# Identification of a Candidate CD5 Homologue in the Amphibian *Xenopus laevis*[1]

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**We identified a novel T cell Ag in the South African clawed toad (*Xenopus laevis*) by a mAb designated 2B1. This Ag is present in relatively high levels on most thymocytes, approximately 65% of splenocytes, 55% of PBL, and 65% of intestinal lymphocytes, but is rarely seen on IgM$^+$ B cells in any of these tissues. Lymphocytes bearing the 2B1 Ag proliferate in response to stimulation with Con A or PHA, whereas the 2B1$^-$ lymphocytes are reactive to LPS. Biochemical analysis indicates that this Ag is a differentially phosphorylated glycoprotein of 71 to 82 kDa. The protein core of 64 kDa bears both *N*- and *O*-linked carbohydrate side chains. The amino-terminal protein sequence of the 2B1 Ag shares significant homology with both the macrophage scavenger receptor type I motif and the mammalian CD5/CD6 family. The biochemical characteristics and cellular distribution of the 2B1 Ag suggest that it represents the CD5 homologue in *X. laevis.* While T cells constitutively express this highly conserved molecule, *Xenopus* B cells acquire the CD5 homologue only when they are stimulated in the presence of T cells.** ***The Journal of Immunology,* 1995, 155: 4218–4223.**

Since mammals and amphibians shared a common ancestor perhaps 350 million years ago, the comparative analyses of their immune systems is of considerable phylogenetic interest. Among amphibian representatives, immune system parameters have been characterized most extensively in the South African clawed frog, *Xenopus laevis* (1, 2). These studies have shown that cellular and humoral immunity are both well developed in *Xenopus,* and the Ig genes are remarkably similar to their mammalian counterparts in structural configuration and utilization (3, 4). *Xenopus* is also the only ectothermic species in which the classical MHC class I and II genes, as well as non-MHC-linked class I genes, have been characterized (5, 6).

*X. laevis* is an especially interesting model for the study of T cell development because of the dramatic changes that occur during metamorphosis (7). These frogs are also one of the rare vertebrate species that can be easily and completely thymectomized very early in their ontogeny without otherwise affecting development (8). The characterization of *Xenopus* T cells is limited, however, in part because of a paucity of *Xenopus* cell markers that would allow T cell identification and isolation.

Our studies define a T cell Ag in *X. laevis*, the structural and functional analysis of which suggests that it is the amphibian homologue of the CD5 lymphocyte differentiation Ag in mammals (9–13). In contrast to its constitutive expression by T cells, B cells acquire this putative CD5 homologue after stimulation with PMA only in the presence of T cells.

## Materials and Methods

### *Animals*

Larval and adult *X. laevis* were purchased from *Xenopus* I Inc. (Ann Arbor, MI) or raised in our laboratories. *X. tropicalis, borealis, amieti, ruwenzoriensis*, and the LG15 clones (*laevis* × *gilli* hybrids) came from the animal colony of the Basel Institute for Immunology (Basel, Switzerland). Thymectomy of 5-day-old larvae was performed as described (8).

### *Antibodies*

The murine mAb D8 (γ3, κ) recognizing *X. laevis* IgM was produced in our laboratories (L. Gartland and M. D. Cooper, unpublished observations). The 2B1 mAb (γ1, κ) was produced by immunizing BALB/C mice with adult *X. laevis* splenocytes depleted of D8$^+$ cells (14). The F17 (μ,κ) and the AM22 (μ,κ) mAb, which are specific for frog CD8, have been described elsewhere (15, 16). The 2B1 mAb was purified from ascites by affinity chromatography and conjugated to phycoerythrin by Southern Biotechnology (Birmingham, AL). All secondary Abs were purchased from Southern Biotechnology.

