

# ISOLATION AND CHARACTERIZATION OF A B LYMPHOCYTE MUTANT WITH ALTERED SIGNAL TRANSDUCTION THROUGH ITS ANTIGEN RECEPTOR

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Activation signals generated through surface Ig (sIg)<sup>1</sup> on B lymphocytes are transduced across the plasma membrane partially or entirely through G protein-linked phospholipase C catalyzed hydrolysis of inositol phospholipids (PI) (1-5). Metabolites of this process act as intracellular "second messengers" to initiate signaling pathways which then synergize to eventually result in transition from the resting state (G<sub>0</sub>) into cell cycle (G<sub>0</sub>-to-G<sub>1</sub> transition) (6).

The linkage of downstream activation events to PI hydrolysis has been inferred from studies using pharmacologic agents that either mimic or inhibit PI hydrolysis. For example, activation of protein kinase C by phorbol diesters upregulates class II antigen expression, induces *c-fos* gene expression (7, 8) and synergizes with Ca<sup>2+</sup> ionophores to drive B lymphocytes into cell cycle (6). Conversely, neomycin inhibition of PI hydrolysis blocks anti-Ig stimulated release of sequestered Ca<sup>2+</sup>, as well as Ca<sup>2+</sup> influx (9). Through such studies, signaling pathways have been constructed that link sIg-generated signals to short-term physiologic changes, changes in gene expression, and long-term adaptive responses (10, 11).

Transformed B cell lines representative of specific maturation stages have proven useful for studies of receptor Ig signal transduction. For example, the murine B lymphoma WEHI-231 displays signal transduction events similar to normal B lymphocytes with respect to G protein-linked PI hydrolysis (2, 12), Ca<sup>2+</sup> mobilization (13-15), protein phosphorylation (16), and induction of *c-fos* (8) and *c-myc* (17, 18) expression. The coupling of later events in WEHI-231 appears different from normal mature B lymphocytes, since sIg-generated signals are translated by WEHI-231 to cause decreased proliferation and eventually cell death (19, 20).

We describe here a system for further exploring sIg signaling and verifying proposed linkages between activation events and downstream processes. We have generated a sIgM signaling variant of WEHI-231 that continues to express high levels of sIgM but is refractory to sIgM-generated downregulatory signals. Coupling of the receptor

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<sup>1</sup> *Abbreviations used in this paper:* PI, inositol phospholipid; PKC, protein kinase C; sIg, surface Ig.

to PI hydrolysis appears altered as do some, but not all, downstream activation events previously believed linked to PI hydrolysis.

### Materials and Methods

**Cell Lines.** The WEHI-231 B lymphoma clone 28 was obtained from Dr. David Scott (University of Rochester, Rochester, NY) and was maintained in DME, high glucose supplemented with 10% FCS (Hyclone, defined), SerXtend (Hana Biologicals, Berkeley, CA), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, and  $5 \times 10^{-5}$  M 2-ME ethanol. VS2.12-cl.2 is a limiting dilution subclone of ethylmethane sulfonic acid mutagenized WEHI-231 selected in goat anti-mouse  $\mu$  antibody (Sigma Chemical Co., St. Louis, MO). Cells were maintained in the above media with 2  $\mu$ g/ml goat anti-mouse  $\mu$ . VS2.12-cl.2 cells to be used for experiments were washed free of anti- $\mu$  48 h before the experiment.

**[<sup>3</sup>H]Thymidine Incorporation Assay.** Triplicate cultures of  $2 \times 10^4$  viable cells were incubated in 200  $\mu$ l of medium in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA). Stimuli were added and cultures were pulsed with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine at times indicated in the experiment. Samples were harvested using a PhD Cell Harvester and washed with 10% TCA. The radioactivity incorporated was measured by liquid scintillation spectrophotometry.

**Measurement of [<sup>3</sup>H]Inositol Phosphate Accumulation.** Cells were loaded with [<sup>3</sup>H]inositol (American Radiolabeled Chemicals, St. Louis, MO) for 4 h in inositol-free DMEM (Biofluids, Inc., Rockville, MD) (40  $\mu$ Ci/ml;  $5 \times 10^6$  cells/ml). After loading, cells were cultured overnight in normal growth media. For the assay, the cells were suspended to  $2.5 \times 10^6$ /ml in inositol-free media and 200  $\mu$ l was aliquoted for each experimental point. To each tube was added 20  $\mu$ l of 0.1M LiCl. Each tube was incubated for 10 min at 37°C, whereupon individual stimuli were added. The cells were harvested at the indicated times and inositol phosphates isolated in total or individually by ion-exchange chromatography exactly as described by Bijsterbosch et al. (2).

