Evidence for Defective Transmembrane Signaling in B Cells from Patients with Wiskott–Aldrich Syndrome

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Abstract

B lymphocytes from patients expressing the X chromosome–linked immune deficiency disorder, Wiskott–Aldrich syndrome (WAS), fail to produce antibodies in response to stimulation with polysaccharides and other type-2 T cell–independent antigens. To investigate whether this abnormality reflects a defect in the signal transduction cascade normally triggered by ligation of surface immunoglobulin (sIg) on B cells, we have examined early signaling events induced by anti-Ig antibody stimulation of EBV B lymphoblastoid cell lines from WAS patients and healthy controls. Despite the expression of comparable levels of sIg and sIgM on WAS and control EBV B cells, WAS cells failed to manifest the increased proliferation in response to anti-Ig treatment observed in the control cell lines. WAS and control EBV B cells also differed in the magnitude of the change in cytosolic free calcium ($[Ca^{2+}]_i$) induced by sIg ligation; WAS cells showed either markedly diminished or no changes in $[Ca^{2+}]_i$ levels whereas control EBV B cells consistently showed increases in $[Ca^{2+}]_i$. Anti-Ig–induced changes in inositol phosphate release were also markedly reduced in WAS compared with control cells. As protein tyrosine phosphorylation is thought to represent a proximal event in the activation of B cells, inducing increases in $[Ca^{2+}]_i$ by virtue of tyrosine phosphorylation of phospholipase C (PLC)-γ, profiles of protein tyrosine phosphorylation and expression of tyrosine-phosphorylated PLC-γ1 were compared between WAS and normal EBV B cells before and after sIg cross-linking. These studies revealed that in addition to defective mobilization of Ca^{2+}, the WAS cells manifested little or no increase in tyrosine phosphorylation of PLC-γ1 or other intracellular proteins after sIg ligation. Together these results indicate the association of WAS with a defect in the coupling of sIg to signal transduction pathways considered prerequisite for B cell activation, likely at the level of tyrosine phosphorylation. The abnormalities observed in these early transmembrane signaling events in WAS EBV B cells may play a role not only in the nonresponsiveness of WAS patient B cells to certain T independent antigens, but also in the genesis of some of the other cellular deficits exhibited by these patients. (*J. Clin. Invest.* 1992. 90:1396–1405.)
Key words: B cell signal transduction • immunodeficiency • Wiskott-Aldrich syndrome

Introduction

The Wiskott–Aldrich syndrome (WAS)[1] is an X chromosome–linked recessive disorder associated with severe thrombocytopenia, eczema, impaired humoral and cellular immunity, and increased susceptibility to lymphoid malignancies (1, 2). The immune abnormalities are variably expressed among affected boys, but the most characteristic include absent antibody responses to polysaccharide antigens and a progressive decline in T cell functions, such as delayed hypersensitivity and allograft rejection (2–4). The molecular and biochemical defects responsible for WAS are unknown and the lack of such information has interfered with precise definition of the cellular basis for the immune deficit. Although this later facet of the syndrome was at one time ascribed primarily to an intrinsic defect of T cells, more recent data suggest that the B cells of WAS patients are also intrinsically abnormal. For example, compared with healthy controls WAS patients manifest reduced numbers of $CD23^+$ and increased numbers of $CD20^+$ $CD21^-$ B cells within their peripheral B lymphocyte populations (5). An intrinsic B as well as T cell defect in WAS is also implied, albeit indirectly, by the detection of nonrandom X chromosome inactivation patterns in both B and T lymphocytes of female WAS carriers (6, 7). Lastly, our recent finding of abnormal expression of a developmentally regulated glycosyltransferase activity in both EBV B lymphoblastoid and T cells from WAS patients suggests that the immune abnormalities observed in these patients reflect both B and T cell dysfunction and possibly a defect in lymphocyte maturation (8).

Although WAS patients are unable to produce antibodies to polysaccharides and other T cell–independent type 2 antigens, their B cells do respond to T cell–independent type 1 antigens, such as TNP–Brucella abortus (9). A similar pattern of immune responsiveness has been described in human neonatal B cells as well as in the B cells of CBA/N mice, a strain that carries an X chromosome–linked immunodeficiency (*xid*) mutation associated with the absence of a major subpopulation of mature B cells (10). In contrast to WAS, the *xid* mutation does not appear to affect T cells and platelets. However, the impaired maturation and hyporesponsiveness to type 2 antigens found in CBA/N *xid* male and homozygous female mice suggest that the effects of the *xid* and WAS mutations on B cells are similar and that data relating to the properties of *xid* B cells may provide clues into the basic cell defects underlying immune dysfunction in WAS.

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1. *Abbreviations used in this paper:* $[Ca^{2+}]_i$, cytosolic-free calcium concentration; PLC, phospholipase C; sIg, surface Ig; TBS, Tris-buffered saline; WAS, Wiskott–Aldrich syndrome; *xid,* X chromosome-linked immunodeficiency.

In recent years increased knowledge of the biochemical pathways linking antigen/mitogen stimulation to lymphocyte activation and proliferation has rendered examination of the molecular mechanisms whereby these cells respond or fail to respond to particular mitogenic stimuli possible. It is now understood, for example, that in normal B cells, cross-linking of surface immunoglobulin (sIg) by anti-Ig antibody triggers the activation of specific tyrosine kinases resulting in the rapid tyrosine phosphorylation of various intracellular proteins including phospholipase C (PLC) (11–13). Ligation of sIg on B cells also evokes hydrolysis of phosphatidylinositol biphosphate by the enzyme PLC with the consequent generation of inositol-triphosphate and diacylglycerol. These latter molecules serve as second messengers in this signal transduction pathway, diacylglycerol inducing the activation and membrane translocation of protein kinase C and inositol-triphosphate stimulating release of ionized calcium (Ca^{2+}) from intracellular stores (14). In normal B cells, the bifurcating phosphoinositide signaling pathway, in conjunction with protein tyrosine phosphorylation, represent key components of a cascade of intracellular events, triggered by sIg receptor engagement and culminating in cell activation. By contrast, recent data from several groups suggest that these early signaling events are impaired in B cells of CBA/N mice, such that cross-linking of sIg receptors induces markedly less inositol phospholipid hydrolysis and intracellular Ca^{2+} mobilization than that observed in normal B cells (15, 16). The basis for this signaling defect is unclear, although it appears to be associated with a quantitative or qualitative defect in PLC activity (16). In any case, these results raise the possibility that hyporesponsiveness of the transmembrane signaling apparatus may also occur in B cells of WAS patients and account for some of their immune dysfunction.

In this report, we have investigated transmembrane signaling events in anti-Ig–treated EBV immortalized B cell lines derived from WAS patients and healthy controls. The effect of anti-Ig stimulation on intracellular Ca^{2+} release, inositol phosphate production, and tyrosine phosphorylation was examined in light of recent data suggesting that the tyrosine phosphorylation of PLC is critical for sIg-induced activation of this enzyme and thus to the transduction of Ca^{2+}-mobilizing signals after cross-linking of B cell sIg (12, 13). The results of our studies indicate that the increases in cell proliferation, inositol phosphate production, and intracellular Ca^{2+} release that normally follow B cell sIg ligation are markedly reduced or absent in B cells of WAS patients. In addition, anti-Ig antibody–stimulated induction of tyrosine phosphorylation on intracellular proteins, including PLC-γ1, was diminished in WAS compared with control B cells. These findings suggest that a defect in the signal transduction cascade is present in WAS B cells and may underlie, at least in part, their unresponsiveness to polysaccharides and certain other mitogenic stimuli.

