Characterization of a CD38-like 78-Kilodalton Soluble Protein Released from B Cell Lines Derived from Patients With X-linked Agammaglobulinemia

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Abstract

Studies on murine B lymphocytes showed that Bruton's tyrosine kinase mediates signal transduction induced via CD38, a nonlineage-restricted 45-kD ectoenzyme. This signaling is defective in B cells from X-linked immunodeficient mice affected with the analogue of human X-linked agammaglobulinemia (XLA). We performed a structural and functional analysis of CD38 in XLA and other immunodeficiencies, using EBV-immortalized B cells derived from such patients. Membrane CD38 was not significantly different from controls in structure, epitope density, enzymatic activity, and internalization upon binding of agonistic mAbs. Meanwhile, an increased release of soluble CD38 from XLA cells was observed: immunoprecipitation from XLA culture media yielded a protein of ~ 78 kD (p78), reacting also in Western blot and displaying both enzymatic activities and a peptide map similar to membrane CD38. Soluble forms and homotypic aggregations of CD38 were documented in different cell models and by crystallographic analysis of the *Aplysia* ADP-ribosyl cyclase, the ancestor of human CD38. p78 might represent the product of an altered turn-over of membrane CD38, a starting point for studying its association with Bruton's tyrosine kinase and its role in XLA and other B cell immunodeficiencies. (*J. Clin. Invest.* 1998. 101: 2821–2830.) **Key words: CD38 • immunodeficiency • ADP-ribosyl cyclase • ectoenzyme • soluble receptor**

Introduction

Human CD38 is a 45-kD type II glycoprotein expressed in different lineages at various stages of differentiation (1). Binding of the CD38 molecule by selected agonistic mAbs is followed by delivery of activation and proliferation signals (2), probably mimicking the interactions occurring physiologically with natural ligand(s) (3, 4); one of these ligands has been recently identified as CD31 (5). The description of these features of a surface receptor ruling cell to cell interactions was enriched recently by the finding that the extracellular domain of the protein bears a catalytic site able to convert NAD^+ to nicotinamide and cyclic adenosine diphosphate ribose (cADPR)[1] (cyclase activity) and, simultaneously, cADPR to ADPR (hydrolase activity) (6). Therefore, human CD38 is an ectoenzyme with peculiar catalytic activities, which result in the synthesis of a product involved in the regulation of cytoplasmic Ca^{2+} currents (7). Further complexity beyond this topological paradox is encountered when one considers the notion that surface CD38 may exist in a soluble form of ~ 39 kD (p39), detectable in in vitro models and in vivo biological fluids from normal or disease conditions (8). In contrast, there is ample evidence that in certain conditions, for instance in vitro upon mAb or ligand binding, the molecule can undergo internalization rather than shedding (9). Furthermore, there are also reports on lateral homotypic and heterotypic organization of the molecule. Indeed, Umar et al. described a high–molecular weight form of CD38, namely p190, on all-*trans*-retinoic acid (ATRA)-induced HL60 cells (10), known to overexpress CD38 upon ATRA treatment (11). Oligomerization processes also take place on erythrocyte membranes, either induced by NAD^+ or GSH (12).

A link between selected diseases (e.g., leukemia and myeloma, Alzheimer's disease, and AIDS [among others]) and CD38 expression and function has been sought with the aim of defining diagnostic and possibly therapeutic markers on one hand and, on the other, of helping to throw light on the functions exerted by the molecule in vivo (13). Interest in B cell immunodeficiencies stems from functional studies on X-linked immunodeficiency (xid) syndrome, the murine analogue of X-linked agammaglobulinemia (XLA), both diseases sharing molecular defects involving Bruton's tyrosine kinase (btk) (14). Mouse btk is an integral component of the CD38 transduction pathway: CD38 ligation not only induces btk phosphorylation, but also triggers proliferation and activation signals on normal murine B cells. None of these effects is seen on B cells obtained from xid mice, in spite of unaltered membrane CD38 levels (15–17).

Currently, not much information is available on altered CD38 expression and function in human congenital immunodeficiencies. This study reports that expression, enzymatic activities, and internalization of membrane CD38 are apparently unaltered in immunodeficient B cells; however, XLA cells are

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1. *Abbreviations used in this paper:* ATRA, all-*trans*-retinoic acid; btk, Bruton's tyrosine kinase; cADPR, cyclic adenosine diphosphate ribose; cGDPR, cyclic guanine diphosphate ribose; ^{125}I-IB4, ^{125}I-labeled IB4; NGD^+, nicotinamide guanine dinucleotide; PE, phycoerythrin; xid, X-linked immunodeficient; XLA, X-linked agammaglobulinemia.

marked by the release of a 78-kD molecule (p78) that shares structural and functional homologies with membrane CD38. The finding of a soluble and active form of a CD38-like structure may reflect either a qualitative or quantitative defect in CD38 turnover, possibly playing an accessory role in the pathogenesis of selected immunodeficiencies involving the B compartment.

Methods

Cell lines. Continuous lymphoblastoid lines were developed by EBV immortalization of B lymphocytes obtained both from patients affected with different immunodeficiencies and healthy donors, and established in long-term cultures. Cell lines considered in this study were from patients affected with XLA, namely, P5.00, P6.30, P8.70 (kindly provided by Dr. M.E. Conley, St. Jude Children Hospital, Memphis, TN), and CAR-EBV. All lines were CD38$^+$. Control immunodeficient lines included severe combined immunodeficiency, common variable immunodeficiency, and Wiskott-Aldrich syndrome. DNS-EBV was obtained from a female patient affected with an agammaglobulinemic syndrome reported previously in other patients as phenotypically identical to XLA (18, 19). Controls included 15 different cell lines obtained from normal B cells with the same procedure (20): out of these, CTR-EBV and LG2 were CD38$^+$, while LG14 was CD38$^-$. All culture supernatants, as well as media from metabolic radiolabeling, were centrifuged and passed through a 0.2 μm syringe filter before undergoing other procedures, to rule out the presence of dead cells or derived debris. Spent medium from cultures of multiple myeloma cells, known to release large amounts of soluble CD38 (p39) (8), was also used as positive control.

Antibodies. mAbs used included affinity-purified IB4 (21), IB6 (22), OKT10 (all anti-CD38), O1.65 (anti-HLA class I heavy chain) (23), and CBT8 (anti-CD8) (24). Anti-CD39 was from Serotec (Oxford, UK), while anti–human IgM antiserum was from Organon Teknika/Cappel (Durham, NC). Phycoerythrin (PE)-conjugated Leu-17 (anti-CD38) was from Becton Dickinson (Milan, Italy). FITC- and PE-labeled A1 (anti-CD39) were kindly provided by Dr. G. Aversa (DNAX, Palo Alto, CA).