**Isolation of Total Cellular RNA and Northern Analysis.** Cells ( $1.2 \times 10^7$ ) were placed in 20 ml of media and equilibrated at 37°C before stimulation. At time 0, 20  $\mu$ g (10  $\mu$ g/ml final) of anti- $\gamma$  antibodies or 200 ng (10 ng/ml final) of PMA was added and incubation was continued at 37°C. At the indicated times, the cells were harvested and total cellular RNA isolated by the guanidium isothiocyanate method with CsCl modification (21). Equal amounts of total RNA were size fractionated by electrophoresis through 1% agarose-formaldehyde gels and transferred to Gene Screen Plus nylon membrane (DuPont Co., Wilmington, DE) as described previously (8). Prehybridization and hybridization in the presence of 100 ng of nick-translated <sup>32</sup>P-labeled *pfos-1* (22) occurred at 42°C in the presence of 50% formamide. Washing at high stringency was performed as described previously (8).

**Determination of [<sup>2+</sup>Ca]; Using Fura-2-dyed Cells.** Cells were washed and resuspended to  $\sim 10^7$  in HBSS containing 1.3 mM Ca<sup>2+</sup>, 0.9 mM Mg<sup>2+</sup>, 10 mM Hepes buffer, and 10% FCS. To each 4-ml cell suspension, 20  $\mu$ g of Fura-2 acetoxymethylester (Fura-2 AM; Molecular Probes, Eugene, OR) was added as an emulsion of 5  $\mu$ l DMSO, 5  $\mu$ l Pluronic F-127 (BASF Wyandotte; Molecular Probes) at 12.5% (wt/vol), and 75  $\mu$ l FCS. The cells were vortexed briefly, then rotated gently at room temperature for 10 min. After this incubation, the cells were washed once, resuspended to the original concentration in HBSS plus 10% FCS, and incubated for 15 min at 37°C to promote hydrolysis of the dye ester. The cells were then washed and resuspended to  $33 \times 10^6$ /ml in HBSS plus 10% FCS without added calcium or magnesium, and stored on ice.

Fluorimetric analysis of dyed cells was performed as follows. At the beginning of each run, cells were diluted in the cuvette to  $3.3 \times 10^6$ /ml in HBSS (total volume, 800  $\mu$ l) so that the final concentration of serum was 1% and, except where specifically noted, calcium and magnesium were 1.3 mM and 0.9 mM, respectively. Excitation was at 340 nm with emission monitored at 510 nm using a fluorescence spectrophotometer (model 650-10S; Perkin-Elmer Corp., Norwalk, CT); sensitivity was set at 3. The fluorometer was fitted with a flow-through water bath set to warm the contents of a cuvette to 37°C. After an initial equilibration of 3-4 min to allow the resting cells signal to stabilize, an excitation spectrum was taken, then

the cells were perturbed with the indicated stimulus. Manual agitation every 1.5 min was sufficient to maintain the cells in suspension. The response of the cells was monitored at an excitation wavelength of 340 nm. At the termination of the response, a second spectrum was taken. Ionomycin was then added (from a 0.75 mM stock solution in ethanol) to a final concentration of 12  $\mu$ M. A final spectrum was then taken after the addition of ionomycin in order to confirm that the dye was fully in the calcium-chelated state.

Data for each determination were digitized and plotted. Light-scattering baselines were subtracted from the 340-nm excitation signals before these values were used to calculate  $[Ca^{2+}]_i$ . For each determination, the fluorescence signal  $S$  was compared to  $S_b$ , the signal from ionomycin-treated cells (the expected fluorescence for 100% chelated dye) and 0.31 times that value. The latter, which we denote  $S_f$ , is the value expected for unchelated dye, as determined on a sample of pentapotassium Fura-2 in the same spectrofluorometer. For each point, a value for  $[Ca^{2+}]_i$  was calculated from the following formula:  $[Ca^{2+}]_i = K_d[S - S_f]/S_b - S_f$ . A value of 224 nM was used for the dissociation constant  $K_d$  as reported by Grynkiewicz et al. (23).