Methods

Media and stock solutions. Complete culture medium was RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 2 mM glutamine (Gibco), 5×10^2 M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO), gentamycin (Sigma Chemical Co.), and 10% FCS (Sterile Systems Inc., Logan, UT). Buffer A had the following composition (mM): 140 NaCl, 3 KCl, 1 $MgCl_2$, 10 glucose, 1 $CaCl_2$, and 20 Hepes, pH 7.23 (Sigma Chemical Co.). Ripa lysis buffer was 50 mM Tris, pH 7.2; 150 mM NaCl; 1% Nonidet P-40; 0.1% SDS; 0.1% sodium deoxycholate; 2 mM EDTA; 10 μM sodium orthovanadate; and 50 μM $ZnCl_2$. [^{3}H]Thymidine (6.7 Ci/mM) was purchased from New England Nuclear (Lachine, Quebec). Indo-1 was from Molecular Probes Inc. (Eugene, OR). Ionomycin was from Calbiochem Corp. (La Jolla, CA). Unless stated otherwise, all other reagents were from Sigma Chemical Co.

Patients and preparation of B cell lines. Peripheral blood was collected in heparin from 9 unrelated WAS patients, ranging in age from 2 to 25 yr, and from 16 age-matched healthy individuals. The nine WAS patients all showed the classical clinical triad of eczema, severe thrombocytopenia, and immune deficiency and each had a family history of this disorder. All patients were studied when not acutely ill or febrile. Mononuclear cells for the studies were obtained by density-gradient centrifugation over Ficoll-Hypaque. Because of difficulties in obtaining adequate numbers of freshly isolated B cells from WAS patients, most of the investigations of transmembrane signaling were performed using EBV-transformed B cell lines. EBV-immortalized cell lines were established by incubating 10^7 mononuclear cells with 5 ml supernatant of the B95-8 marmoset cell line at 37°C for 2 h. The cells were distributed in 24-well culture plates and cultured in complete culture medium and 0.5 mg/ml cyclosporine A. The cell lines were subsequently maintained in complete culture medium. Cells were cultured in RPMI 1640 without FCS for 24 h before analysis of tyrosine phosphorylation.

Immunofluorescent analysis. EBV B cells (1×10^6) were washed twice with staining solution (PBS plus 0.1% BSA), and then incubated with either rabbit anti–human Ig (IgM, IgG, IgA, IgG-fraction; Organon Teknika, West Chester, PA), monoclonal anti–human IgM (Becton Dickinson Immunocytometry Systems, Mountain View, CA), or rabbit anti–human CD45 (IgG-fraction; Hybritech, Inc., San Diego, CA) antibody. After the first incubation (30 min, 4°C), cells were washed twice with staining solution and were then incubated for 30 min at 4°C with FITC-conjugated goat anti–rabbit antibody (Gibco) or with FITC-conjugated goat anti-mouse antibody (Becton Dickinson Immunocytometry Systems). Background staining was assessed using a nonspecific primary antibody control (anti-TdT; Gibco) and was subtracted from values obtained on test samples. Alternatively, 1×10^6 EBV B cells were washed twice and stained directly by 30-min incubation at 4°C with either FITC-conjugated monoclonal anti-CD20 antibody or FITC-conjugated monoclonal IgG1 control antibody (Becton Dickinson Immunocytometry Systems). Cells were then washed twice, resuspended in 0.5 ml staining solution, and examined by flow cytometry (EPICS-C; Coulter Electronics Inc., Hialeah, FL). For cell sorting, 20×10^6 mononuclear cells separated from peripheral blood of each of two WAS patients and two controls were incubated with monoclonal FITC-conjugated anti-CD20 antibody in 0.5 ml staining solution and then applied to the cell sorter.

Proliferation assays. EBV B cells from patients and controls were resuspended at a concentration of 5×10^4 cells/ml in RPMI 1640 containing 1% FCS and cultured in 0.2-ml aliquots in flat-bottom microliter plates in the presence or absence of anti-Ig (at a final concentration of 5 μg/ml). Cultures were maintained for either 24, 48, 72, 96, or 120 h and the cells were pulsed with [^{3}H]thymidine (2 μCi/well) 4 h before termination of the incubation. [^{3}H]thymidine incorporation was measured using an automated β liquid-scintillation counter (Beckman). The maximal stimulation index was calculated as: cpm[^{3}H]-thymidine incorporation anti-Ig–treated cells/cpm[^{3}H]thymidine incorporation untreated cells.

Intracellular calcium measurements. Intracellular ionized calcium concentration ($[Ca^{2+}]_i$) was measured as previously described (17). Briefly, either EBV-transformed B cells or freshly isolated unseparated or sorted CD20-positive peripheral blood lymphocytes were suspended at $5–10 \times 10^6$/ml in RPMI 1640 with 10% FCS and 2.5 μg/ml indo-1 acetoxymethyl ester for 30 min at 37°C. Cells were then washed twice and resuspended at 1×10^6/ml in RPMI with 10% FCS. Immediately before analysis of $[Ca^{2+}]_i$, cells were washed in buffer A and suspended (10^6 cells in a volume of 1.7 ml) in a thermostatically controlled (37°C) cuvette. Fluorescence emission was measured in a spectrophotometer (Hitachi F 4000; Hitachi Ltd., Tokyo, Japan) using an excita-

tion wavelength of 331 nm and an emission wavelength of 410 nm. After equilibration of fluorescence to a stable baseline, the cells (10^6) were stimulated with anti-Ig (10 μl of a 1 mg/ml anti-Ig solution) and fluorescence assessment was continued. Dependence on extracellular Ca^{2+} was analyzed using EBV B cells suspended in nominally Ca^{2+}-free buffer A with 100 μM EGTA, a solution calculated to contain < 10 nM free Ca^{2+}. Each analysis was calibrated by addition of 5 μM ionomycin followed by 1 mM Mn^{2+}. $[Ca^{2+}]_i$ was calculated based on a K_m for Indo-1 at pH 7.2 of 180 as previously described (17).

In EBV B cells from healthy controls, peak $[Ca^{2+}]_i$ levels were generally attained within 1–2 min after anti-Ig antibody addition and were considered representative of the anti-Ig–induced change in cytosolic free calcium ($\Delta[Ca^{2+}]_i$). As this initial peak was frequently not detected after cross-linking of sIg on EBV B cells from WAS patients (see below), the peak anti-Ig–induced $\Delta[Ca^{2+}]_i$ in these latter cells was considered equivalent to the highest $[Ca^{2+}]_i$ value observed in the first 2 min after antibody addition (see Table II).