### *Cells*

The B3B7 thymoma cell line was cultured at 27°C in a humidified air-$CO_2$ atmosphere (4% $CO_2$) as described (17). Fresh lymphocyte suspensions were prepared in amphibian PBS (65% mammalian PBS + 35% water) containing 10 U/ml heparin (Elkins-Sinn, Cherry Hill, NJ). Single-cell suspensions were obtained by teasing thymus or spleen through a steel mesh, layered over a Ficoll-sodium ditrizoate density gradient (Pharmacia Biotech., Piscataway, NJ) and then centrifuged at room temperature for 10 min at 200 × *g*. Cells recovered from the interphase layer were washed by centrifugation (three times) at 300 × *g* for 10 min at 4°C. Blood was obtained from the dorsal tarsus vein in adult anesthetized frogs with the addition of a heparin solution (20 U/ml in amphibian PBS) to avoid clotting (18), and lymphocytes (PBL) were enriched as described for splenocytes. Peritoneal cells were obtained from tadpoles by rinsing their peritoneal

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cavity with amphibian PBS and subsequent centrifugation (18). Intraintestinal epithelial lymphocytes (IEL)[4] were prepared according to the procedure of Coligan et al. (19). Briefly, the small intestine was rinsed extensively, transected, and cut into 1- to 2-cm pieces that were placed into a flask with an EDTA solution and stirred at 37°C for 30 min. The cell suspension was filtered through a nylon column and then layered on a Percoll gradient. Cells collected from the interphase were washed well before staining. Cell viability was determined by trypan blue exclusion.

### Mitogen activation and proliferation assays

All assays were performed in equal amounts of AIM V medium, Leibovitz L15 medium, and tridistilled water, supplemented with sodium bicarbonate (final concentration 18 mmol/l; all reagents were from Life Technologies, Grand Island, NY) and 10% FCS (HyClone, Logan, UT) at 27°C, in a humidified air-$CO_2$ (4%) atmosphere. For cell activation studies, splenocytes from adult frogs (5 to 8 mo old) were cultured in 24-well plates (Costar, Cambridge, MA) at 2.5 × $10^6$/ml (1 ml/well) with or without PMA (50 ng/ml; Sigma Chemical Co., St. Louis, MO). After 16 h, the PMA was diluted by changing the medium. Proliferation assays were performed on negatively sorted splenocyte subpopulations. Specifically, splenocytes incubated with either the 2B1 mAb or the D8 mAb were washed, stained with biotinylated goat-anti-mouse Ig, and washed again before incubation with MACS-streptavidin-microbeads (10 μl/$10^7$ cells; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min on ice. The cells were washed again and stained with FITC-conjugated streptavidin. After a final wash in amphibian PBS containing 0.01% sodium azide, 5 mM EDTA and 1% BSA, cells were resuspended in 500 μl of this buffer and applied to the top of a Mini-MACS separation column (Miltenyi Biotec) in a MACS separator (flow rate defined by a 23-gauge needle). The nonmagnetic fraction (purity >95% as judged by flow cytometry) was collected and washed three times in culture medium. Cells (2 × $10^5$ well) were incubated for 72 h in 96-well flat-bottom plates (Costar) with either 5 μg/ml Con A, 1 μg/ml PHA (both from Sigma Chemical Co.) or 100 μg/ml LPS (S. typhimurum; Difco, Detroit, MI). [$^3$H]Thymidine (1 μCi; Amersham, Arlington Heights, IL) was added during the final 16 h of culture. Cells were collected using an automated cell harvester and counted by standard liquid scintillation; results obtained from triplicate cultures were expressed as mean counts per minute.

### Immunofluorescence

Indirect and direct immunofluorescence staining were employed for single- and two-color analysis. PMA-activated cells were incubated with normal goat serum before staining to minimize background staining. Cells (1 × $10^6$) were incubated with hybridoma supernatants of either the D8 mAb or an isotype-matched control mAb (20 min on ice), washed, and then reacted with FITC-conjugated goat-anti-mouse-γ3 serum. After washing, the cells were blocked with normal mouse serum and then stained with phycoerythrin-conjugated 2B1 mAb. Relative immunofluorescence intensities of cells with lymphocyte light-scatter characteristics were determined by flow cytometry using a FACScan (Becton Dickinson, Mountain View, CA).