## Results

*Generation of WEHI-231 sIg Signaling Variant VS2.12-cl.2.* WEHI-231 was cultured for 17 h with the base-pair substitution mutagen ethyl methanesulfonate (EMS), washed, and placed in mutagen-free media for 48 h, and then selected in the presence of anti- $\mu$  antibodies and cloned by limiting dilution. Signaling variants were chosen based upon their ability to grow in the presence of anti- $\mu$ . VS2.12-cl.2 was among 10 variants selected. None of the variants were negative for sIgM expression, although one expressed extremely low levels. Variant VS2.12-cl.2 was chosen for the studies reported here. sIgM expression by this variant was  $\sim$ 80% that of the parental line (Fig. 1). Furthermore, iodination and immunoprecipitation followed by SDS-PAGE analysis of IgM from the surface of each cell line did not show detectable differences in protein structure of the  $\mu$  heavy or  $\kappa$  light chains (Monroe, J., preliminary studies). Therefore, although sIgM expression is slightly lower on VS2.12-cl.2 than WEHI-231, the variant is still a high sIgM expressor, and importantly, 100% of the cells were observed to be sIgM<sup>+</sup>. Equally important, the primary anti- $\mu$  antibody used for cytofluorimetric analysis in Fig. 1 is also the same reagent used for stimulation in the studies described below.

*VS2.12-cl.2 Is Not Inhibited by Anti- $\mu$  Antibodies.* Previous studies have shown that crosslinking of sIgM on WEHI-231 by anti- $\mu$  antibodies inhibits proliferation of this cell line in a dose-dependent manner (8, 19, 20). Clearly shown in Fig. 2, VS2.12-cl.2 is refractory to the inhibitory signal generated through sIgM crosslinking. No inhibition of VS2.12-cl.2 by anti- $\mu$  was observed at any concentration of anti- $\mu$  tested,

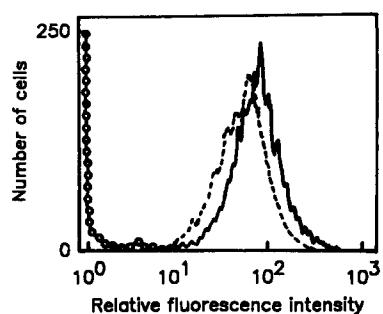


FIGURE 1. Cytofluorimetric analysis of surface IgM expression on WEHI-231 and VS2.12-cl.2. WEHI-231 (—) or VS2.12-cl.2 (---) was stained with goat anti-mouse  $\mu$  followed by FITC-conjugated rabbit anti-goat antibodies and analyzed by flow cytometry. Cells minus the primary antibody (O). For each analysis, 10,000 cells were analyzed and dead cells were omitted by gating on narrow angle light scatter.

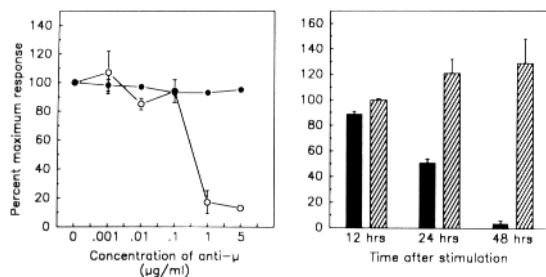


FIGURE 2. VS2.12-cl.2 is refractive to growth-inhibitory signals associated with the wildtype WEHI-231. Values are expressed as [ $^3\text{H}$ ]thymidine incorporation relative to cultures without anti- $\mu$  (100%). Dose-dependent effects of anti- $\mu$  on VS2.12-cl.2 ( $\bullet$ ) or WEHI-231 ( $\circ$ ) are shown in the left panel. Temporal studies (right panel) on VS2.12-cl.2 (hatched bars) or WEHI-231 (solid bars) were performed with 2  $\mu\text{g/ml}$  of anti- $\mu$ . In studies shown in the left panel, stimulation occurred for the last 16 h. In studies shown in the right panel, stimulation occurred for the last 6 h. Depicted values in both panels are means of triplicate cultures  $\pm$  SEM.

For the left panel, [ $^3\text{H}$ ]thymidine incorporation by unstimulated cells was  $271,443 \pm 7,387$  and  $303,356 \pm 18,102$  for WEHI-231 and VS2.12-cl.2, respectively. For the right panel, [ $^3\text{H}$ ]thymidine incorporation for unstimulated cells was:  $144,063 \pm 1,176$  and  $192,774 \pm 7,371$  at 12 h;  $119,444 \pm 15,226$  and  $122,065 \pm 18,275$  at 24 h; and,  $126,339 \pm 8,470$  and  $158,908 \pm 4,558$  at 48 h for WEHI-231 and VS2.12-cl.2, respectively.

although the wildtype is profoundly inhibited by 1 and 5  $\mu\text{g/ml}$  of anti- $\mu$ . Inhibition of WEHI-231 is detectable by 12 h after stimulation and appears to affect all cells by 48 h. VS2.12-cl.2 does not exhibit any negative response to sIgM crosslinking insofar as its proliferation is concerned.