Assay for inositol phosphate production. Analysis of inositol phosphate release was performed as previously described (18, 19). Briefly, 5×10^7 EBV B cells were incubated at 37°C in RPMI 1640 and labeled with [^{3}H]myoinositol (100 μCi/cells; New England Nuclear, Boston, MA). After a 16-h incubation, cells were washed twice and incubated for a further 15 min at 37°C in DME containing 20 mM Hepes (pH 7.5), 25 mM LiCl, and 1 mg/ml BSA. Samples were then stimulated with 20 μg anti-Ig or medium for 3 min at 37°C. The reaction was terminated by addition of cold 5% perchloric acid and the inositol phosphates were extracted, diluted in 5 ml of H_2O, and applied to AG1-X8 (Bio-Rad, Mississauga, Ontario) columns. Columns were washed with 20 ml H_2O and a 20-ml solution of 5 mM sodium tetraborate and 60 mM ammonium formate. [^{3}H]inositol phosphates were eluted with a solution of 1.0 M ammonium formate and 0.1 M formic acid and quantified in duplicate samples by liquid-scintillation counting. Anti-Ig–induced release of inositol phosphates was calculated as: [^{3}H]inositol phosphate formation in anti-Ig–treated cells/[^{3}H]inositol phosphate formation in untreated cells.

Immunoprecipitation of PLC-γ1. After stimulation with 100 μg anti-Ig antibody, 1.7×10^8 EBV B cells were suspended in 1.5 ml cold Ripa lysis buffer and centrifuged at 10,000 *g* for 5 min (because this assay involved analysis of more cells than did the studies of Ca^{2+} levels, a greater amount of antibody was used to stimulate the cells). Supernatants were precleared with 0.8% (wt/vol) *Staphlyococcus aureus*-Cowan/strain A (Staph A; Calbiochem, San Diego, CA) for 30 min at 4°C and incubated for 1 h at 4°C with 30 μl of polyclonal rabbit anti-human PLC-γ1 antibody (a kind gift of Dr. T. Pawson, Samuel Lunenfeld Research Institute, Toronto, Ontario). Protein A-Sepharose 4B (Pharmacia, Baie d'Urfé, Quebec), prewashed three times in RIPA lysis buffer, was then added (1.3% vol/vol) and the samples incubated for another 1 h at 4°C. The immune complexes were washed in lysis buffer and pelleted by centrifugation three times at 10,000 *g* for 2 min at 4°C. The pellets were then boiled for 10 min and frozen.

Western blotting analysis. EBV B cells (3×10^6) were suspended in 500 μl of RPMI and incubated with 20 μg anti-Ig. Cells were then pelleted, lysed in Ripa lysis buffer, and boiled for 10 min. These lysates as well as the anti-PLCγ1 immunoprecipitates obtained as described above were then subjected to Western blotting as previously described (20). Briefly, the frozen pellets were thawed, centrifuged at 10,000 *g* for 5 min, and the proteins were fractionated electrophoretically in polyacrylamide–SDS gels. Proteins were then transferred overnight onto nitrocellulose papers. The unreacted sites on nitrocellulose blots were blocked overnight in a blocking solution of 10 mM Tris, 140 mM NaCl, and 0.01% NaN_3, pH 8.2, containing 5% BSA (Calbiochem). Blots were then incubated overnight with 1 μg of monoclonal PY-20 antiphosphotyrosine antibodies (ICN Biomedicals Inc., Costa Mesa, CA) in 4 ml of blocking solution. After washing, the nitrocellulose sheets were incubated for 1 h with rabbit anti–mouse antibodies (Western Blotting Associates, Mississauga, Ontario, Canada) and then subjected sequentially to two 10-min washes in 1× Tris-buffered saline (TBS), two 15-min washes in TBS containing 0.5% Nonidet P-40, and two 10-min washes in TBS. Finally, the filters were incubated for 1 h with 1 μCi of ^{125}I-labeled protein A (Amersham Corp. Oakville, Ontario, Canada) in 4 ml of blocking solution for 1 h, rewashed as before, dried, and exposed to XAR-5 film.

Results

Proliferative responses to anti-Ig are impaired in WAS B-lymphoblastoid cells. Before analysis of sIg-mediated intracellular signal transduction in WAS EBV B cells, B cell lines derived from WAS patients and healthy controls were compared with regards to their proliferative responses to anti-Ig antibody. As measured by [^{3}H]thymidine incorporation, proliferation of normal control EBV B cells increased markedly over the baseline level during the 5-d period after stimulation with anti-Ig antibody (Fig. 1). By contrast, over this same time period, EBV B cells from WAS patients showed little or no change in proliferation rate. Therefore, WAS EBV B cells appear refrac-

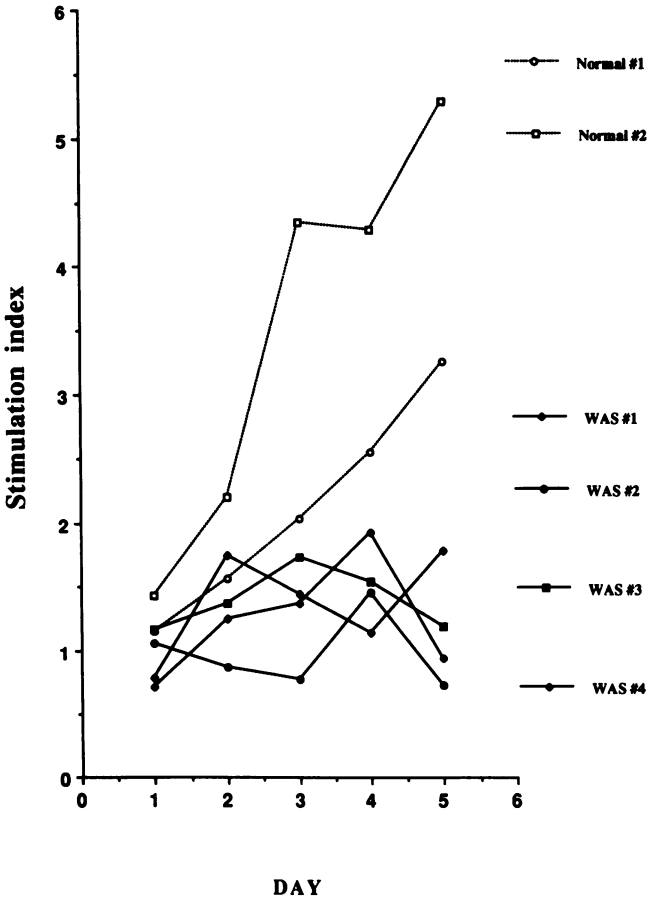

Figure 1. Effect of anti-Ig on proliferation of EBV-transformed B cells from normal individuals and from patients with WAS. Cells cultured in RPMI + 1% FCS at a density of 10^4 cells/well were stimulated with 5 μg/ml anti-Ig (day 0). Proliferation was measured after 1, 2, 3, 4, and 5 d of culture by [^{3}H]thymidine incorporation. Stimulation indices (see Methods) were calculated using the arithmetic mean values of triplicate cultures (SEs were < 10% of means). Results of one representative experiment out of two repetitions are shown. The WAS patient cell lines shown here (1, 2, and 3) are the same three used to derive the data shown in the other figures and correspond to patients 1, 5, and 6 in Table II.

tory to anti-Ig–induced mitogenesis as is consistent with their manifesting a functional defect similar to that described in CBA/N *xid* B cells.

Expression of surface immunoglobulin and CD45 is similar on WAS and normal B lymphoblastoid cells. To determine whether the failure of anti-Ig stimulation to induce increased proliferation in WAS EBV B cells reflects altered expression of sIg on these cells, WAS and normal EBV B cell sIg expression was assessed by immunofluorescence staining. As shown in Fig. 2, levels of sIg detected on EBV B cells from WAS patients were comparable to those apparent on cells from control individuals. WAS and normal EBV B cells also showed no differences in levels of sIgM expression (Table I) and in intensity of sIgM staining (data not shown).