Flow cytometry analysis. Cells were incubated with the primary antibody for 20 min at 4°C. After washing, an FITC-conjugated anti–mouse Ig was added for 20 min. Cells were then washed again and analyzed on a FACSort® (Becton Dickinson) using Lysis II software.

Modulation experiments and shedding, and endocytosis assays with ^{125}I-labeled IB4 (^{125}I-IB4). Cells for modulation experiments were cultured for 48 h in the presence of either IB4 mAb or isotype-matched CBT8 mAb, washed twice, and analyzed by direct immunofluorescence and flow cytometry analysis. Residual mAb bound to the cell surface was monitored by incubation with FITC-conjugated goat anti–mouse Ig.

^{125}I-IB4 was used in acid elution assays, as described previously (25). Briefly, cells were resuspended in RPMI 1640 with 0.2% BSA and 10 mM Hepes (pH 7.4), and incubated with ^{125}I-IB4 for 30 min on ice, followed by the addition of anti–mouse IgG mAb. Cells were washed twice and incubated for 4 h at 37°C. Supernatants were then collected and counts per minute measured with a Cobra II γ-counter (Packard, Meriden, CT). After two washings, cells were either incubated in RPMI 1640 medium containing 10 mM Hepes (pH 7.4) or RPMI 1640 buffered to pH 2.0 with 20 mM glycine–HCl for 5 min, washed again, and counted in a γ-counter. The proportions of internalized and shed ^{125}I-IB4 were determined as the ratio between acid resistant (pH 2.0) and supernatant, respectively, and total counts (pH 7.4 plus shed fraction). Each sample was assayed in triplicate.

Binding inhibition experiments. IB4 mAb was iodinated as described previously (26). Spent media from XLA, multiple myeloma, or control cell cultures were preincubated overnight with the radiolabeled mAb. CD38$^+$ cells were then added for 2 h, washed twice, and counts per minute of bound mAb counted.

Immunoprecipitation and electrophoretic analysis. Cells ($\sim 2 \times 10^7$) were washed in PBS, preincubated in methionine/cysteine–free medium for 20 min, and then pulsed for 4 h with 50 μCi/ml of [^{35}S]methionine/[^{35}S]cysteine (specific activity 1,174 Ci/mmol) (ICN, Irvine, CA). For subsequent immunoprecipitation, cells were washed with ice-cold PBS and lysed in PBS containing 3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and 1mM PMSF, as described previously (10). Labeling media were filtered and then immunoprecipitated; supernatants from unlabeled cells were concentrated (to approximately one-fourth of the initial volume) using Centricon filters (Amicon, Beverly, MA). Samples were successively precleared by means of an irrelevant mAb covalently conjugated to Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Anti-CD38 mAb (IB4, IB6, or OKT10) was then added (15 μg), followed by the addition of a rabbit anti–mouse Ig mAb. Immunocomplexes were then recovered with protein G–Sepharose (Pharmacia) for 3 h at 4°C, washed twice, and extracted by boiling in SDS sample buffer. Proteins were analyzed on a 10% SDS-PAGE, and the gels were either silver-stained using Quick-silver (Amersham Italia, Milan, Italy) or exposed for autoradiography. Sequential immunoprecipitation experiments were performed by substituting the irrelevant mAb in the preclearing step with either anti-CD39 mAb or anti–human IgM antiserum.

Western blot analysis. Partially purified p39, p78, and purified gp120 (Chiron, Emeryville, CA) were separated by electrophoresis and transferred onto nitrocellulose. After overnight blocking with 5% low-fat dried milk in 0.1% Tris–buffered saline Tween, the membrane was probed with IB4 mAb and the reaction detected using a horseradish peroxidase–conjugated anti–mouse Ig mAb and ECL reagents (Amersham).

p78 purification. The protein was quantitatively immunoprecipitated from culture supernatants as described above, and run on an 8% SDS-PAGE. The gel was stained with 0.25 M KCl in 1 mM DTT (27), and the bands of interest were excised and eluted in PBS, with sequential incubations at 4°C and 37°C. As controls, membrane CD38 was purified from U937 cells and p39 from a peritoneal ascites obtained from a patient with multiple myeloma (8). Purified gp120 was added as a negative, soluble control.

Comparative peptide mapping by limited proteolysis. Membrane CD38- and p78-purified proteins were ^{125}I-labeled with the chloramine T method (26) and free ^{125}I was eliminated by dialysis against PBS: the specific activity achieved was $\sim 10^5$ cpm/μg. Approximately 200,000 cpm of each protein was boiled in SDS sample buffer and loaded on a 15% SDS-PAGE. *Staphylococcus aureus* V8 protease (Sigma Italia, Milano, Italy) was prepared in a buffer consisting of 125 mM Tris (pH 6.8), 1 mM EDTA, 2.5 mM DTT, and 0.1% SDS at a final concentration of 0.5 μg/ml and directly overlaid on samples in the gel wells (28). When the dye line had migrated half the distance in the stacking gel, the power was disconnected and digestion was allowed to proceed for 5 min. The run was then completed and the gel soaked in 1 M salicylic acid for 30 min, dried, and exposed for fluorography.

Determination of cyclase and hydrolase activity. p78 and p39 proteins were immunoprecipitated from supernatants (see above), concentrated previously using a 50-kD cut-off for XLA samples, and a 30-kD cut-off for the myeloma sample: thus, possible activity due to undetectable amounts of p39 in XLA culture media was excluded by ultrafiltration. As negative controls, culture media from multiple myeloma cells were concentrated with a 50-kD cut-off membrane before immunoprecipitation. Both the conventional and the high–molecular weight forms of CD38 (p45 and p190, respectively) were partially purified from DNS-EBV lysates. Immune complexes were gently eluted with 100 mM glycine–HCl (pH 2.8) and immediately neutralized with 1 M Tris. Approximate amounts of the partially purified protein were estimated by running parallel samples on SDS-PAGE. After silver staining, the bands of interest were scanned and compared to known amounts of protein standards. Cyclase and cADPR hydrolase activities of either immunoprecipitated proteins or total concentrated supernatants were assayed at 37°C in the presence of 10 mM Tris-HCl

(pH 7.4) and 0.2 mM nicotinamide guanine dinucleotide (NGD^+) or 0.1 mM cADPR, respectively, in a total volume of 200 μl. At various times (15, 30, and 60 min), 50 μl-aliquots were withdrawn, deproteinized with TCA, briefly centrifuged, and the TCA was removed from the supernatants with diethyl ether. The samples were then subjected to reverse phase HPLC as described previously (29). Cyclase activity was also determined on whole cells. The incubation was done for 1 h at 37°C in the presence of 100 μM NGD^+ (30). The reaction was stopped by adding 5% SDS and fluorescence intensity measured with a spectrophotometer at 310 nm excitation and 400 nm emission.