### Immunoprecipitation

Cell surface proteins of 5 × $10^7$ cells (viability >95%) were labeled with 1 mCi $Na^{125}I$ (Amersham) by the lactoperoxidase catalyzed method. After extensive washing, the cells were lysed in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% $NaN_3$, 1 mM pefabloc (Boehringer Mannheim, Indianapolis, IN), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 100 μg/ml chymostatin, 100 μg/ml trypsininhibitor, 20 mM *e*-amino-*n*-caproic-acid, 2 μg/ml antipain, 2 μg/ml aprotinin, 20 mM iodoacetamide (all reagents were from Sigma Chemical Co.), and 0.5 or 1.0% Nonidet P-40 (Calbiochem, La Jolla, CA) for 45 min at 4°C. Following centrifugation (100,000 × *g*, 45 min, 4°C), the lysate was subjected to immunoprecipitation by a solid-phase method (14), and absorbed molecules were eluted with 1% SDS in 50 mM Tris-HCl with or without 5% 2-ME. For dephosphorylation of $^{125}$I-labeled molecules, immunoprecipitates in microwells were incubated in reaction buffer containing 4 U of calf intestinal alkaline phosphatase (Boehringer Mannheim) for 30 min at 37°C (20). For glycolytic enzyme digestions, the immunoprecipitates were eluted with 0.5% SDS and 0.1 M 2-ME for 20 min at 80°C. The reaction conditions were adjusted according to manufacturer's suggestions and the immunoprecipitates were incubated overnight at 37°C in the presence of 50 U/ml N-glycanase. After changing the pH to 6.3 with 1 M glacial acidic acid, the samples were incubated with 3 U/ml neuraminidase for 2 h at 37°C and then incubated overnight with 82 mU/ml *O*-glycanase (all enzymes were from Genzyme, Boston, MA). Undigested controls for each enzyme digestion were treated similarly but without the addition of enzymes. Samples were prepared for electrophoresis by addition of 6 × Laemmli sample buffer (21) containing 2-ME. $^{32}$P-Labeling of the B3B7 cell line was performed essentially as described (22). Briefly, cells (5 × $10^7$/2 ml) were starved for 2 h in phosphate-free RPMI (Life Technologies) diluted 1:3 with tridistilled water containing 2% BSA. After phosphate starvation, 1.25 mCi/ml [$^{32}$P]orthophosphate (HCl free; Amersham) was added and the cultures were incubated for 3 h at 27°C. Cells were then washed and incubated in 1% Nonidet P-40 lysis buffer, containing protease inhibitors and phosphatase inhibitors (0.1 mM $Na_3VO_4$, 0.4 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 0.1% $NaN_3$). Immunoprecipitates were electrophoresed on 7.5% linear or 5 to 15% gradient SDS-PAGE as described (21). For two-dimensional gel analysis, the immunoprecipitates were resuspended in 9.5 M urea, 2% Nonidet P-40, 2% ampholites (pH 3.5 to 10), and 5% 2-ME; separated in a tube gel containing ampholites; and then subjected to a 5 to 15% gradient SDS-PAGE as described (23).

### Protein purification

Cell lysates from $10^{10}$ B3B7 thymoma cells (1 × $10^8$ cells/ml lysis buffer) were obtained as described above. After preclearing twice on Sepharose beads coupled to mAb of irrelevant specificity, the lysate was incubated with 2B1 coupled Sepharose beads (8 mg mAb/ml beads). Following extensive washing, the 2B1 Ag was eluted with 0.2 M glacial acetic acid and the fractions containing protein were combined, evaporated under vacuum, redissolved in Laemmli sample buffer, and separated on a 7% SDS-PAGE. Proteins were electroblotted and visualized by Coomassie blue staining. Bands corresponding to the $M_r$ range of the 2B1 Ag were excised and subjected to protein sequencing.

## Results

### Tissue distribution and ontogeny of 2B1$^+$ cells

Immunofluorescence analysis of lymphoid tissues from young frogs revealed that the 2B1 Ag is expressed on the vast majority of thymocytes (85% ± 3.5; mean ± SD, $n = 3$), and at relatively high levels on 63% ± 4.1 ($n = 6$) of the splenocytes, 52% ± 3.6 ($n = 6$) of PBL, and 65% ± 4.7 ($n = 6$) of the IEL (Fig. 1*A*). The IgM$^-$ B3B7 thymoma cell line expressed high levels of the 2B1 Ag (data not shown). The IgM$^+$ B cell population constituted approximately 30%, 20% and 10% of the lymphocytes in spleen, blood, and intestine, respectively, and these rarely expressed the 2B1 Ag at higher than background levels. B cells in the peritoneal fluid of tadpoles were also examined with the same result (Fig. 1*B*).