Expression of the CD45 tyrosine phosphatase on WAS and normal EBV B cells was also assessed in view of the recognized role of this molecule in regulating the cytoplasmic calcium concentration increases that occur in both T and B cells after receptor cross-linking (21). Again, no difference in level of CD45 surface expression (Table I) or staining intensity (data not shown) was apparent between WAS and healthy control EBV B cells.

Anti-Ig–induced intracellular calcium mobilization is markedly decreased or absent in WAS EBV B cells and peripheral blood B cells. The lack of any abnormality in sIg expression on WAS EBV B cells implied that their defective proliferative responses to anti-Ig stimulation reflected a defect in the ability of these cells to transduce signals through their sIg receptors. To determine the nature of this signal transduction defect, the effect of anti-Ig on intracellular Ca^{2+} mobilization was studied in EBV B cells from WAS patients and healthy controls. Anti-Ig was added at a concentration of 6 μg/ml based on results of preliminary titration studies that indicated that anti-Ig concentrations in the range of 3–5 μg/ml induce maximal calcium responses in EBV B cells. As shown in Fig. 3 *A*, cross-linking of sIg on B lymphoblastoid cells from normal controls resulted in an immediate increase in $[Ca^{2+}]_i$. The peak changes in $[Ca^{2+}]_i$ ranged between 31 and 92 nM (Table II). After this initial rapid response, the $[Ca^{2+}]_i$ decreased to a level that was still significantly higher than the basal $[Ca^{2+}]_i$ and plateaued at this or a slightly increased level for a 15-min time period.

Increased $[Ca^{2+}]_i$ after ligand-membrane receptor interaction may reflect uptake of extracellular Ca^{2+} as well as release of Ca^{2+} from intracellular stores. To ascertain the source of the biphasic Ca^{2+} response observed after sIg cross-linking of normal EBV B cells, anti-Ig–stimulated changes in $[Ca^{2+}]_i$ were also studied using calcium-depleted medium (free $[Ca^{2+}] < 10$ nM), a context that obviates the possibility of extracellular Ca^{2+} influx but does not affect intracellular Ca^{2+} release. As shown in Fig. 3, ligation of sIg on EBV B cells in the absence of extracellular Ca^{2+} was followed by rapid elevation of $[Ca^{2+}]_i$, comparable to that initially observed using buffers containing normal levels of Ca^{2+}. However, in contrast to the biphasic profile of increased $[Ca^{2+}]_i$ seen in this latter context, the rise in $[Ca^{2+}]_i$ observed under conditions of extracellular Ca^{2+} depletion was not prolonged, the cytosolic Ca^{2+} levels returning almost immediately to basal levels. These results suggest that the initial transient rise in $[Ca^{2+}]_i$ observed after anti-Ig stimulation of EBV B cells reflects release of intracellular Ca^{2+} stores, whereas the subsequent prolonged plateauing of the Ca^{2+} response is due to delayed transmembrane calcium influx.

In contrast to the rapid increase in $[Ca^{2+}]_i$ triggered by anti-Ig stimulation of normal EBV B cells, cross-linking of sIg on EBV B cells from WAS patients resulted in a relatively di-

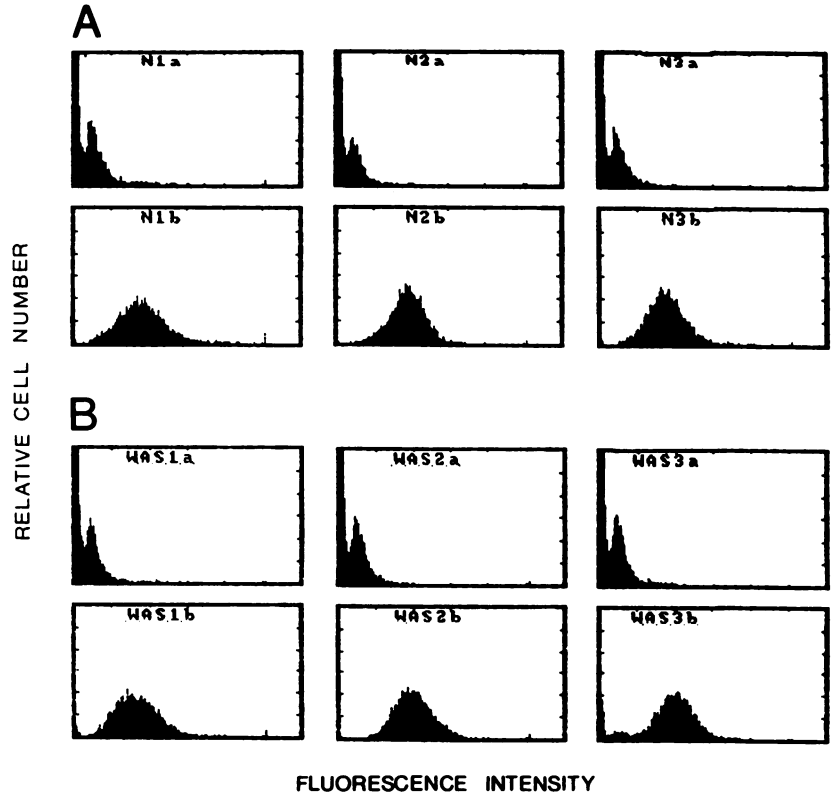

Figure 2. Surface Ig expression on EBV-transformed B cell lines. EBV-transformed B cells from (*A*) normal individuals and (*B*) patients with WAS were stained with either nonspecific rabbit antibody (*top*) or rabbit anti-human Ig (*bottom*) followed by a second step reagent (FITC-conjugated goat anti-rabbit antibody) as described in Methods. For each analysis, Ig expression on 10,000 cells was assessed using a flow cytometer.

Table I. Surface Expression of IgM and CD45 on EBV-transformed B Cells from Normal Individuals and from Patients with WAS

Antigen recognized	Percent positive cells*	
	Healthy controls	WAS patients
sIgM	32±17 (8)	29±8 (6)
CD45	94±5 (4)	97±1 (4)

* Values are means±SD of the percentage of positively stained lymphocytes; value in parentheses is no. of determinations.

minished or absent increase in $[Ca^{2+}]_i$ (Fig. 3 *B*). The results of repeated assays of anti-Ig–induced intracellular calcium responses in EBV B cell lines derived from nine individual WAS patients are shown in Table II. As may be seen, no measurable change in intracellular $[Ca^{2+}]$ was found in three WAS cell

