower proliferative response to anti-Ig stimulation than did HCL cells.

Immune Complex Kinase Assay. Raf-1 was immunoprecipitated from leukemic cells of HCL Case 1 before and 1, 15, and 30 min after treatment with anti-IgG, and then kinase activity was assayed with histone type V-S as an exogenous kinase substrate (Fig. 5). An increase of kinase activity was detected at 15 min and reached its highest point at 30 min, despite a slight decrease after 1-min stimulation. Autophosphorylation of Raf-1 was not detected before or after stimulation with anti-IgG (data not shown).

PKC Inhibitor Inhibits Anti-Ig-induced Proliferative Response and Phosphorylation of Raf-1. Leukemic cells from HCL (Case 1) were cultured with anti-IgG or anti-Ig Poly in the presence or absence of the PKC inhibitor, H7, and the effect of H7 on anti-IgG-induced proliferation was determined by [3H]thymidine incorporation. As shown in Table 1, anti-IgG and anti-Ig Poly had a similar effect on induction of HCL cell proliferation, and anti-IgG-induced proliferation of HCL cells was partially or completely inhibited by H7 at a concentration of 10 μM or 20 μM, respectively.

In addition, in order to assess whether or not PKC is involved in the phosphorylation of Raf-1, the cells were pretreated for 2 h with H7 and stimulated with anti-Ig; Raf-1 immunoblot was conducted (Fig. 6). H7 reduced the density of the slower migrating bands at concentrations of 10 and 20 μM. Further, HCL cells were treated with PMA for 24 h to downregulate PKC, and anti-Ig-induced phosphorylation of Raf-1 was examined. Anti-IgG induced Raf-1 phosphorylation after cultivation with control medium, whereas Raf-1 phosphorylation was not detectable in PMA-treated cells (Fig. 7). These results suggest that PKC may be involved, at least partially, in phosphorylation of Raf-1 as well as in anti-Ig-induced proliferation.

**DISCUSSION**

Raf-1, the product of c-raf-1 protooncogene, is known to encode a Mr 74,000 cytoplasmic serine/threonine protein kinase (8, 9, 10). Raf-1 protein is now considered to be phosphorylated and activated by kinases, which have been activated by early signals, and to transduce further signals which lead to the cellular responses (10). However, the expression, function, and phosphorylation of Raf-1 have not been studied in B-cells in any detail.

In the present studies, we have shown that Raf-1 protein is expressed in human B-cells and that it is rapidly phosphorylated by the proliferative signal through s-Ig in human B-cells. We previously examined the proliferative response of neoplastic B-cells to anti-Ig and showed a striking proliferative response of neoplastic B-cells from two patients with HCL and poor response of those from 19 B-CLL patients (7). The neoplastic cells from these patients will serve as a monoclonal model of the B-cell subset which proliferates in response to anti-Ig. In most experiments, therefore, we used neoplastic cells of HCL as a monoclonal model of a B-cell subset which proliferate in response to anti-Ig.

![Anti-IgG](image_url)
Fig. 4. Effects of anti-Ig stimulation on phosphorylation of Raf-1 in normal and neoplastic B-cells. Neoplastic B-cells (HCL, 2 cases; B-CLL, 2 cases) and normal tonsillar B-cells were stimulated with anti-Ig (100 µg/ml of anti-IgG in HCL cells or anti-Ig Poly in B-CLL and tonsillar cells) for 15 min at 37°C. The cells were then lysed, and Raf-1 was detected by immunoblot.

![Raf-1 in Human B-Cells](Image)

**Fig. 5.** Immune complex kinase assay for Raf-1-associated kinase activity. Raf-1 was immunoprecipitated from HCL cells (Case 1) treated with anti-Ig (100 µg/ml) for 0 to 30 min. The immune complexes were collected on Protein A-Sepharose beads, washed, and incubated with histone type VS (5 µg/sample in 50 µl) and 10 µCi of [γ-32P]ATP for 20 min at 37°C. The immune complex-histone mixture was separated by SDS-PAGE. Incorporation of 32P in histone was visualized by autoradiography. An increase of kinase activity was detected at 15 min and reached its highest point at 30 min. Autophosphorylation of Raf-1 was not detected up to 30 min.