As early as the 17th day of larval life, almost 90% of the thymocytes were found to express the 2B1 Ag, and this level of 2B1 expression was maintained beyond metamorphosis. In contrast, the frequency of 2B1$^+$ cells in the spleen gradually increased from approximately 20% of the splenocytes in 17-day-old larvae to reach adult levels of approximately 65% in 90-day-old frogs (Fig. 2). All of the CD8$^+$ cells in the spleen, blood, and IEL populations expressed the 2B1 Ag at relatively high levels irrespective of donor age.

### Species specificity of the 2B1 epitope

Spleen cells from five other *Xenopus* species were examined for reactivity with the 2B1 mAb. Cells from *X. tropicalis*, *borealis*, *amieti*, and *ruwenzoriensis* were nonreactive, whereas spleen cells from LG15 frogs (*laevis* × *gilli* hybrid) expressed the 2B1 Ag at the same intensity and in the same frequency as *X. laevis*. This result suggests that *X. gilli* T cells also express the 2B1 epitope expressed by *X. laevis* T cells and is in agreement with evidence suggesting that these are closely related *Xenopus* species (24).

### Biochemical characterization of the 2B1 reactive molecule

When the 2B1 Ag on B3B7 thymic tumor cells was analyzed by SDS-PAGE, a single major band with the apparent $M_r$ of 71 kDa

[4] Abbreviations used in this paper: IEL: Intraintestinal epithelial lymphocytes; pI, isoelectric point.

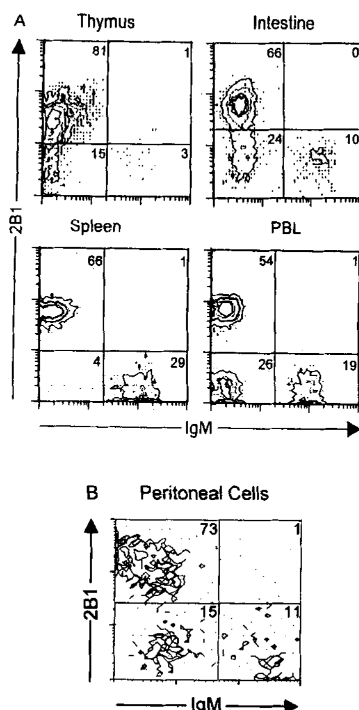



**FIGURE 1.** Distribution of $2B1^+$ cells in lymphoid tissues. Viable thymocytes, IEL, splenocytes, and PBL from adult frogs (*A*) or peritoneal cells from tadpoles (*B*) were analyzed by two-color immunofluorescence for 2B1 and IgM expression. Note that $IgM^+$ lymphocytes in all of these tissue sites do not express 2B1 Ag at high levels, and few express even low levels of Ag.

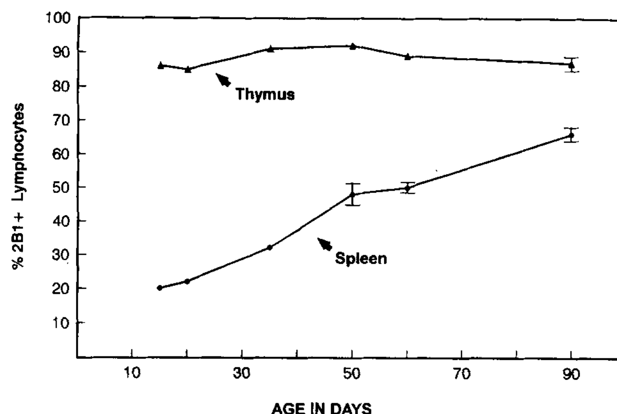



**FIGURE 2.** Ontogeny of 2B1 Ag expression by thymocytes and splenocytes. Lymphocytes of larvae, tadpoles, and young frogs were isolated and stained for cell surface 2B1 expression. Early points represent values for pooled samples from at least 10 animals, whereas individual values (mean ± SD) were determined for larger animals. Metamorphosis occurred between 65 and 90 days.

was observed under nonreducing conditions, and an 82-kDa protein under reducing conditions (Fig. 3*A*). In most experiments, a faint band of approximately 170 kDa was also detected, suggesting a tendency to dimerize under these experimental conditions. Similar results were obtained when the 2B1 Ag was examined on splenocytes from adult frogs (data not shown). In two-dimensional gels, the 2B1 Ag migrated as a broad band with an isoelectric point (pI) range of 5.2 to 6.0 (Fig. 4). To determine the relative $M_r$ of the protein core of this cell surface Ag, the 2B1 immunoprecipitates were digested with glycolytic enzymes. Treatment with neuraminidase and *O*-glycanase yielded a protein band of 78 kDa under reducing conditions (Fig. 3*B*). After *N*-glycanase treatment, two protein bands of 71 and 67 kDa were observed. Digestion with all three enzymes resulted in the appearance of discrete bands of 71 and 64 kDa (Fig. 3*B*).