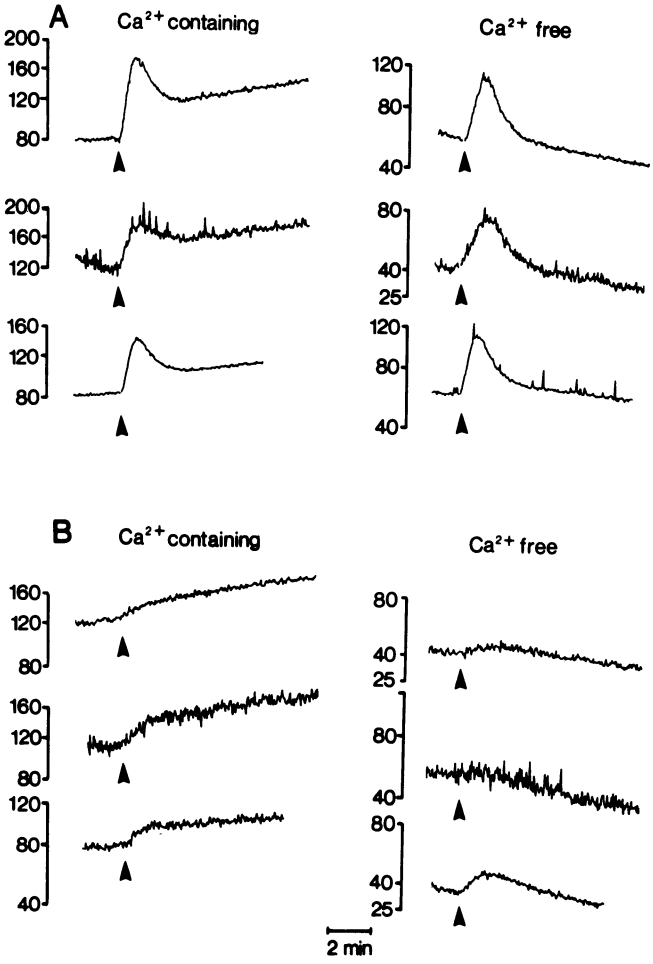

Figure 3. Changes in $[Ca^{2+}]_i$ induced by sIg cross-linking. EBV B cell lines from (*A*) three normal individuals and (*B*) three representative patients with WAS were loaded with Indo-1 and monitored for changes in $[Ca^{2+}]_i$ in response to stimulation with 10 μg anti-Ig antibody (*arrowhead*). As described in Methods, anti-Ig–induced $[Ca^{2+}]_i$ changes were followed in 10^6 Indo-1 loaded cells suspended in 1.7 ml of either Ca^{2+}-containing or nominally Ca^{2+}-free (< 10 nM $[Ca^{2+}]$) buffer. Scales at the left of each tracing indicate intracellular free $[Ca^{2+}]$(nM).

Table II. Anti-Ig–induced Increases in $[Ca^{2+}]_i$ in EBV-transformed B Cells from Normal Individuals and from Patients with WAS

Experiment number	Peak Δ$[Ca^{2+}]_i$*						
	1	2	3	4	5	6	7
	nM						
Healthy controls							
1	41	59	72	45	60		
2	42	64	37	49	50		
3	69	42	75	—	—		
4	40	31	—	—	—		
5	60	82	—	—	—		
6	53	64	—	—	—		
7	45	53	—	—	—		
8	64	—	—	—	—		
9	40	—	—	—	—		
10	92	—	—	—	—		
11	88	—	—	—	—		
12	46	—	—	—	—		
13	50	—	—	—	—		
14	84	—	—	—	—		
15	90	—	—	—	—		
16	70	—	—	—	—		
Mean±SD (n = 30)	58±17						
Range	31–92						
WAS patients							
1	20	20	14	8	0	0	15
2	19	7	0	15	—	—	—
3	0	0	0	—	—	—	—
4	20	24	11	—	—	—	—
5	24	15	10	—	—	—	—
6	24	0	—	—	—	—	—
7	0	0	—	—	—	—	—
8	18	11	—	—	—	—	—
9	0	—	—	—	—	—	—
Mean±SD (n = 27)	10±9						
Range	0–24						

* Values represent highest levels of cytosolic free Ca^{2+} measured in Indo-1–loaded EBV B cells (10^6 cells in 1.7 ml) within 3 min after stimulation with 10 μg anti-Ig antibody. Each data point represents the result of an independent experiment. n is the total number of independent experiments.

lines consistently, and on at least one occasion in four others. In WAS B cell lines that did show increased $[Ca^{2+}]_i$ in response to sIg ligation, the $[Ca^{2+}]_i$ did not peak immediately after stimulation but instead rose slowly over the following 15-min period. This latter profile of Ca^{2+} mobilization is illustrated in Fig. 3 *B* in which changes in $[Ca^{2+}]_i$ are shown for the three WAS EBV B cell lines manifesting the most substantial increases in $[Ca^{2+}]_i$ in response to sIg ligation. However, as is evident from the data included in Table II, maximal intracellular Ca^{2+} concentrations attained after sIg cross-linking were significantly less in 9 consecutively studied EBV B cell lines of WAS patients compared with those observed in 16 consecutively studied B cell lines from healthy controls ($P < 0.001$, Student's t test). Although the absolute Δ$[Ca^{2+}]_i$ detected in given cell lines varied somewhat in repeat assays, the distinc-

tion between WAS and normal responses to anti-Ig was consistently apparent such that even the highest $\Delta[Ca^{2+}]_i$ (24 nM) observed among the WAS cell lines was lower than the lowest $\Delta[Ca^{2+}]_i$ detected in the control lines (31 nM; Table II). Furthermore, in contrast to normal EBV B cells, the WAS EBV B cell Ca^{2+} response to sIg ligation was either entirely abrogated or markedly diminished in the absence of extracellular Ca^{2+} (Fig. 3 *B*). Therefore, extracellular Ca^{2+} influx rather than intracellular Ca^{2+} release appears largely responsible for the increases in $[Ca^{2+}]_i$ observed in some WAS patient B cells after sIg cross-linking.

sIg-induced Ca^{2+} responses were also assessed in freshly isolated peripheral blood lymphocytes obtained from three affected boys (WAS patients 1, 2, and 3) and three controls. As was observed in the EBV B cell lines, peak changes in $[Ca^{2+}]_i$ were consistently lower in WAS compared with control anti-Ig–stimulated cells (Table III). This finding does not appear related to a relative reduction in peripheral blood B cell number in WAS patients, since the percentage of circulation B cells, as defined by staining with the pan-B-cell marker, CD20, was comparable between the patients and controls. (Table III). Furthermore, similar results were obtained by analysis of Ca^{2+} responses in sorted $CD20^+$ peripheral blood mononuclear cells. As is evident from the representative example shown in Fig. 4, anti-Ig–stimulated $CD20^+$ B cells from WAS patients did not manifest either the rapid rise or the magnitude of $\Delta[Ca^{2+}]_i$ apparent in similarly treated control $CD20^+$ cells.

Taken together these results reveal that the ability to mobilize Ca^{2+} from intracellular stores in response to sIg ligation is either lacking or markedly reduced in WAS patient compared with normal B lymphocytes. Accordingly, it appears likely that at least some facets of B cell dysfunction in WAS patients may reflect abnormalities in signal transduction, and in particular, in the signaling pathways that elicit intracellular calcium mobilization. Lastly, it should be noted that neither the profile nor level of WAS EBV B cell intracellular Ca^{2+} mobilization was

Table III. Surface Expression of CD20 and Anti-Ig–induced Increases in $[Ca^{2+}]_i$ in Peripheral Blood Cells from Normal Individuals and WAS Patients

	Percent $CD20^+$ peripheral blood cells	Peak $\Delta[Ca^{2+}]_i$*
		nM
Healthy controls		
1	5	75
2	6	75
3	12	48
Mean±SD	7.7±3.8	66.0±15.6
Range	6–12	48–75
WAS patients		
1	4	18
2	2	5
3	18	19
Mean±SD	8.0±8.7	14.0±7.8
Range	2–18	5–19

* Values represent highest levels of cytosolic free Ca^{2+} measured in Indo-1–loaded peripheral blood mononuclear cells (10^6 cells in 1.7 ml) within 3 min after stimulation with 10 μg anti-Ig antibody.