Results

Membrane CD38 expression. Immunodeficient B cell lines did not display significant differences in CD38 expression when compared to EBV lines derived from healthy individuals, even though CD38 levels were higher in some of them (e.g., P5.00 and P8.70) than in their normal counterparts (Fig. 1 *A*). CD21, which is the EBV and CRII receptor, was included as control because of its proven lateral association with CD38 on B cells (31), and it demonstrated comparable levels of expression (data not shown). CD38 levels were also comparable in terms of surface cyclase activity (Fig. 1 *B*).

CD38 shedding and internalization. Modulation of the CD38 molecule from the membrane was initially studied by FACScan® analysis (Fig. 2 *A*). The binding of IB4 mAb to CD38 expressed on B cell membranes induced a consistent downmodulation of the molecule, quantitatively comparable in immunodeficient and normal B cells. The observed effects cannot be secondary to epitopic competition between IB4 and PE-conjugated Leu17, since very low levels of surface IB4 mAb were detectable after 48 h, as highlighted by staining with an FITC-labeled goat anti–mouse Ig (data not shown).

Shedding and internalization ratios of bound ^{125}I-IB4 were further studied by acid elution assay (Fig. 2 *B*). This procedure allows the removal of $\sim$ 95% of membrane-bound radioactivity, without significantly affecting cell viability or the percentage of internalized ^{125}I-labeled mAbs (32). The differences in endocytosed fractions between immunodeficient and control cell lines proved negligible. On the contrary, shedding was more prominent in cell lines derived from patients. In conclusion, internalization and shedding processes occur both in normal or pathological B cells, while shedding rates are increased in XLA and related conditions. Moreover, these events can be triggered by CD38 ligation by specific mAbs, likely mimicking the interactions occurring with the natural ligand(s) (32).

Binding inhibition and supernatant cyclase activity. The test selected relied upon the ability of different culture media to inhibit ^{125}I-IB4 mAb binding to $CD38^+$ cells (Fig. 3 *A*). The

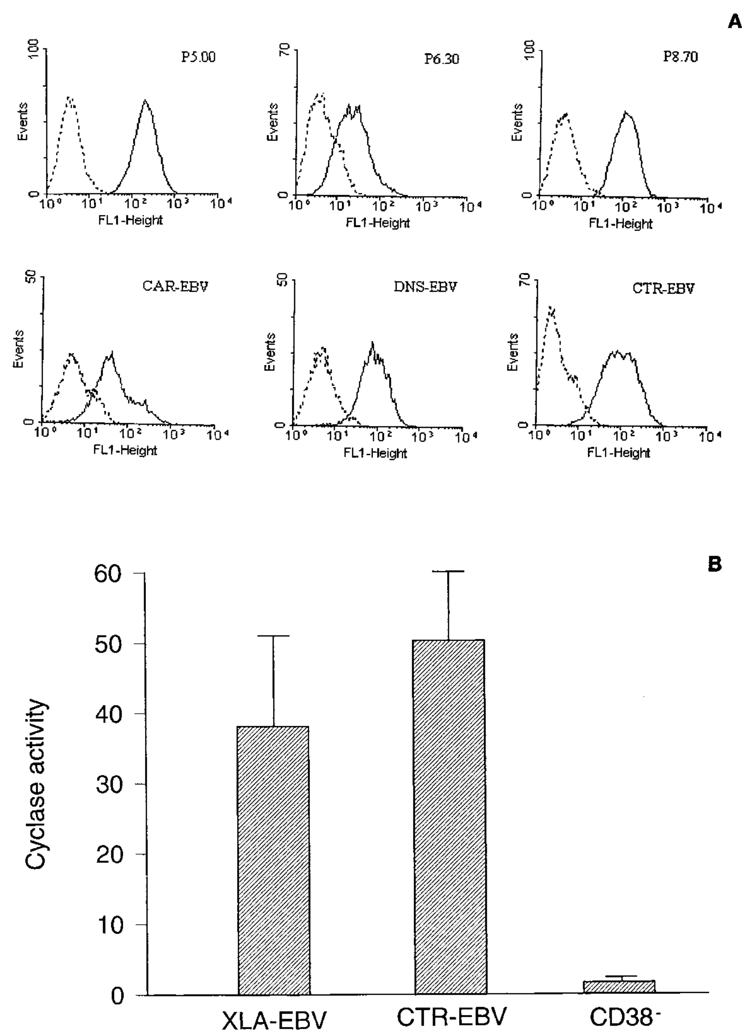
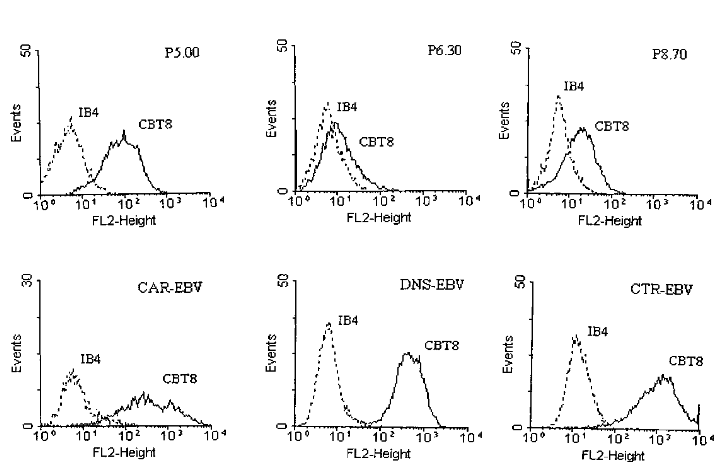


Figure 1. Comparison of membrane CD38 expression and enzymatic activity between XLA and control EBV lines. (*A*) One-parameter flow cytometric (FACScan®) analysis of CD38 expression on immunodeficient and control lines. Cells were stained with an FITC-conjugated anti–mouse Ig after incubation with IB4 mAb (*dashed lines*) or isotype-matched control (*solid lines*). *x* axis = *fluorescence intensity/cells*; *y* axis = *number of cells registered/channel. Number of cells tested* = 10,000. (*B*) ADP-ribosyl cyclase activity detected on whole cells, using NGD^+ as a substrate. Enzyme activity is expressed as absorbance at 410 nm emission and 300 nm excitation. Results refer to a representative experiment performed in duplicate with different XLA–EBV cell lines.