When B3B7 thymic tumor cells were metabolically labeled with $[^{32}P]$orthophosphate, a single 2B1 reactive molecule of approximately 82 kDa was detected under reducing conditions (Fig. 3*C*). To further elucidate the nature of the two proteins observed after deglycosylation, the 2B1 immunoprecipitates were treated with phosphatase before digestion with the glycolytic enzymes and a single band of 64 kDa was detected under these conditions (Fig. 3*D*).

### *2B1 amino-terminal protein sequence is homologous to mammalian CD5 sequences*

To further characterize the 2B1 Ag, it was purified from lysates of B3B7 cells by affinity chromatography. Following SDS-PAGE separation, two protein bands migrating in the 2B1 $M_r$ range were visualized by Western blot analysis, which presumably represented the phosphorylated and nonphosphorylated forms. Identical 25 residue amino acid sequences were obtained for both bands. When this amino-terminal sequence was compared with the known mammalian CD5 sequences, 10 of 25 residues were shared, including four amino acids that are conserved in all mammalian CD5 proteins (Fig. 5). Alignment of homologous sequences suggested that the amino terminus of the 2B1 Ag is slightly shorter than the mammalian CD5 counterparts. Analysis of the amino-terminal 2B1 sequence also revealed significant homology with the macrophage scavenger receptor type I motif (Fig. 5, *top line*), multiple repeats of which characterize the family of cell surface molecules to which CD5 belongs (25).

### *Mitogen responsive patterns of the $2B1^+$ and $IgM^+$ splenocytes*

Magnetic cell separation was employed to isolate the $2B1^-$ and $IgM^-$ subpopulations of splenic lymphocytes for functional analysis. This negative isolation strategy was employed because it yields relatively pure populations of viable $IgM^+$ and $2B1^+$ (95% purity) cells without deliberate stimulation during the isolating procedure. In the presence of Con A or PHA, the $IgM^-$ cells showed vigorous proliferative responses, whereas the $2B1^-$ were relatively unresponsive to these classical T cell mitogens (Fig. 6). In contrast, the $2B1^-$ cells could be stimulated by LPS, whereas the $IgM^-$ cells were unresponsive. These results confirm the T cell nature of the $2B1^+$ cells.

### *B cells can express the 2B1 Ag when stimulated with PMA in the presence of T cells*

When expression of the 2B1 Ag was examined following PMA stimulation of splenocytes, the $IgM^+$ cells began to express the 2B1 Ag as early as 4 h after PMA stimulation, at first in relatively low levels that increased over a 40-h period of PMA stimulation (Fig. 7*A*). Even at maximal levels, however, the density of the 2B1 Ag on activated B cells was consistently lower than on resting T cells, which acquired even higher 2B1 levels following PMA activation. At the same time that B cells acquired cell surface 2B1 Ag, their IgM Ag receptors were down-modulated by PMA stimulation, presumably as a consequence of protein kinase C-induced phosphorylation and internalization of the B cell receptor complex (26). These dramatic effects of PMA activation were observed when *Xenopus* B cells were stimulated in the presence of T cells