*sIgM and Hydrolysis of Inositol Phospholipid Are Uncoupled in VS2.12-cl.2.* One of the primary signaling events associated with sIg crosslinking on WEHI-231 as well as untransformed B cells is phospholipase C-catalyzed hydrolysis of inositol phospholipids (PI) (1, 2, 12, 24). Recent evidence suggests that PI hydrolysis is coupled to sIg via a GTP-binding protein intermediary (4, 5). To begin to define the stage in the sIgM signaling process where uncoupling occurs in VS2.12-cl.2, we investigated association between sIgM crosslinking and subsequent changes in inositol phospholipid hydrolysis as determined by measuring accumulation of inositol phosphates. Shown in Fig. 3, crosslinking of sIgM on WEHI-231 causes PI hydrolysis. In sharp contrast, identical stimulation through sIgM on VS2.12-cl.2 was completely ineffective in this regard. To attempt to compensate for the fewer receptors on VS2.12-cl.2 relative to WEHI-231, we used increasing concentrations of ligand. Despite the fact that significant responses were observed in WEHI-231 at 5  $\mu\text{g/ml}$  and maximum responses here at 50  $\mu\text{g/ml}$ , anti- $\mu$  at 100 and 10 times these concentrations, respectively, elicited no detectable response over background in VS2.12-cl.2.

Crosslinking of sIgM on normal B cells as well as WEHI-231 results in direct production of  $\text{IP}_1$ ,  $\text{IP}_2$ , and  $\text{IP}_3$ , each with different kinetics of production. To evaluate whether the uncoupling of sIgM from the PI response in VS2.12-cl.2 similarly affected each of these species, we measured individual production of each inositol phosphate. The results of these studies are shown in Fig. 4. Crosslinking sIgM on WEHI-231 elicited marked responses by each inositol phosphate species. Similar to that observed by others (2),  $\text{IP}_3$  appears earliest after stimulation, followed quickly by  $\text{IP}_2$ , and somewhat later by  $\text{IP}_1$ . Corroborating that observed by measuring total inositol phosphate production (Fig. 3), no measurable increases relative to unstimu-

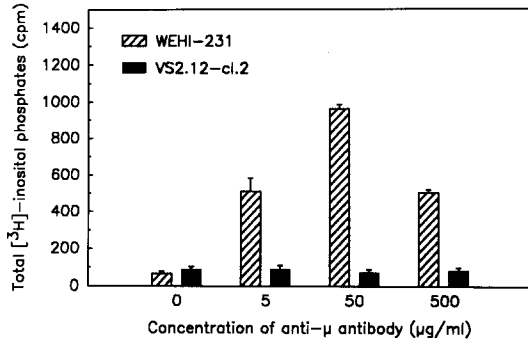


FIGURE 3. Crosslinking of sIgM on VS2.12-cl.2 does not stimulate inositol phospholipid hydrolysis. Inositol phospholipid hydrolysis was determined by measuring accumulation of [<sup>3</sup>H]inositol phosphates at 45 min in the presence of LiCl as described in Materials and Methods. Values represent means of triplicate determinations ± SEM.

lated cells were detected in any of the three inositol phosphate species following anti-μ stimulated VS2.12-cl.2. It should be noted that this lack of a measurable response occurred even though LiCl was present in all assays to prevent breakdown of the inositol phosphates thereby enhancing the signal.

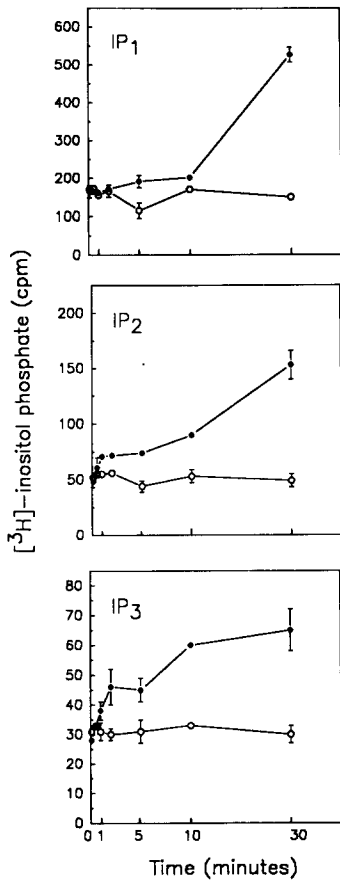


FIGURE 4. Comparison of WEHI-231 and variant VS2.12-cl.2 for kinetics and levels of production of individual inositol phosphates. [<sup>3</sup>H]inositol phospholipid-labeled cells were stimulated for the indicated period of time with 10 μg/ml of anti-μ antibody in the presence of LiCl. In studies not shown, this concentration gave PI responses equivalent or greater than that resulting from stimulation by 50 μg/ml. Individual inositol phosphate species were sequentially eluted from an ion-exchange column exactly as described by Bijsterbosch et al. (2). Values represent means of triplicate determinations ± SEM.