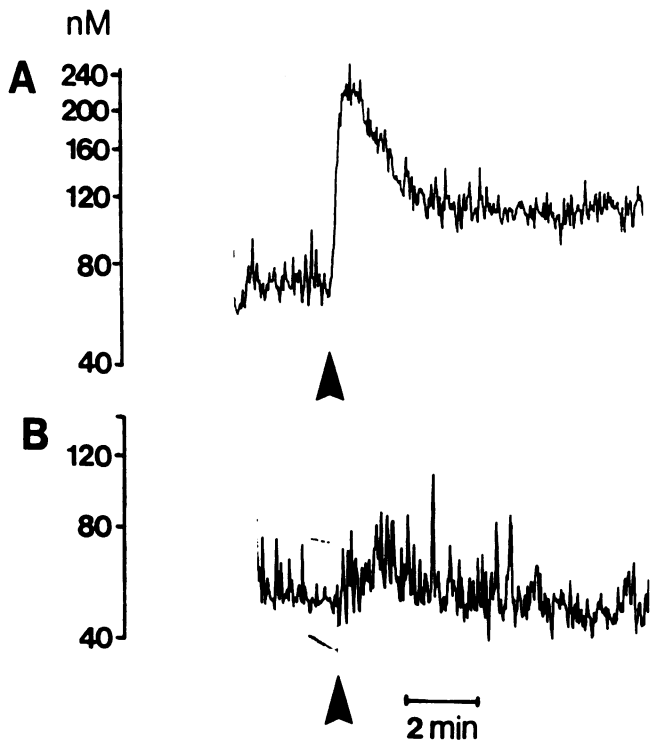

Figure 4. Changes in $[Ca^{2+}]_i$ induced by ligation of sIg on freshly isolated $CD20^+$ B cells. Peripheral blood mononuclear cells from a normal individual (*A*) and a WAS patient (*B*) were stained with FITC-conjugated anti-CD20 antibody and the $CD20^+$ cells collected by sorting on a flow cytometer. As detailed in Methods, the selected cells (90% $CD20^+$) were loaded with Indo-1 and monitored for changes in $[Ca^{2+}]_i$ in response to stimulation with 10 μg anti-Ig antibody (*arrowhead*). The data shown are representative of two independent determinations.

altered by the use of higher anti-Ig dose or anti-Ig cross-linked by rabbit anti–mouse antibody (data not shown).

Anti-Ig–induced increases in phosphatidylinositol hydrolysis are diminished in WAS compared with control EBV B cells. In view of the correlation between anti-Ig–induced intracellular Ca^{2+} release and PLC-mediated triggering of phosphoinositide hydrolysis, the induction of inositol phosphate production after sIg ligation was measured in [^{3}H]myoinositol-loaded EBV B cells from three WAS patients and three controls. As shown in Table IV, release of inositol phosphates was evident in anti-Ig–stimulated WAS EBV B cells but was reduced by about 50% in these cells compared with the levels observed in similarly treated control cells. As > 95% of the [^{3}H]inositol incorporated into inositol phosphates after cellular activation appear to be derived from phosphatidylinositol (22, 23), this result suggests that a defect in anti-Ig–induced PLC-dependent phosphoinositide hydrolysis occurs in WAS EBV B cells and may account for their diminished degree of intracellular Ca^{2+} release in response to sIg ligation.

Tyrosine phosphorylation is not increased by cross-linking sIg on WAS EBV B cells. In previous studies we have shown that induction of protein tyrosine phosphorylation is a prerequisite for the mobilization of Ca^{2+} from intracellular stores after sIg ligation in B cells (13). This observation appears to reflect a requirement for tyrosine phosphorylation in the activation of PLC-γ and suggests that protein tyrosine phosphory-

Table IV. Induction of Inositol Phosphate Release by Ligation of sIg on WAS and Control EBV B cells

	Increase in [^{3}H]inositol phosphates	
	Fold increase‡	Baseline levels of inositol phosphate formation§
		cpm
Healthy controls		
1	3.64	(85)
2	1.87	(99)
3	1.86	(156)
Mean±SD	2.46±1.02	
Range	1.86–3.64	
WAS patients		
1	1.18	(60)
2	1.10	(60)
3	0.80	(389)
Mean±SD	1.03±0.20	
Range	0.80–1.18	

* [^{3}H]inositol phosphate formation was measured in 5×10^7 [^{3}H]myoinositol-loaded EBV B cells 3 min after stimulation with 20 μg anti-Ig antibody. ‡ Results are expressed as [^{3}H]inositol phosphate formation in anti-Ig-treated cells/[^{3}H]inositol phosphate formation in untreated cells; each value represents the mean of determinations done on duplicate samples in six independent experiments. § Numbers in parentheses represent the levels of inositol phosphate incorporation in untreated cells.

lation is induced before increases in $[Ca^{2+}]_i$ in anti-Ig-stimulated B cells. Accordingly, we next examined the possible relevance of a defect in tyrosine phosphorylation to the differences in sIg-stimulated intracellular Ca^{2+} release apparent between EBV B cells of WAS patients and normal individuals.

In preliminary studies of EBV B cells from normal individuals, we established that the degree of intracellular protein tyrosine phosphorylation begins to increase within seconds after ligation of sIg, peaks in ~ 3 min, and declines thereafter (data not shown). Furthermore, as has been previously observed in nontransformed B cells, anti-Ig-provoked increases in tyrosine phosphorylation occurred in parallel or before the initial rise in cytosolic $[Ca^{2+}]$, a finding that is consistent with the contention that tyrosine phosphorylation represents a more proximal signaling event than intracellular Ca^{2+} release. Interestingly, the plateau phase of increased $[Ca^{2+}]_i$ that persists after anti-Ig stimulation occurs despite the decrease in the amount of protein tyrosine phosphorylation.

Based on these findings, tyrosine phosphorylation of WAS and normal EBV B cell proteins was assayed 3 min after treatment of these cells with anti-Ig antibody. As illustrated by the representative examples shown in Fig. 5, sIg ligation of EBV B cells from five normal individuals was consistently associated with increased tyrosine phosphorylation of proteins with molecular masses of 110, 55, and 34 kD. Tyrosine phosphorylation of 85- and 39-kD proteins was also observed in some of the normal cell lines. Among these protein bands, the anti-Ig-induced increase in tyrosine phosphorylation was most obvious in relation to the 34-kD species, a band that was almost not detectable before sIg cross-linking. As noted in relation to the total protein tyrosine phosphorylation, maximal phosphorylation of this 34-kD band occurred at 3 min after sIg ligation and declined to baseline level within 10 min (data not shown).