A



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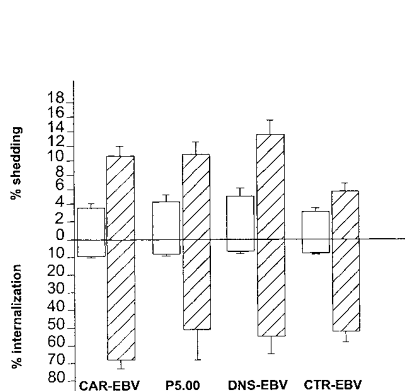

Figure 2. IB4 mAb endocytosis and shedding. (*A*) CD38 downmodulation after a 48-h culture in the presence of either IB4 or irrelevant idiotype-matched CBT8 mAb, as highlighted by PE-conjugated Leu-17. *x* axis = *fluorescence intensity/cells*; *y* axis = *number of cells registered/channel. Number of cells tested* = 5,000. (*B*) Internalized and shed fractions of ^{125}I-IB4 mAb were compared between different cell lines, either at 0 h (*white bars*) or 4 h (*hatched bars*). The graphic shows the ratios of acid-resistant or supernatant counts to the total cell and supernatant-associated label (as described in Methods). Results refer to a representative experiment performed in triplicate.

presence of supernatants (2 d-culture, range 200–400 μl) from XLA–EBV cells reduced the binding of ^{125}I-IB4 mAbs to the cell surface by ~ 50%, as compared to controls; moreover, the effect was dose-dependent. The observation that p78-rich supernatants inhibit ^{125}I-IB4 mAb binding more efficiently than those containing p39 (e.g., myeloma culture media) is in line with the possibility that p78 might contain twice the amount of epitopes available for mAb ligation. Further, cyclase activity was determined on different concentrated supernatants (Fig. 3 *B*), showing a clear correlation between the degree of enzymatic activity and the ability to inhibit ^{125}I-IB4 mAb binding.

Identification of the soluble molecule. Immunoprecipitation experiments performed with IB4 mAb on culture supernatants from metabolically labeled XLA cells showed a 78-kD band, both under reducing and nonreducing conditions (Fig. 4 *A*, lane *2*; nonreducing not shown). Thus, the 78-kD soluble form is, indeed, a product of cellular synthesis, being obtained after internal radiolabeling. Additionally, IB4 mAb immunoprecipitated both the 45- and 190-kD high–molecular mass form of CD38 from the cell membrane fraction, both in XLA (Fig. 4 *A*, lane *1*) and control B-EBV cell lysates (Fig. 4 *A*, lane *3*), as well as in other related B cell immunodeficiency samples (e.g., DNS–EBV). The main difference was that the culture medium obtained after metabolic labeling of the EBV lines from healthy individuals and control immunodeficiencies did not yield any detectable amount of the soluble p78 molecule (Fig. 4 *A*, lane *4*). Immunoprecipitation from LG14 cells (CD38^{-}) did not show any band either from the membrane fraction or the labeling supernatant (data not shown). The 78-kD band was also observed by immunoprecipitating culture media of unlabeled XLA cells with three different anti-CD38 mAbs, namely IB4, IB6, and OKT10 (Fig. 4 *B*, lanes *1–3*). Cross-specificity was excluded by using the isotype-matched irrelevant O1.65 mAb (anti-HLA class I heavy chain) (Fig. 4 *B*, lane *4*). Cross-reactivity with bovine components of the medium was ruled out by treating normal culture media with IB4 mAb under the same conditions (Fig. 4 *B*, lane *5*).

Anti-CD38 mAbs recognized the partially purified p78 in Western blots (Fig. 4 *C*, lane *1*). A reaction was also observed with partially purified p39 (Fig. 4 *C*, lane *2*), while no signals were reported using either IB4 mAb on an irrelevant protein (Fig. 4 *C*, lane *3*) or the isotype-matched O1.65 mAb on partially purified p78 (Fig. 4 *C*, lane *4*). This finding indicates that IB4 mAb recognizes a linear epitope on p78, as is the case for CD38, offering further proof that the two molecules display a similar immunoreactivity (33).

CD39 and surface IgM were further investigated as sources of possible pitfalls to the correct interpretation of the results by unexpected cross-reactions with CD38 mAbs. CD39 is a 78-kD surface protein and a marker of EBV-transformed cell

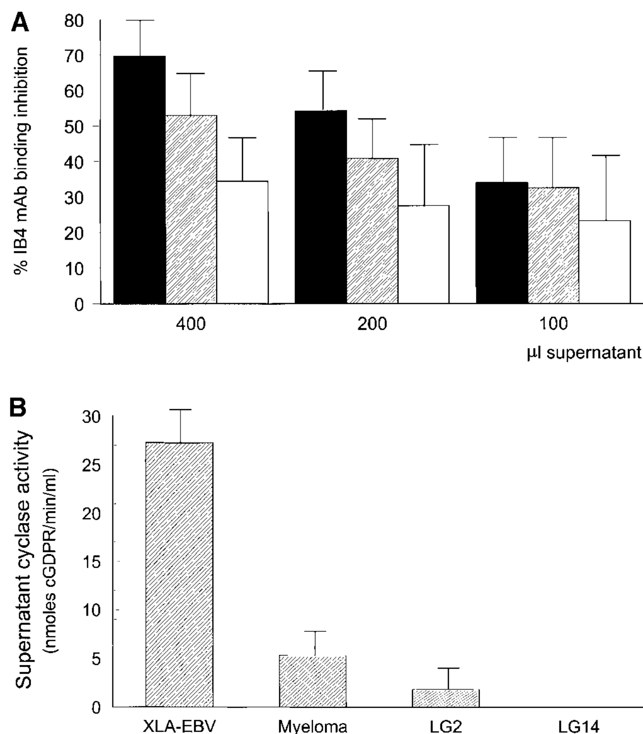

Figure 3. Inhibition of ^{125}I-IB4 mAb binding to target cells and cGDPR production by supernatants of XLA, myeloma, and control cultures. (*A*) Increasing volumes of XLA supernatant (*black bars*), myeloma supernatant (*hatched bars*), and control supernatant (*white bars*) were preincubated with ^{125}I-IB4 mAbs and subsequently added to U937 cells. The percent binding inhibition was calculated after background subtraction. Results refer to a representative experiment performed in triplicate. (*B*) The same supernatants were assayed for cyclase activity after concentration. Results refer to a representative experiment performed in duplicate.