*Stimulation of VS2.12-cl.2 with  $AlF_4^-$  Promotes Inositol Phospholipid Hydrolysis.* To investigate whether the inability to initiate PI-hydrolysis by anti- $\mu$  stimulation in VS2.12-cl.2 reflected a functional defect in phospholipase C or some component more proximal to sIgM, we stimulated the cells with  $AlF_4^-$ .  $AlF_4^-$  binds to G proteins at the site normally involved in binding GTP. By mimicking GTP, it can then directly activate the G protein in the absence of receptor-generated signals (25). Direct activation of the G protein by  $AlF_4^-$  elicited similar responses in both WEHI-231 and VS2.12-cl.2 with respect to PI hydrolysis (Fig. 5). Whereas anti- $\mu$  was effective only in WEHI-231, identical dose response effects with respect to the  $AlF_4^-$  concentration were observed. Interestingly, somewhat higher levels were observed for VS2.12-cl.2.

These results suggest that uncoupling of sIgM from downstream activation events reflects the uncoupling of this receptor from PI-hydrolysis. Thus, the signal transduction component involved appears to be localized more proximal to sIgM than PI-specific phospholipase C.

*Anti- $\mu$  Is Able to Stimulate Increased  $[Ca^{2+}]_i$  in Both VS2.12-cl.2 and the Wildtype.* Crosslinking sIg on WEHI-231 as well as normal, untransformed B lymphocytes have been shown to cause rapid elevations in intracellular free  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) (13–15). Previous studies have suggested that at least some of this increase is derived from  $IP_3$ -stimulated release from intracellular pools (14, 15). Regulation of the other component, that derived from extracellular sources, is unknown but has been suggested to require PI hydrolysis (9) and may involve sIg-linked conductive  $Ca^{2+}$  channels (26). In Fig. 6 we show that the magnitude and kinetics of the  $Ca^{2+}$  response to anti- $\mu$  is equivalent in VS2.1-cl.2 and WEHI-231. As can be seen, sharp increases in  $[Ca^{2+}]_i$  are observed for both VS2.12-cl.2 and wildtype WEHI-231. The increase in  $[Ca^{2+}]_i$  observed for WEHI-231 is very similar in magnitude to that reported by LaBaer et al. (15) using Fura-2.

Studies shown in Figs. 3 and 4 indicate uncoupling of sIgM signaling from PI hydrolysis and specifically inositol phosphate production in VS2.12-cl.2. To evaluate whether release of  $Ca^{2+}$  from intracellular stores was occurring in VS2.12-cl.2 despite the lack of inositol phospholipid hydrolysis, we stimulated cells in the presence of the calcium chelator EGTA. EGTA was present at levels sufficient to chelate all

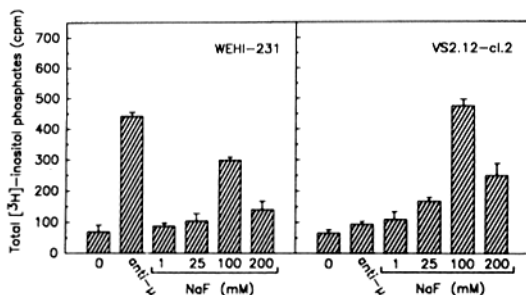


FIGURE 5. Direct G protein activation by  $AlF_4^-$  promotes inositol phospholipid hydrolysis in both VS2.12-cl.2 and WEHI-231. Cells preloaded with [<sup>3</sup>H]inositol as described in Materials and Methods were stimulated with medium (negative control), 10  $\mu$ g/ml anti- $\mu$ , or varying concentrations of NaF. The latter stimuli were in the presence of 25  $\mu$ M  $AlCl_3$ . Stimulation occurred after 45 min in the presence of LiCl, at which time stimulation was terminated and total [<sup>3</sup>H]inositol phosphates was determined as described in Materials and Methods. Values represent means of triplicate determinations  $\pm$  SEM.