In contrast to normal EBV B cell lines, cross-linking of sIg on EBV B cells from five WAS patients failed to increase tyrosine phosphorylation of any protein species. As indicated by the three examples shown in Fig. 5 (which represent the same WAS cell lines used to derive data shown in Fig. 3 *B*) and confirmed by the results of laser densitometry, the anti-Ig-induced increases in tyrosine phosphorylation of 110-, 55-, and 34-kD proteins that were detected in normal EBV B cells were not apparent in any WAS EBV B cell line. Moreover, increases in tyrosine phosphorylation of these molecules were not observed even after prolonged incubation (up to 60 min) of the WAS cells with anti-Ig antibody (data not shown). These results indicate that anti-Ig-induced increases in tyrosine phosphorylation are markedly diminished in WAS compared with normal EBV B cells and may even be absent in cells from some of these patients.

To specifically determine whether surface Ig ligation induces tyrosine phosphorylation of PLC in WAS B cells, phosphorylation of the γ1 isozyme of PLC was assessed in WAS and control EBV B cells by immunoprecipitation and Western blot analysis. For analysis of tyrosine phosphorylation of PLC-γ1,

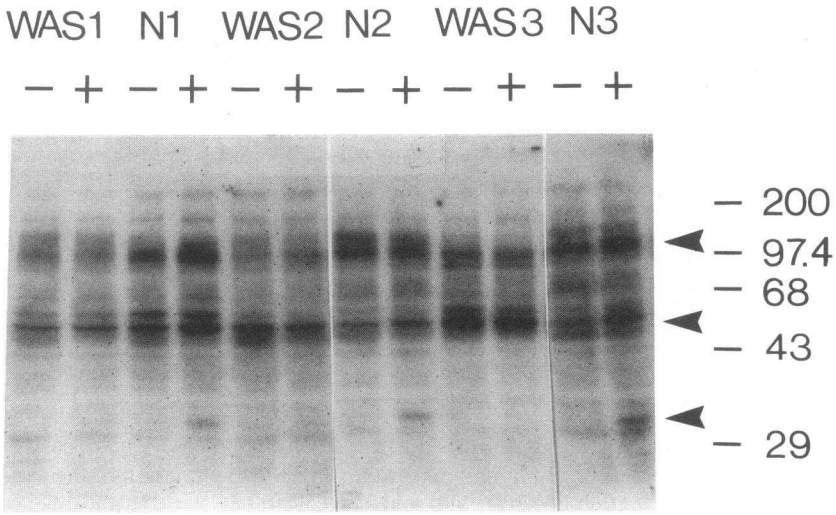

Figure 5. Immunoblots showing the effect of sIg cross-linking on protein tyrosine phosphorylation in EBV B cells. EBV-transformed B cells (3×10^6) from three normal individuals and from three patients with WAS (same as those presented in Figs. 1–3) were cultured in serum-free medium for 24 h and then incubated with (+) or without (−) 20 μg anti-Ig antibody. Cells were lysed in Ripa buffer and centrifuged, and the supernatant proteins were electrophoretically separated in SDS–polyacrylamide gels. After transfer to nitrocellulose, proteins were probed with a monoclonal antibody to phosphotyrosine. The migration positions of prestained molecular mass standards (in kD) are shown on the right. The arrows indicate protein bands showing net increases in tyrosine phosphorylation after sIg cross-linking. Results of one representative experiment out of three are shown for each individual.

cells were lysed 3 min after anti-Ig stimulation and the lysate proteins were immunoprecipitated with a rabbit anti-PLC-γ1 antisera, resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibody. As is evident from the representative example shown in Fig. 6, tyrosine phosphorylated PLC-γ1 is essentially undetectable in unstimulated control or WAS EBV B cells but is apparent in the control EBV B cells after anti-Ig treatment. By contrast, tyrosine phosphorylated PLC-γ1 was not detected in anti-Ig–stimulated WAS EBV B cells. This latter observation cannot be ascribed to differences in PLC-γ1 expression in WAS compared with control cells, as the results of immunoblot and Northern analyses indicated that levels of PLC-γ1 mRNA and protein, respectively, are similar in WAS and control EBV B cell lines (data not shown). Together these results suggest that induction of tyrosine phosphorylation on PLC-γ1, a key event in the activation of this enzyme after sIg ligation, is impaired in WAS EBV B cells, as is consistent with the finding that anti-Ig–induced increases in intracellular protein tyrosine phosphorylation and Ca^{2+} mobilization are diminished in these cells compared with EBV B cells from controls.

Discussion

The molecular basis for the severe immune deficiency expressed by patients with WAS remains undefined, but recent data suggest that B cell dysfunction occurs in these patients and resembles that described in X chromosome–linked immunodeficient CBA/N mice. Accordingly, the reported association of the *xid* mutation with impaired anti-Ig–induced B cell proliferation and intracellular Ca^{2+} mobilization prompted us to investigate signaling via sIg on B cells from WAS patients. The results of the studies reported here reveal that EBV-transformed B cells from WAS patients do not manifest increased proliferation in response to sIg ligation, despite their expression of sIg at levels comparable to those found on control EBV B cells. In addition, our findings indicate that several key sequelae of sIg ligation, i.e., increase in protein tyrosine phosphorylation and most notably PLC-γ1 tyrosine phosphorylation, production of inositol phosphates, and induction of Ca^{2+} release from intracellular stores, are markedly diminished in WAS compared with normal control EBV B cells. These data provide further evidence of an intrinsic defect in WAS B cells and suggest that at least some of the immune abnormalities found in these patients may reflect impaired lymphocyte competency to transduce activation signals.

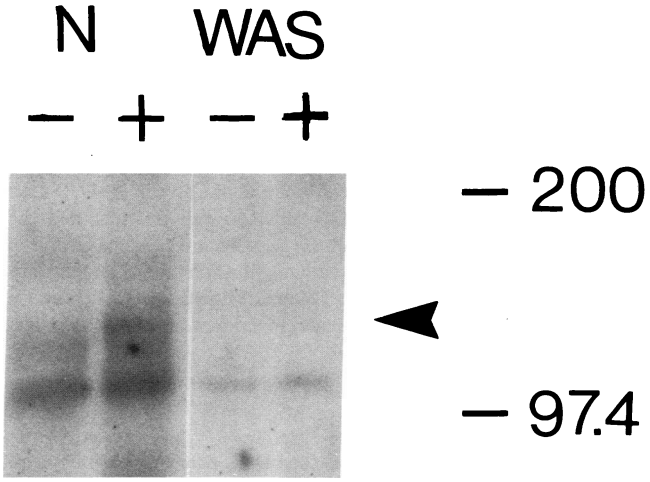

Figure 6. Immunoblot showing the effect of sIg cross-linking on the tyrosine phosphorylation of PLC-γ1 in EBV B cells. Cell lysates were prepared from 1.7×10^8 anti-Ig antibody–stimulated (+) or unstimulated (−) EBV B cells and were incubated with PLC-γ1–specific antiserum followed by Protein A-Sepharose 4B. The immunoprecipitated proteins were eluted, electrophoresed through SDS-PAGE, transferred to nitrocellulose, and probed with antiphosphotyrosine antibody. The migration positions of prestained molecular mass standards (in kD) are shown on the right. The arrow indicates the position of tyrosine-phosphorylated PLC-γ1.