![Immune Complex Kinase Assay](Image)

**Table 1** Effects of H7 on anti-immunoglobulin-induced proliferation of HCL cells

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>cpm</th>
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<tr>
<td>Medium</td>
<td>143 ± 43*</td>
</tr>
<tr>
<td>Anti-Ig Poly</td>
<td>16,399 ± 1,873</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>18,523 ± 1,754</td>
</tr>
<tr>
<td>Anti-IgG + H7 (10 µM)</td>
<td>3,908 ± 468</td>
</tr>
<tr>
<td>Anti-IgG + H7 (20 µM)</td>
<td>122 ± 39</td>
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* Mean ± SD of triplicate values.
* One hundred µg/ml.

The immune complex kinase assay showed that treatment of HCL cells with anti-Ig resulted in an increase in phosphorylation of Histone V-S which was used as an exogenous substrate. This result is comparable to previous findings in murine 3T3 fibroblasts treated with other growth factors (8, 9). How Raf-1 might function in this increase in phosphorylation of Histone V-S remains to be elucidated at this time, since it is not fully understood yet how Raf-1 is activated, what substrate(s) is phosphorylated by activated Raf-1, nor what molecule(s) is associated with Raf-1. It is possible that Raf-1-associated kinase immunoprecipitated with anti-Raf-1 antibody may be responsible for the phosphorylation of the exogenous substrate. Nevertheless, the fact that phosphorylation of Raf-1 is roughly associated with an increase in Raf-1 or Raf-1-associated kinase suggests that Raf-1 might be involved, at least in part, in the sIg-induced signal transduction pathway.

Although anti-Ig stimulation can rapidly activate tyrosine kinase, the mechanism by which this occurs is still not fully understood. The results presented here suggest that Raf-1 may play a role in this process, although further studies are needed to confirm this hypothesis.

![Figure 6](Image)

![Figure 7](Image)
kinase in murine B-cells (5, 6), tyrosine residues were not major sites of phosphorylation. The increase in phosphorylation of Raf-1 was primarily because of an increase in phosphoserine. This result is similar to previous findings in other growth factors such as GM-CSF and IL-3 (14). Interestingly, the PKC inhibitor, H7, partially inhibited Raf-1 phosphorylation as well as the proliferation induced by anti-Ig stimulation. In addition, anti-Ig could not induce Raf-1 phosphorylation in PKC-depleted HCL cells. These results suggest that PKC might be one of the kinases responsible for Raf-1 phosphorylation in human B-cells. Recently, similar findings were reported in T-cells, that the proliferative signal mediated by T-cell antigen receptors induces phosphorylation and activation of Raf-1 via a PKC-dependent pathway but not via tyrosine kinase associated with T-cell antigen receptors (17). In the signal transduction pathway of PDGF, however, it was reported that Raf-1 is associated with PDGF receptor and directly phosphorylated by the tyrosine kinase of the PDGF receptor (8, 9). The difference in the mechanisms of Raf-1 phosphorylation might be attributable to the characteristics of receptor or cell type.

In addition to neoplastic B-cells from HCL patients, normal B-cells obtained from tonsils also proliferated in response to anti-Ig, and phosphorylation of Raf-1 protein was induced in these cells with anti-Ig treatment. In this study, we used tonsillar cells obtained from patients with chronic tonsillitis, and consequently some activation of B-cells might already have taken place. Indeed, a faint phosphorylated band was already seen in these cells before stimulation with anti-Ig. The weakness in the induction of Raf-1 phosphorylation in tonsillar B-cells may be attributable to the activation state of the cells before the stimulation with anti-Ig or to the low proportion of cells which react with anti-Ig. In contrast to HCL cells, anti-Ig did not stimulate proliferation of B-CLL cells (7), nor induce prominent phosphorylation of Raf-1 in these cells following treatment with anti-Ig. The unresponsiveness of B-CLL cells to anti-Ig could be explained by their derivation from a B-cell subset which does not respond to anti-Ig or by a neoplastic change which made them refractory to anti-Ig stimulation because of a defect in the signal transduction pathway.

In summary, our results implicate Raf-1 as part of a kinase cascade involved in signal transduction through s-Ig on human B-cells. It is not known yet how Raf-1-associated kinase are regulated, or which residues are critical for Raf-1 activation. Identification of the in vivo substrates of Raf-1 kinase will provide a clue to further understanding of growth regulation and differentiation of B-cells.

REFERENCES

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