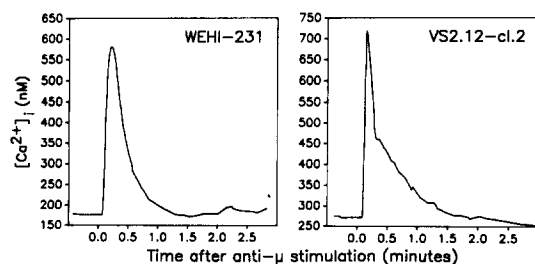


FIGURE 6. Comparison of  $\text{Ca}^{2+}$  responses in anti- $\mu$  antibody-stimulated wildtype WEHI-231 and variant VS2.12-cl.2. Cells were loaded with Fura-2 as described in Materials and Methods. Identical numbers of cells were stimulated with  $10 \mu\text{g/ml}$  of anti- $\mu$  antibody. Relative levels of calcium were determined from which absolute levels were calculated and presented here.

extracellular  $\text{Ca}^{2+}$ . Under these conditions, any elevation in  $[\text{Ca}^{2+}]_i$  must be derived from intracellular pools. Shown in Table I, maximal  $\text{Ca}^{2+}$  responses to anti- $\mu$  stimulation in both wildtype and VS2.12-cl.2 occurred in the presence of added  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . However, elevation in  $[\text{Ca}^{2+}]_i$  was observed for both cell lines in the absence of extracellular  $\text{Ca}^{2+}$ . Note that the responses are lower than that observed in the presence of extracellular  $\text{Ca}^{2+}$ . This difference reflects both that the extracellular influx component has been eliminated and that EGTA treatment results in gradual leaching of  $\text{Ca}^{2+}$  from intracellular stores (15). Nonetheless, it is clear that a significant increase in  $[\text{Ca}^{2+}]_i$  is occurring in VS2.12-cl.2 despite the total absence of detectable inositol phospholipid hydrolysis and  $\text{IP}_3$  generation.

*Bypassing PI Hydrolysis by Direct Activation of Protein Kinase C Results in Inhibition of Proliferation in VS2.12-cl.2.* Previous studies have shown that pharmacologic activation of protein kinase C (PKC) by phorbol diesters causes inhibition of WEHI-231 proliferation, suggesting that inhibition by anti- $\mu$  is linked to the PKC component of sIg signaling (8). If, as our above results suggest, the defect in sIgM signaling in VS2.12-cl.2 occurs before PI hydrolysis, bypassing the biochemical process by di-

TABLE I  
Effect of EGTA on Anti- $\mu$ -induced  $[\text{Ca}^{2+}]_i$  Increase in WEHI-231 and VS2.12-cl.2

Cells	$\text{CaCl}_2$	$\text{MgCl}_2$	$[\text{Ca}^{2+}]_i$		
			Initial	Final	$\Delta$
				<i>nM</i>	
WEHI-231	+	+	$218 \pm 4$	$576 \pm 16$	358
	-	-	$131 \pm 3$	$248 \pm 7$	117
	-	+	$122 \pm 8$	$233 \pm 9$	111
VS2.12-cl.2	+	+	$323 \pm 11$	$725 \pm 25$	402
	-	-	$132 \pm 7$	$212 \pm 5$	80
	-	+	$138 \pm 10$	$225 \pm 12$	87

Stimulation with anti- $\mu$  antibodies ( $10 \mu\text{g/ml}$ ) and determination of  $[\text{Ca}^{2+}]_i$  was accomplished as described in Materials and Methods. Values are representative of the peak response to anti- $\mu$  stimulation (at 15 s after anti- $\mu$  stimulation; see Fig. 6). EGTA was used at 2 mM and was present in all determinations. Where indicated,  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  was added to give final concentrations of 1.3 and 0.9 mM, respectively.

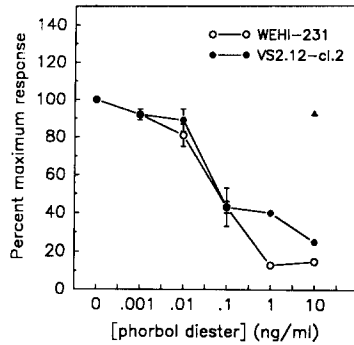


FIGURE 7. PMA downregulates proliferation of VS2.12-cl.2. VS2.12-cl.2 (●) or WEHI-231 (○) ( $2 \times 10^4$ /well) were cultured for 24 h in the presence of varying doses of PMA. At this time, the cells were pulsed with [ $^3$ H]thymidine and cells were harvested 16 h later. Values are the percent response relative to unstimulated cells of each type. Each point represents the mean of triplicate cultures  $\pm$  SEM. Unstimulated values were  $271,443 \pm 7,387$  for WEHI-231 and  $303,356 \pm 13,802$  for VS2.12-cl.2. VS2.12-cl.2 stimulated with 10 ng/ml of 4 $\alpha$ PDD is also shown (▲).