lines highly expressed by all the EBV lines studied (34), which displays lateral association with CD38 (35). No soluble forms are reported for this molecule. Other instances include functional complementarities with CD38, as is the case for PC-1 (36). Lack of cross-reactivity was established by the dissociation in terms of expression between CD39 (expressed by all the EBV lines) and CD38 (whose expression is only present in the majority of EBV lines). Indeed, LG2, a CD38$^+$ EBV line used as a model for the study of CD39 (37), did not generate any p78 band in immunoprecipitation experiments. Further, sequential immunoprecipitation ruled out any epitopic similarity between CD38 and CD39 (Fig. 5 *A*, lanes *1* and *2*).

Mistaking p78 for an IgM heavy chain was also ruled out by sequential immunoprecipitation (Fig. 5 *A*, lanes *3* and *4*). Further evidence of the differences between p78 and IgM heavy chains came from XLA lines virtually negative for surface IgM (e.g., CAR-EBV) (Fig. 5 *B*), whose culture supernatants yielded significant amounts of an immunoprecipitable 78-kD band (Fig. 5 *C*). Moreover, IB4 mAb immunoprecipitation from metabolically radiolabeled peripheral blood lymphocytes obtained from a patient with Waldenstrom macroglobulinemia (with 78% CD19$^+$ lymphocytes and producing high amounts of IgM) did not show any soluble form released in the medium; however, both p190 and p45 were precipitated from the membrane fraction (data not shown). Similar results were obtained from multiple myeloma cell lines (e.g., U266 and SK-MM1) as well as from pre-B leukemia lines (e.g., Nalm-6 [38] and BV173 [39]), and from preparations of lymphocytes obtained from umbilical cord vein.

Comparative peptide mapping between membrane CD38 and p78. The results of peptide mapping of p78 and CD38 with the serine protease *S. aureus* V8 indicate that the two proteins share several peptides (Fig. 6, *1–4*). Furthermore, the digestion of both molecules results in a 39-kD band. This result reconfirms data reported by Funaro et al. (8), who demonstrated a partial blockage of p39 production from membrane CD38 by the serine protease inhibitor *N*-α-*p*-tosyl-L-lysine chloromethyl ketone. As a 39-kD band is also observed after p78 digestion by *S. aureus* V8, a reasonable inference is that the two molecules may undergo similar cleavage processes. Peptides 1 and 3 (Fig. 6) migrate with apparent masses of 30 and 22 kD, respectively, as observed earlier by Alessio et al. (40). Moreover, Funaro et al. reported that p39 coprecipitates with a soluble 30-kD form (8), probably a major degradation product; this is a further, indirect evidence that serine proteases are involved in the production of soluble forms, the digestion with *S. aureus* V8 giving a prominent 30-kD band.

Determination of cyclase and hydrolase activities. The p78 protein was characterized in terms of catalytic activities. Cyclase activity was measured by using NGD$^+$, an NAD$^+$ analogue that is converted to the corresponding cyclic product cyclic guanine diphosphate ribose (cGDPR), which in turn is

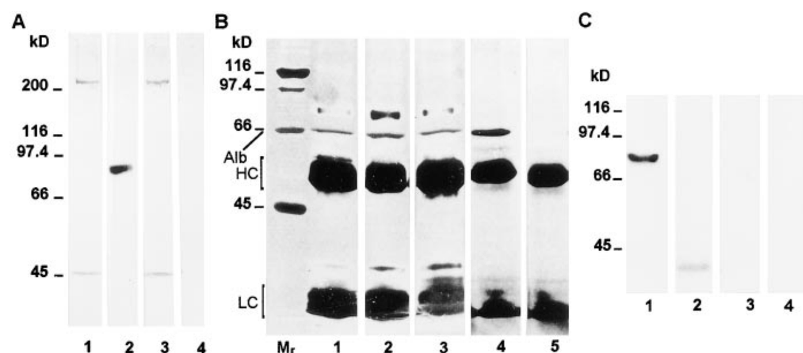

Figure 4. Immunoprecipitation from supernatants and lysates of immunodeficient B cells with anti-CD38 mAbs and Western blot analysis. (*A*) CD38 immunoprecipitated with IB4 mAbs from metabolically labeled XLA and LG2 cells, respectively (lanes *1* and *3*); immunoprecipitate with IB4 mAb from XLA and LG2 supernatants, respectively (lanes *2* and *4*). (*B*) Immunoprecipitation from unlabeled XLA spent media either with different anti-CD38 mAbs, IB4, IB6, and OKT10 (lanes *1–3*, respectively) or an irrelevant mAb (lane *4*); immunoprecipitation with IB4 mAb from unspent culture medium (lane *5*). HC and LC extrabands correspond to the heavy and light chains of the IgG used for immunoprecipitation, and Alb to the albumin present in all the culture media tested. (*C*) Partially purified p78 immunoreactivity with IB4 mAb in Western blot (lane *1*), compared to p39 (lane *2*), gp120 (lane *3*), and p78 probed with the isotype-matched 01.65 mAb (lane *4*).