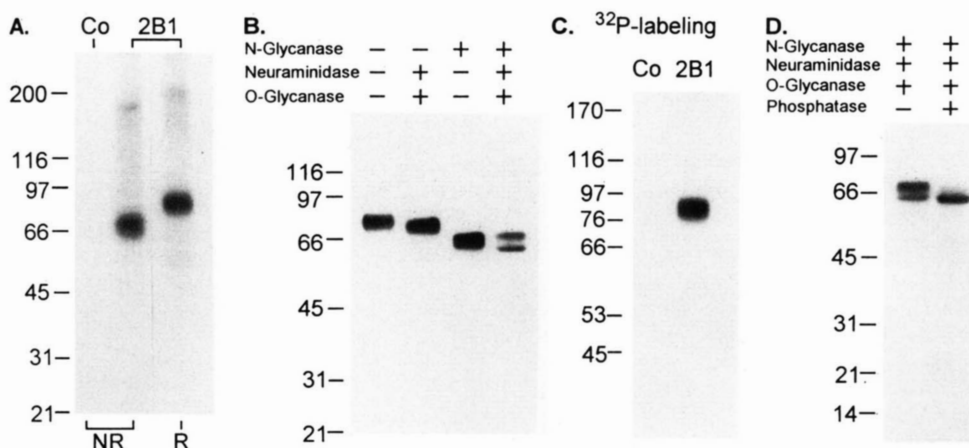



**FIGURE 3.** Biochemical analysis of the 2B1 Ag. B3B7 thymic tumor cells were surface iodinated (*A*, *B*, and *D*) or metabolically labeled with [$^{32}$P]orthophosphate (*C*), and cell lysates incubated with either isotype-matched control mAb (Co) or 2B1 mAb. After the indicated enzyme treatments, immunoprecipitates were analyzed by SDS-PAGE on a 5 to 15% (*A*, *B*, and *D*) or a 7.5% gel under nonreducing (NR; *A*) or reducing (R; *A–D*) conditions. The $M_r$ ($\times 10^3$) of protein standards is indicated.

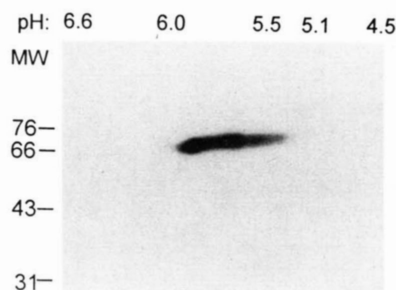



**FIGURE 4.** Two-dimensional gel analysis of the 2B1 Ag. 2B1 immunoprecipitates were separated first by isoelectric focusing and then by electrophoresis on a 5 to 15% SDS-PAGE. pI and $M_r$ ($\times 10^3$) of protein standards is indicated.

and other cell types in the normal spleen. To determine the possible effects of T cells on the B cell activation response, splenocytes were tested from frogs that were thymectomized as 5-day-old larvae. While B cells in these T cell-deficient splenocytes responded normally to PMA stimulation with regard to down-modulation of their cell surface IgM, they failed to express the 2B1 Ag by 16 h (Fig. 7*B*) or after 40 h (data not shown) of PMA treatment.

## Discussion

The *Xenopus* 2B1 Ag appears to have all of the essential characteristics expected for an amphibian CD5 homologue. Mammalian CD5 was initially characterized as a pan-T cell Ag that serves as a thymocyte differentiation marker (9–13, 27). With further study, it became evident that a distinct B cell subpopulation in humans and mice may also express CD5 (28), and recently the CD72 molecule present on all human B cells has been identified as a CD5 ligand (29). CD5$^+$ B cells are relatively abundant in the peritoneal cavity, have remarkable self-renewal capacity, and are considered potential mediators of autoimmune diseases on the basis of their production of polyreactive Abs (28). Interestingly, however, all of the B cells in rabbits are CD5$^+$ (30), whereas CD5 is absent on rat B cells (31). Structurally, the CD5 Ag belongs to a newly defined family characterized by the macrophage scavenger type I motif, which includes the CD6 Ag and the WC1 Ag found on ruminant $\gamma\delta$ T cells (25, 32, 33). In spite of their relatively extensive characterization, the function of these molecules is still largely unknown.

The *Xenopus* 2B1 molecule is expressed on virtually all thymocytes from early larval stages onward, and thus, like mammalian CD5 (34), is an early thymic differentiation Ag. In peripheral tissues, lymphocytes belonging to the IgM$^-$ lymphocyte population express the 2B1$^+$ Ag in relatively high levels. The 2B1$^+$ population includes both CD8$^+$ and CD8$^-$ lymphocytes, thus suggesting that both cytotoxic and helper T cells express the 2B1 Ag.

To test the idea that the 2B1 Ag is a pan-T cell marker in *Xenopus*, we examined the response of lymphocyte subpopulations to classical T or B cell mitogens. When enriched by removal of IgM$^+$ splenocytes, the 2B1$^+$ cells proliferated well in response to classical T cell mitogens, Con A and PHA, but were unresponsive to the B cell mitogen LPS. In contrast, *Xenopus* B cells, enriched by removal of the 2B1$^+$ cells, responded with proliferation to LPS stimulation but were unresponsive to Con A and PHA. These results further attest to the T cell nature of the IgM$^-$ population of *Xenopus* 2B1$^+$ lymphocytes.