rect activation of PKC should restore the inhibitable phenotype in VS2.12-cl.2. This is indeed what was observed. Both WEHI-231 and VS2.12-cl.2 proliferation is inhibited by PMA (Fig. 7). The inactive phorbol diester analog 4 $\alpha$ PDD was ineffective in inhibiting proliferation of VS2.12-cl.2 further supporting that the observed effect is the result of PKC activation. These results are consistent with the only signaling defect in VS2.12-cl.2 being that before PI hydrolysis. Furthermore, this result is supportive of a direct role for PI hydrolysis in initiating processes culminating in inhibition of proliferation in WEHI-231.

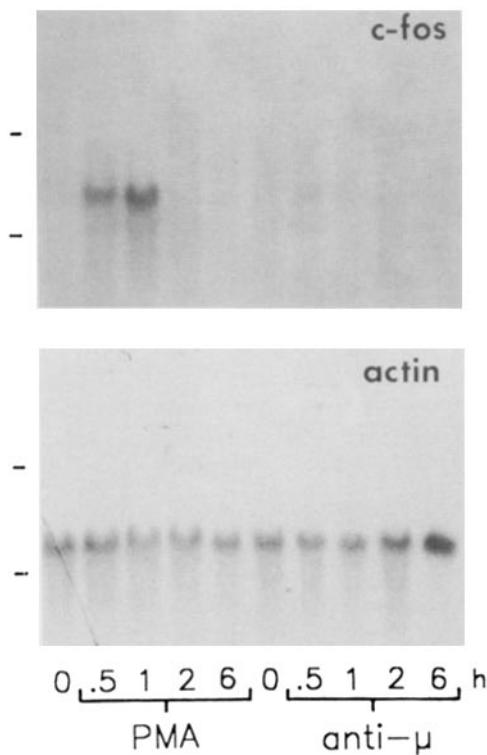


FIGURE 8. PMA but not anti- $\mu$  upregulates *c-fos* expression by VS2.12-cl.2. Total RNA isolated from VS2.12-cl.2 stimulated for varying lengths of time with either PMA (10 ng/ml) or anti- $\mu$  (10  $\mu$ g/ml) was subjected to Northern analysis for *c-fos* or  $\beta$ -actin mRNA expression. The marks on the right side of the figure depict the positions of the 28S and 18S rRNA.



*Upregulation of c-fos Expression and sIg Signaling in VS2.12-cl.2 Are Uncoupled.* Our previous studies (8) have linked induction of the proto-oncogene *c-fos* to transduction of sIg generated signals. Although proliferation is downregulated in WEHI-231 as a result of sIg-generated signals, *c-fos* expression is nonetheless upregulated (8). Consistent with the notion that stimulatory signals transduced via PI hydrolysis are critical to induction of *c-fos* expression after sIg crosslinking are studies shown in Fig. 8. While direct stimulation of VS2.12-cl.2 by phorbol diester causes upregulation of *c-fos* mRNA expression, anti- $\mu$  stimulation is ineffective in this regard. Not shown here, both PMA and anti- $\mu$  stimulation are equally effective in inducing *c-fos* expression in wildtype WEHI-231 (8). These results show that while direct stimulation through sIg is unable to cause increases in *c-fos* expression, bypassing PI hydrolysis by direct stimulation of PKC can restore this coupling in VS2.12-cl.2.

### Discussion

The signaling variant VS2.12-cl.2 was isolated to provide another tool with which to identify critical components linked to sIg signaling as well as to verify linkages between specific activation events and downstream cellular processes. Characterization of this variant indicates that the signaling defect is manifest in some component before PI-specific PLC as direct activation of G protein could potentiate PLC-mediated inositol phospholipid hydrolysis. Furthermore, downstream signaling-related events such as *c-fos* induction and inhibition of proliferation could be potentiated by direct activation of PKC with phorbol diester whereas direct signaling through sIg was ineffective in this regard. Thus, our results corroborate those using pharmacologic agents and temporal studies in postulating direct linkage between PI-hydrolysis and *c-fos* induction, as well as downregulation of proliferation in WEHI-231 (8).