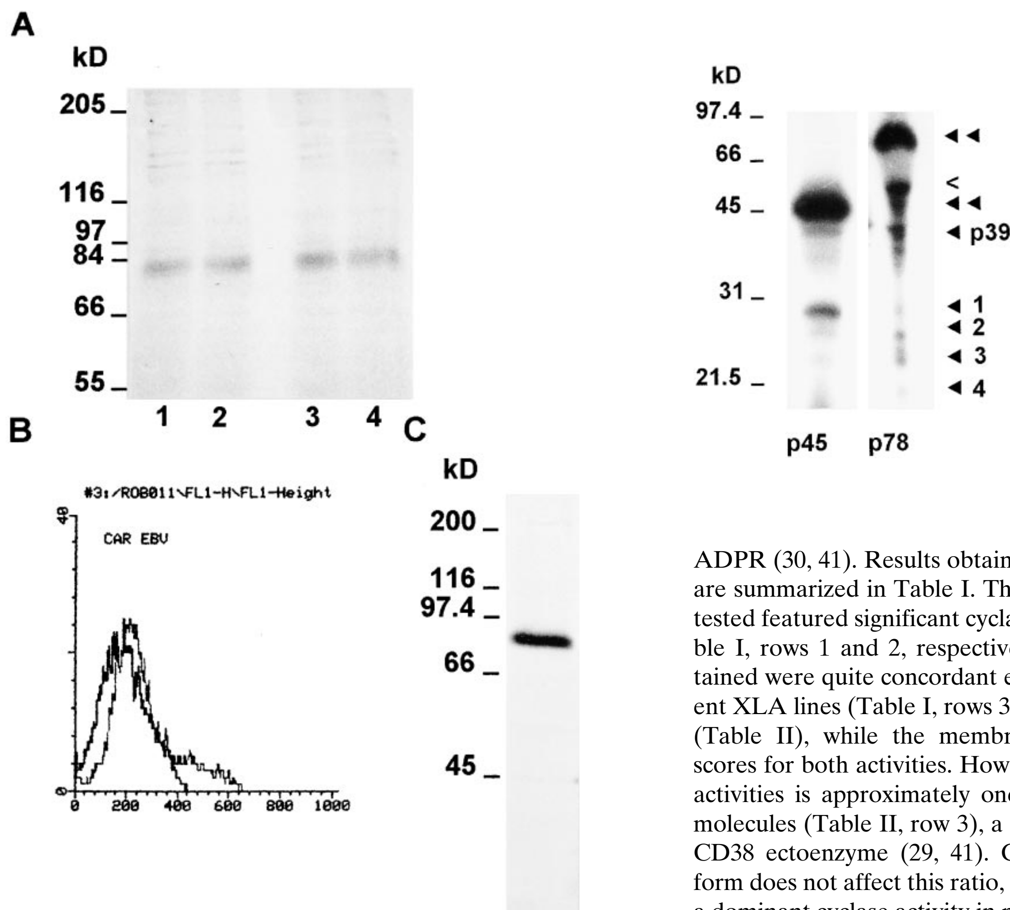

Figure 5. Sequential immunoprecipitation with anti–human IgM antiserum and IB4 mAb. (*A*) Sequential immunoprecipitation experiments were performed by using an irrelevant mAb (lanes *1–3*), anti–human IgM antiserum (lane *4*), or anti-CD39 mAb (lane *2*) in the preclearing step before incubation with IB4 mAb. (*B*) XLA lines with low-to-nil expression of surface IgM are positive, as well, for p78 release in the supernatant (*C*) *x* axis = *fluorescence intensity/cells*; *y* axis = *number of cells registered/channel. Number of cells tested* = 10,000.

much more resistant to hydrolysis than cADPR. This feature allows a more precise quantitative assay, since cyclase scores are not influenced by the correlated cADPR hydrolase activity (30). In addition, this substrate is much more specific for CD38, since it is not degraded by possible contaminating NADase activities, which instead convert NAD^+ directly to

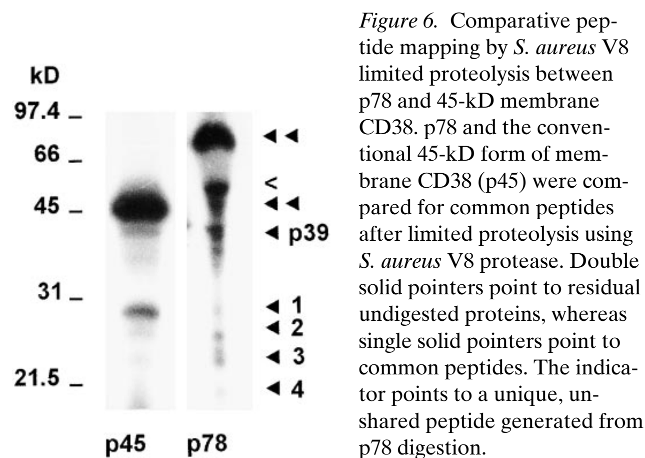

Figure 6. Comparative peptide mapping by *S. aureus* V8 limited proteolysis between p78 and 45-kD membrane CD38. p78 and the conventional 45-kD form of membrane CD38 (p45) were compared for common peptides after limited proteolysis using *S. aureus* V8 protease. Double solid pointers point to residual undigested proteins, whereas single solid pointers point to common peptides. The indicator points to a unique, unshared peptide generated from p78 digestion.

ADPR (30, 41). Results obtained after testing the 78-kD form are summarized in Table I. The p78 protein from all the lines tested featured significant cyclase and hydrolase activities (Table I, rows 1 and 2, respectively). The specific activities obtained were quite concordant either in the group of the different XLA lines (Table I, rows 3 and 4) or between p78 and p39 (Table II), while the membrane p45 form showed higher scores for both activities. However, the ratio between the two activities is approximately one for both surface and soluble molecules (Table II, row 3), a ratio reported as typical for the CD38 ectoenzyme (29, 41). CD38 modification to a 78-kD form does not affect this ratio, which is instead shifted towards a dominant cyclase activity in p190 (Table II), as reported previously (10). A negative control, obtained by concentrating multiple myeloma culture media with a 50-kD cut-off membrane, did not yield detectable enzymatic activities (Table II).

Discussion

As for many other receptors of unknown function, the study of diseases has represented a twofold valence: on one side, it is aimed at defining the role of such receptors in the pathogenesis of the disease and, on the other, it provides hints about their functions in physiological conditions. One of the few instances where CD38 is clearly involved is in murine xid, a pathology model where the signal transduction and btk phosphorylation induced by the molecule are defective (15–17). The inference derived from the murine model cannot be entirely transferred to XLA for two reasons. (*A*) Murine CD38 displays relevant differences in comparison to its human counterpart (42). For

Table I. Comparative Analysis of p78 Enzymatic Activities Between Different XLA Cell Lines

	p78 (P5.00)	p78 (P6.30)	p78 (DNS–EBV)
GDP-ribosyl cyclase (nmol cGDPR/min/ml)	0.524±0.131	0.345±0.017	0.855±0.410
cADPR hydrolase (nmol ADPR/min/ml)	0.699±0.125	0.511±0.056	0.834±0.187
Cyclase specific activity (nmol/min/mg)	17.449±4.405	13.760±0.712	24.360±11.651
Hydrolase specific activity (nmol/min/mg)	23.285±4.207	20.405±2.215	23.771±5.295
Cyclase/Hydrolase ratio	0.740±0.055	0.687±0.109	0.964±0.275

p78, partially purified from different immunodeficient B lines, was tested for both cyclase and hydrolase activities. Values are expressed as the mean±SD of three different experiments.