Because of the difficulties inherent to obtaining sufficient numbers of fresh B cells from young children, and in particular from frequently ill and anemic WAS children, most of the analyses of sIg-mediated signaling described in this paper were carried out using EBV-transformed B cell lines. Although we cannot be certain of the extent to which the functional and phenotypic properties of these immortalized cells are representative of those manifested by nontransformed B cells in vivo, the finding of impaired intracellular Ca^{2+} release in freshly isolated B ($CD20^+$) cells from two WAS patients suggests strongly that WAS B cells are defective with regards to signal transduction. This contention is supported by the distinct and consistent differences apparent between WAS and normal EBV B cells in relation to several of the other biochemical sequelae of sIg ligation, including tyrosine phosphorylation of PLC-γ1 and phosphatidylinositol hydrolysis.

Accordingly, it appears that EBV-transformed B cells provide a valuable model system for studying the functional characteristics of WAS and perhaps other immunodeficient patient B cells. In addition, as has been previously found in other studies (24), our data indicate that immortalized cell lines can be successfully exploited for characterization of cell signaling mechanisms. For example, the results of our studies on WAS EBV B cells indicate that transmembrane uptake of extracellular Ca^{2+} during lymphocyte activation can occur in the absence of intracellular Ca^{2+} mobilization or detectable protein tyrosine phosphorylation. Therefore, in contrast to data garnered using other cell types, including T cells, the results presented here suggest that extracellular Ca^{2+} influx does not depend on the tyrosine phosphorylation and activation of PLC-γ or on the generation of IP_3, IP_4, or other inositol phosphates produced consequent to PLC-γ activation.

Although the capacity of sIg receptors to transduce activation signals in B cells is well established, the mechanisms whereby they do so have not been clearly elucidated. Neither sIgM nor sIgD, the predominant sIg isotypes on mature B cells, have sufficient cytoplasmic structure to interact directly with the other intracellular molecules, such as G proteins and tyrosine kinases, that mediate signal transduction (25, 26). The resolution of this dilemma, however, appears to be forthcoming as the result of the recent identification of several sIg-associated glycoproteins that do have intracellular domain structure suitable for delivery of activation signals (25–29). Among these newly identified molecules are two sIgM-associated proteins, designated as IgM-α and IgM-β, which have molecular masses of 34 and 39 kD, respectively (25, 26). These proteins, which represent the products of the mb-1 (IgM-α) and B29 (IgM-β) genes, comprise a disulfide-linked heterodimer that associates with sIgM to form a sIgM receptor complex. Al-

though the functions of these molecules are not fully understood, recent data showing their inducible tyrosine phosphorylation after sIg ligation (30) imply that these proteins are involved in signal transduction and hence may be relevant to defective signaling in WAS B cells. As expression of IgM-α seems to be required for the transport of IgM to the cell surface (28) and levels of sIgM expression appear comparable on WAS and normal EBV B cells, it seems unlikely that the hyporesponsiveness of WAS cells to anti-Ig relates to insufficient quantity of IgM-α protein. Furthermore, a constitutively tyrosine-phosphorylated protein with a size comparable to that of IgM-α (34 kD) was detected, albeit at low levels, in both the WAS and normal EBV B cells studied here. Therefore, in contrast to CBA/N mice, in which impaired membrane expression of a sIg-associated protein has been described (31), altered signaling in WAS B cells appears unrelated to IgM-α expression per se. However, after sIg ligation, the WAS EBV B cells failed to manifest the increased tyrosine phosphorylation of either the 34-kD or several other protein species that was observed in anti-Ig–treated EBV B cells from controls.

In view of the putative role for IgM-α in signal transduction, this latter finding raises the possibility that failure to phosphorylate IgM-α may be directly responsible for reduced activation of PLC and Ca^{2+} release in WAS EBV B cells. In B cells, coupling of sIg to the phosphoinositide signaling pathway is associated with and appears to require activation of G-proteins (32–35). Accordingly, it is possible that defective generation of tyrosine-phosphorylated IgM-α in anti-Ig–stimulated WAS B cells interrupts transduction of Ca^{2+}-mobilizing signals by interfering with an IgM-α–G-protein interaction required for PLC activation. However, our data also reveal anti-Ig–stimulated WAS EBV B cells to be defective with regards to the generation of tyrosine phosphorylated PLC-γ1. Therefore, it is also possible and perhaps more likely that altered Ca^{2+} release after sIg cross-linking in the WAS cells is, at least in part, a direct consequence of defective tyrosine phosphorylation of PLC isozymes, a phenomenon that normally accompanies and is considered necessary for sIg-induced activation of these enzymes and stimulation of phosphatidylinositol hydrolysis (24). In this context, the link between G-proteins and PLC activation may not be through IgM-α, but instead, as we have previously shown in neutrophils (36), may involve G-protein modulation of a tyrosine kinase or phosphatase activity required for phosphorylation and activation of PLC. One other point worth noting with regards to anti-Ig–induced tyrosine phosphorylation of PLC is that tyrosine phosphorylated PLC-γ1, while clearly identifiable in anti-Ig–treated control EBV B cells (Fig. 6), was not detected in antiphosphotyrosine antibody immunoblots of total cell lysates (Fig. 5). This finding suggests that among the various isozymes of PLC, the γ1 form of the enzyme does not represent one of the major substrates for anti-Ig–induced tyrosine phosphorylation.

Studies of the signaling events initiated by engagement of the antigen receptor on T cells have revealed that transduction of Ca^{2+}-mobilizing signals in these cells requires their expression of a membrane tyrosine phosphatase, CD45 (37, 38). In addition, the pattern of Ca^{2+} mobilization as well as cellular activation and proliferation induced by cross-linking of the antigen receptors on either B or T lymphocytes is markedly altered in the context of coincident cross-linking of CD45 (21). Although these data suggest a role for CD45 in sIg-mediated signal transduction, our finding of similar levels of membrane CD45 expression by WAS and control EBV B cells indicates that defective signal transduction in the WAS cells cannot be ascribed to the absence or reduced expression of this phosphatase. These results do not, however, preclude the possibility that CD45 may be functionally defective in WAS B lymphocytes.

In summary, the data reported here indicate that WAS EBV B cells are defective with regards to their ability to proliferate and to transduce Ca^{2+}-mobilizing and tyrosine phosphorylation signals in response to sIg cross-linking. The results suggest that disruption of early signaling events in these cells reflects a defect at a point upstream of tyrosine phosphorylation of PLC, possibly related to dysfunction of tyrosine kinases such as the lyn or blk kinases that have recently been implicated in sIgM-triggered signal transduction (37, 38). As anti-Ig–induced cross-linking of sIg is thought to represent a model for the intracellular events triggered by B cell stimulation with T independent antigens (39), our findings suggest that aberrant transmembrane signal transduction may account for the nonresponsiveness of WAS patient B cells to certain T independent antigens and may also underlie or contribute to other abnormalities of WAS B cell function. Although the extent to which cell signaling is altered in other hemopoietic lineages of WAS patients remains to be determined, defective signal transduction provides an attractive and plausible explanation for the impaired T cell and platelet activation observed in these patients and may also underlie other facets of WAS cellular dysfunction. Thus, although it is unclear whether impaired B cell signal transduction in WAS represents a direct or indirect consequence of the primary gene defect, continued investigation of the process whereby signal transduction is altered in WAS EBV B cells should provide valuable insights into the molecular basis for expression of the WAS phenotype. Analysis of the structural elements and level of the signal abnormalities that interfere with delivery of normal sIg-mediated activation signals in WAS B cells also represents a promising avenue toward delineating the nature and sequence of the molecular interactions that mediate lymphocyte signal transduction.

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