One could argue that the lack of an sIg-coupled response in VS2.12-cl.2 with respect to PI-hydrolysis, *c-fos* induction, and inhibition of proliferation reflects fewer receptors on the mutant versus the wildtype. We feel this explanation is unlikely for several reasons. First, VS2.12-cl.2 is refractory to anti- $\mu$  stimulation of PI hydrolysis even at anti- $\mu$  concentrations as high as 500  $\mu\text{g/ml}$ . Second, Mizuguchi et al. (24) have shown that a  $\gamma$  chain transfectant of WEHI-231 could function as a signal-transducing molecule despite expressing much lower levels (relative to wildtype sIgM levels) of sIg than that observed in VS2.12-cl.2. Last, and most definitive,  $\text{Ca}^{2+}$  responses in VS2.12-cl.2 and WEHI-231 were of similar magnitude, demonstrating that sIgM on VS2.12-cl.2 is able to generate and transduce at least some signals in VS2.12-cl.2.

Regarding  $\text{Ca}^{2+}$  responses in VS2.12-cl.2, we were surprised to observe equivalent responses to that of WEHI-231 despite the fact that we were unable to detect any PI hydrolysis in VS2.12-cl.2. Others have argued that  $\text{Ca}^{2+}$  fluxes resultant from both extracellular influx and intracellular release following sIg signaling require PI hydrolysis (9). These studies involved the use of neomycin to block PI hydrolysis. Whether neomycin, at the concentrations used, has effects on the B lymphocyte other than that of blocking PI hydrolysis is not known. Our results using VS2.12-cl.2 suggest that PI-hydrolysis is not necessary for elevation of  $[\text{Ca}^{2+}]_i$  from extracellular as well as intracellular sources following anti- $\mu$  stimulation or at least the level required is very much less than that normally observed during sIg signaling. It is somewhat surprising that the magnitude of the  $\text{Ca}^{2+}$  response was equivalent in the mu-

tant with respect to the wildtype cell despite the lack of PI hydrolysis, especially given that products of PI hydrolysis have been shown to be capable of causing elevation of  $[Ca^{2+}]_i$  in B lymphocytes (14, 15). These studies suggest that  $Ca^{2+}$  fluxes resulting from products of PI hydrolysis may not represent a significant component of the  $Ca^{2+}$  response in WEHI-231 and perhaps untransformed B lymphocytes as well. Perhaps related to our findings are those of Woldemussie et al. (27). Using a variant of the basophilic leukemic cell line RBL-2H3, they were able to show increased PI hydrolysis in the absence of detectable  $[Ca^{2+}]_i$  increases. One interpretation of this work is that a pathway distinct from PI hydrolysis is mainly responsible for increased  $[Ca^{2+}]_i$  in wildtype RBL-2H3. This conclusion would be consistent with our findings in VS2.12-cl.2 as well.

A molecular definition of the signaling defect in VS2.12-cl.2 is not known. Our observation that aluminum fluoride-mediated G protein stimulation causes PI hydrolysis in this cell suggests that the defect lies proximal to PI-specific phospholipase C activation. That direct activation of PKC by PMA in VS2.12-cl.2 restores the inhibitable phenotype associated with WEHI-231 supports this notion. Therefore, the defect must lie either in a component of the G protein involved in receptor interaction, an intermediary coupling protein between sIg and G protein, or within the signaling domains of sIgM itself. We are currently exploring each of these possibilities. Thus, VS2.12-cl.2 will prove to be a valuable tool with which to analyze sIg signal transduction at the molecular level.

### Summary

A receptor surface Ig (sIg) signaling variant of WEHI-231 was constructed to investigate components and linkages between various signaling events associated with signal transduction through sIg. Unlike the wildtype, crosslinking of sIgM on VS2.12-cl.2 did not result in downregulation of proliferation. Similarly, receptor crosslinking was uncoupled from inositol phospholipid (PI) hydrolysis and upregulation of *c-fos* expression in the variant. The signaling defect in VS2.12-cl.2 appears to be proximal to phospholipase C activation as direct G protein activation by  $AlF_4^-$  triggers PI hydrolysis and bypassing PI hydrolysis using phorbol diester stimulation of protein kinase C restores the inhibitable phenotype and the ability to upregulate *c-fos*. Even more interesting, sIg-linked  $Ca^{2+}$  responses by VS2.12-cl.2 are equivalent to those observed in the wildtype WEHI-231. These latter results suggest that contrary to current thought, sIg-generated signals may not be coupled to  $Ca^{2+}$  fluxes entirely via inositol phospholipid hydrolysis. Thus, VS2.12-cl.2 is a new and powerful tool with which to analyze signaling through sIg at the molecular level.

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