Table II. Comparative Analysis of Enzymatic Activities Between p78, p45, p39, and p190

	p78	p39	p45	p190	Myeloma 50-kD cut-off
Cyclase specific activity (nmol/min/mg)	18.523±4.393	17.025±0.923	89.591±7.696	133.917±9.568	0
Hydrolase specific activity (nmol/min/mg)	22.487±1.485	30.187±7.512	103.695±12.832	50.117±6.095	0
Cyclase/Hydrolase ratio	0.797±0.120	0.597±0.121	0.864±0.181	2.672±0.515	0/0

Partially purified molecules were assayed for both cyclase and hydrolase activities. Scores reported for p78 are expressed as the mean of the values reported in Table I; the other scores are expressed as the mean±SD of three different experiments. The last column shows a negative control obtained by concentrating myeloma culture supernatant with a 50-kD cut-off membrane, thus eliminating the p39 fraction.

instance, murine CD38 pattern of expression throughout B cell differentiation from follicular B cells ($CD38^+$) to germinal center B cells ($CD^{38dim/-}$) and plasma cells ($CD38^-$) is exactly opposite to that reported for the human model (43). (*B*) In spite of sharing the same molecular defect, xid shows, at least in the majority of mouse strains, normal levels of peripheral B cells (although phenotypically immature) and Igs of all isotypes (with only reduced levels of IgM and IgG_3) (44). On the contrary, XLA displays extremely low levels of both Ig (45) and B cells (46). To circumvent the drawback, we used EBV-immortalized cells derived from such patients.

Our initial analysis of CD38 in selected immunodeficiencies was restricted to some structural aspects. The comparative analysis with EBV lines obtained from healthy donors did not highlight relevant differences in the membrane density of CD38. Further, the molecule maintains enzymatic activities almost overlapping with that of normal counterparts, and the ability to be downmodulated upon ligation of agonistic mAbs via internalization is also maintained, even if the modulation is reported to be reduced or absent in xid B cells (15). The novelty of this study is the identification of a protein released from XLA cells. The characteristics of the protein are the M_r of 78,000 and catalytic properties strikingly similar to that featured by membrane CD38. Further, XLA–EBV cells, B cells from other related immunodeficiences, and normal controls, express the high–molecular weight membrane form of CD38 (p190), initially reported only in differentiated HL-60 (10). These observations confirm that the posttranslational process leading to the production of p190 is not confined to selected cell lines or differentiation conditions (Mallone, R., unpublished observations).

Interestingly, the p78 protein was also isolated from lymphocytes immortalized from a female patient affected with agammaglobulinemia. This disease, although reported in other patients to be phenotypically identical to XLA (18), does not show any mutation on the *btk* gene (19). Moreover, several congenital XLA-like agammaglobulinemias affecting both males and females display an autosomic recessive pattern of inheritance and absence of *btk* alterations (47). Mutations in the μ heavy chain gene have been recently identified as responsible for the majority of these syndromes (48). Thus, p78 release apparently is not specific for the XLA condition, but seems to be a feature of B lymphocytes with severe differentiation defects. Whether the mechanism underlying p78 release is common to both conditions or rather involves different pathways remains to be determined. Indeed, an association between CD38 and membrane Ig in human B cells has been demonstrated (31): therefore, the altered processing observed in this related condition might also be due to a defective interaction between these two molecules.

Two distinct hypotheses can be formulated to explain p78 synthesis. The first is that p78 is produced ab initio as a 78-kD soluble form, either due to alternative mRNA splicing (49) or increased expression of a presently unknown soluble protein belonging to the CD38 family (50). A second hypothesis is that CD38 undergoes oligomerization, a posttranslational process that could take place either within the cell or on the membrane. Such a mechanism has been described in ATRA-induced HL-60 cells (10) and erythrocyte membranes (12). More recently, the crystallographic analysis of *Aplysia* ADP-ribosyl cyclase (51), a soluble protein that is a phylogenetic ancestor of human CD38 (52), has revealed two forms of crystals, one consisting of a dimer (two molecules) and the other of a double dimer (four molecules). Further, indirect evidence was obtained by production of sCD38 in *Pichia pastoris* (53) that yielded, besides the expected 30-kD protein, a second species exhibiting a molecular weight of twice the conventional form. Based on this body of data, three different models can be proposed (Fig. 7). In the most simple one (*A*), two p45 molecules could undergo dimerization and then be proteolytically cleaved, giving rise to p78. Against this speculation is the fact that no relevant bands of $\sim$ 78 kD are isolated from XLA membrane fractions. A second model (*B*) could involve membrane CD38 acting as a docking site for its own soluble 39-kD form and then undergoing cleavage, but no consistent 39-kD band is highlighted from XLA supernatants. Thus, the model best supported by our data is that of CD38 oligomerization, leading to p190 production and subsequent release of p78 molecules in the supernatant (*C*).

Release of cell surface molecules is a common finding in many disease states. The released molecules may be eliminated or included in a regulatory network, either by competing in cell–cell adhesions or triggering signals in cells expressing the relevant ligands (54). CD38 is known to perform key roles both as an adhesion molecule (3) and as a signaling structure (2, 55), with different biological effects. For instance, CD38 signaling prevents apoptosis of human germinal center $CD38^+$ B cells (56), while it transduces negative signals on the growth of immature B lymphoid cells in the bone marrow (57). Both functions are probably mediated by binding with one or more ligands (4), one of which is identified as CD31 (5). Evidence that the signaling role of CD38 is mediated by the interaction with its ligand came from a study on the role of these molecules in the activation of MHC nonrestricted T cell cytotoxicity (58). Almost identical observations were made in murine models, where binding of a CD38 homologue by a mAb not only