In ontogenetic studies, thymocyte expression of the 2B1 Ag was consistently high at all ages, whereas the frequency of 2B1$^+$ cells in the spleen increased steadily from approximately 20% in 17-day-old tadpoles to reach adult levels of approximately 65% around the time of metamorphosis. The immune system repertoire of *X. laevis* has to be reshaped during metamorphosis to accommodate novel self and environmental Ag. This is accomplished by, as yet, poorly understood mechanisms that temporarily render peripheral T cells unresponsive to stimulation. In addition, an adult population of T cells is generated via a new wave of progenitor cells entering the thymus during metamorphosis (35). Although the total number of T cells in both thymus and spleen declines during metamorphosis (1), our findings, together with more detailed observations of thymocytes and splenocytes during the metamorphic period, indicate that the relative frequency of 2B1$^+$ cells remains unaffected, perhaps reflecting the gradual replacement of larval T cells by newly formed adult lymphocytes.

Stimulation of mammalian B cells with the protein kinase C activator PMA leads to CD5 expression (36, 37). When *Xenopus* splenocytes were activated with PMA, IgM$^+$ B lymphocytes were also induced to express the 2B1 Ag, albeit at lower levels than on T cells, while down-modulating their cell surface IgM. Interestingly, PMA-activated B cells from athymic frogs exhibited IgM down-modulation, but they did not express the 2B1 Ag. This suggests that *Xenopus* B cells require T cell influences to express the

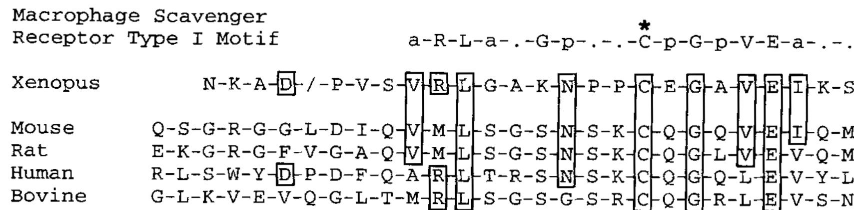



**FIGURE 5.** 2B1 amino-terminal sequence is homologous to mammalian CD5 and macrophage scavenger receptor type I motif. Twenty-five amino acids obtained by peptide sequencing of 2B1 immunoprecipitates were aligned to macrophage scavenger receptor type 1 motif (Ref. 25) and CD5 sequences of mouse (39), rat (31), human (40), and bovine (41). Gaps (slash) are introduced to maximize homology and periods (period) denote nonconserved amino acids. a, Aliphatic residue, *p* = polar residue; *, Cysteine, only present in CD5/CD6 subfamily.

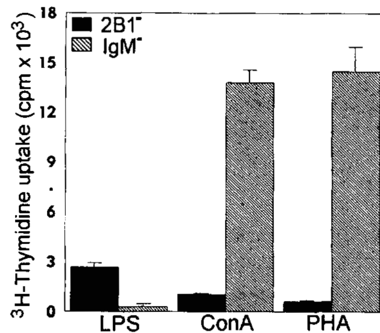
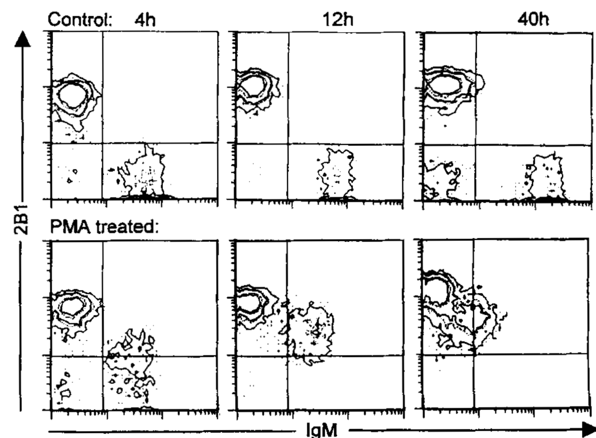



**FIGURE 6.** Mitogen responsiveness of $IgM^-$ and $2B1^-$ splenocytes. 2B1 or IgM depleted populations were obtained by negative magnetic sorting. After staining with respective mAb and cell separation on a mini-MACS column as described in *Materials and Methods*, splenocytes ($2 \times 10^5$/well) were incubated in triplicate with mitogens indicated. Proliferation was measured in cpm after 72 h in culture by [$^3$H]thymidine incorporation and expressed as mean ± SD (minus background).