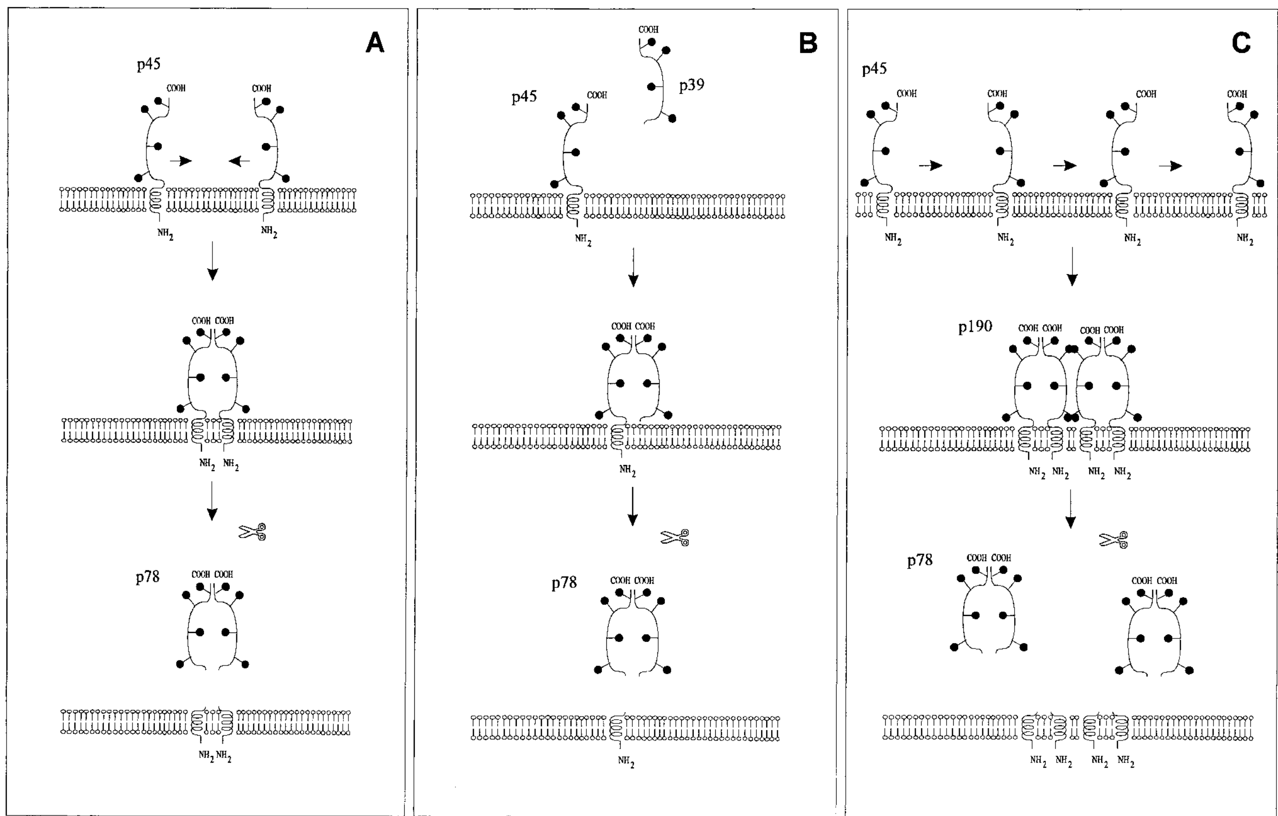

Figure 7. Possible models of CD38 oligomerization and p78 production. (*A*) p45 dimerization and subsequent proteolytic cleavage. (*B*) p45 acting as a docking site for p39, giving rise to a complex subsequently released in the supernatant. (*C*) p45 oligomerization and p190 production; the high–molecular weight form could then be cleaved to give p78 molecules.

induces B lymphocytes to proliferate, but also partially prolongs in vitro survival and protects against apoptosis. The same mAb fails to deliver effective signals to xid B cells (59). These observations raise the possibility that membrane and soluble CD38 forms might compete for binding the ligand(s), with detrimental effects in cell–cell interactions. The resulting impaired cross-talk could inhibit the progression in the B-lineage pathway. p78 could exert this in vivo hypothetical role mainly by operating in discrete microenvironments. Even if circulating or tissue immunodeficient B lymphocytes do release some amounts of p78, the protein would be highly diluted in the bloodstream, as a result of the very low number of peripheral B cells; nonetheless, the effects secondary to shedding could reach physiological significance (e.g., block of adhesion and signal triggering on cells expressing the ligands) in closed environments, such as lymph nodes or bone marrow. Moreover, the XLA phenotype may be heterogeneous, ranging from a nearly agammaglobulinemic state with complete lack of circulating B cells to milder conditions with substantial levels of IgM, IgG, and IgA, and/or a low, but detectable number of peripheral blood B cells (45, 46). One explanation for this phenotypic heterogeneity lies in the many different types of *btk* mutations described so far. Alternatively, some B cell precursors manage to escape the block in the development and are able to undergo further, though incomplete, differentiation and to synthesize Ig. This is in line with the loss of surface CD38 physiologically occurring during progression of B maturation, where the molecule is reexpressed only upon activation in the terminal stages (61).

Whether p78 release is peculiar to XLA and, more generally, immunodeficient B lymphocytes, or indeed is exacerbated in the model is not answered in full at the moment. Control supernatants tested by immunoprecipitation for the presence of p78 were obtained from (*a*) EBV lines derived from patients with common variable immunodeficiency, severe combined immunodeficiency, and Wiskott–Aldrich syndrome; (*b*) continuous human tumor cell lines of B, multiple myeloma, T and monocytic origin; and (*c*) cultured PBMC from healthy donors and patients with myeloma and Waldenstrom's macroglobulinemia. In all instances, the p78 protein was undetectable, at least in these analytical assays. Similarly, EBV lines from healthy donors did not display any relevant 78-kD band immunoprecipitated from the supernatant, even if minimal enzymatic activities were detected and acid elution assays highlighted some degree of IB4 mAb shedding. A second possibility is that p78 release might be peculiar to some stage of differentiation, i.e., the pre-B stage, rather than the immunodeficiency condition. However, immunoprecipitation experiments performed on pre-B leukemia cell lines as well as lymphocytes obtained from umbilical cord veins, known to contain a large percentage of immature B cells, did not show any 78-kD protein in the supernatant, although the membrane fractions were positive for both p45 and p190. A third possibility could be that immunodeficient B cells were, in some way preselected

by the EBV immortalization so that the continuous cell lines obtained would represent only a discrete B subset. This cannot be ruled out, given the observation that the EBV receptor CD21 is found on a lower percentage of XLA B cells compared to normal B cells (46), this molecule being expressed later in ontogeny. Many of these questions will be answered by the imminent availability of a sensitive assay for the detection of soluble CD38 circulating in the biological fluids.

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