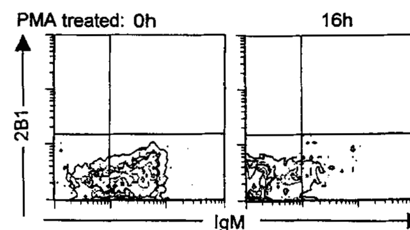




**FIGURE 7.** B cells can express 2B1 Ag after PMA stimulation in the presence of T cells. Splenocytes ($2.5 \times 10^6$/ml) from normal adult frogs (*A*) or thymectomized frogs (*B*) were incubated with 50 ng/ml PMA (PMA treated) or with media (Control) and then analyzed for 2B1 (PE) and IgM (FITC) cell surface expression as a function of time.

2B1 Ag following activation. The selective acquisition of the 2B1 Ag by B cells activated together with T cells, other T cell Ag not being acquired, suggests that the activated B cells make their own 2B1 Ag. However, we have not yet performed a biosynthetic analysis of the activated B cells to test this assumption.

Biochemical characterization of the 2B1 Ag indicates that it is a single-chain transmembrane glycoprotein of approximately 71 kDa under nonreducing conditions. The native molecule has a considerable charge heterogeneity reflected by its isoelectric distribution from pI 5.2 to 6.0. The $M_r$ of mammalian CD5 Ag are reported to be 62 to 72 kDa and also exhibit considerable charge heterogeneity (9–13).

The higher $M_r$ observed after reduction of the 2B1 molecule (82 kDa) probably reflects the presence of intramolecular disulfide bonds. Another important characteristic of CD5 is constitutive phosphorylation (38), which was also demonstrated for the 2B1 Ag. In our experiments, glycosidase treatment of the 2B1 Ag resulted in the appearance of two distinct bands of approximately 71 and 64 kDa. Treatment of the deglycosylated proteins with phosphatase revealed that the 71-kDa protein was the phosphorylated form of the 64-kDa band. Thus, after removal of carbohydrates and dephosphorylation, the protein core size of the 2B1 determinant is approximately 64 kDa. An abundance of N-linked sugars apparently accounts for approximately 18 kDa of the mature glycoprotein, as is the case for the mouse and human CD5 molecules (39, 40).

Since mammalian CD5 and CD6 comprise a subfamily of the molecules containing the macrophage scavenger motif, we have considered the possibility that the *Xenopus* lymphocyte-specific 2B1 Ag could be a CD6 homologue rather than a CD5 homologue. However, several characteristics suggest that the 2B1 Ag more closely resembles mammalian CD5. 1) The 2B1 expression pattern for *Xenopus* thymocytes is very characteristic for mammalian CD5, whereas CD6 is found only in low density on a subpopulation of mammalian thymocytes (42). 2) While the $M_r$ of 2B1 is very close to that of mammalian CD5, it is quite different from that of mammalian CD6, which is 130 kDa (43). 3) CD6 has not been found on activated B cells. While the sequence comparison of the N-terminal amino acids of 2B1 cannot discriminate between mammalian CD5 and CD6 relationships, current efforts to clone the 2B1 gene could formally resolve this issue. Interestingly, the 2B1 N-terminal sequence lacks two serines and a glutamine that are present in mammalian CD5 sequences. While this difference is not surprising given the great phylogenetic distance between amphibians and mammalians, it should be informative in determining the most highly conserved elements of these lymphocyte-specific proteins.

In conclusion, our analysis of tissue distribution, biochemical properties, protein sequence, and functional studies suggest that

the 2B1 molecule represents the homologue of the CD5 Ag. The 2B1 mAb that recognizes this *Xenopus* CD5 candidate also serves as a pan-T cell marker that can be used to study development of the *Xenopus* immune system. As in mammals, *Xenopus* B cells can also express this CD5 homologue provided that they are activated in the presence of T cells. The remarkable conservation of this molecule over 350 million years points to its functional importance, the precise nature of which remains unknown.

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