1996

BP-3, a novel member of the CD38/ADP-ribosyl cyclase family: Its function and regulation in immune system development.

Chen Dong
University of Alabama at Birmingham

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BP-3, A NOVEL MEMBER OF THE CD38/ADP-RIBOSYL CYCLASE FAMILY: ITS FUNCTION AND REGULATION IN IMMUNE SYSTEM DEVELOPMENT

by

CHEN DONG

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of Microbiology
in the Graduate School, The University of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1996

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D Major Subject Microbiology

Name of Candidate Chen Dong

Title BP-3, A Novel Member of the CD38/ADP-Ribosyl Cyclase Family: Its Function and Regulation in Immune System Development

Murine BP-3 cell surface antigen is a variably glycosylated glycosyl-phosphatidylinositol-linked molecule that is expressed by early B and T lineage cells, myeloid cells, and discrete reticular cells in the peripheral lymphoid organs. It is also expressed on the brush border of intestinal epithelial cells and the lumenal surface of renal collecting tubules, suggesting that it may be an ectoenzyme. As a first step toward understanding the physiological role(s) of the BP-3 molecule, the BP-3 cDNA was cloned, sequenced, and expressed. Two *BP-3* gene transcripts were found to share the same open reading frame, but to utilize different polyadenylation sites.

A search for BP-3-related gene sequences in available databases indicated that BP-3 is a novel gene that shares significant homology with CD38 and molluscan ADP-ribosyl cyclase, enzymes that generate the calcium mobilizing agent cyclic ADP-ribose from NAD. The recombinant BP-3 molecule has relatively low ADP-ribosyl cyclase enzyme activity, measurable only at pH 4.0. The *BP-3* gene was mapped to mouse chromosome 5, very near the gene for CD38, supporting the view that they arose by gene duplication. Analysis of genomic clones indicates that the *BP-3* gene consists of 9 exons and spans approximately 27

kb. The overall exon organization of the *BP-3* gene is very similar to that reported for the *ADP-ribosyl cyclase* gene in the mollusc, *Aplysia kurodai*.

To study the regulatory mechanism of the *BP-3* gene expression, the transcription start sites of the *BP-3* gene were determined in a pro-B cell line. The major transcriptional start site (-17 from the ATG start codon) contains a weak initiator sequence. The upstream region lacks a TATA box, but has recognition sequences for PU.1, Ikaros/LyF-1, E2A, and TCF-1, transcriptional factors that regulate gene expression in lymphoid and myeloid cells. Binding sites for interleukin 6 (IL6) responsive factors (NF-IL6 and H-APF-1) were also found, and IL6 treatment significantly enhanced BP-3 expression by the M1 myeloblastoid cell line.

To understand BP-3 function in the immune system development, a BP-3 knockout vector was constructed and used for gene targeting in embryonic stem cells. These cells have been implanted into blastocytes to create a *BP-3* gene-deficient mouse model.

Abstract Approved by: Committee Chairman _____

Program Director _____

Date 9/4/96 Dean of Graduate School _____

DEDICATION

To my beloved family.

ACKNOWLEDGMENTS

Foremost, I would like to thank my mentor, Dr. Max Cooper, for his patience, guidance and support. He not only instructed me how to be successful in research, but also set an example of a respectful, decent scientist. I also appreciate the help and contribution from Dr. Peter Burrows, who has kindly read nearly everything I wrote and was always willing to answer questions. I thank other members of my thesis committee, Drs. Eric Hunter, Etty Benveniste and John Volanakis for their support and suggestions. Finally, I thank my colleagues and friends in Tumor Institute: John, Chen-lo, Lanier, Le-hong, Hiromi, Robin, Fengyu, Lirong, Fan-kun, Qun Lin and others for providing a stimulating and joyful scientific environment, which has made the past five years a memorable part of my life.

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ABBREVIATIONS

ADA	adenosine deaminase
APN	aminopeptidase N
BSAP	B-cell specific activation protein
BTK	Bruton's tyrosin kinase
cADPR	cyclic ADP-ribose
CALLA	acute lymphocytic leukemia antigen
cDNA	complementary DNA
DPP IV	dipeptidyl peptidase IV
EAP	glutamyl aminopeptidase
EBF	early B-cell factor
ES cells	embryonic stem cells
FCS	fetal calf serum
GCG	genetics computer group
GPI	glycosyl-phosphatidylinositol
H-APF-1	human acute phase factor 1
HIV	human immunodeficiency virus
HSC	hematopoietic stem cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin

ABBREVIATIONS (Continued)

IP_3	inositol 1, 4, 5-triphosphate
kb	kilobase
kDa	kilo-Daltons
Mab	monoclonal antibodies
MGD	Mouse Genome Database
NAADP	nicotinic acid adenine dinucleotide phosphate
NAD	nicotinamide adenine dinucleotide
ε-NAD	1, N^6-ethano-NAD
NADP	nicotinamide adenine dinucleotide phosphate
NF-IL6	IL-6-resonsive nuclear factor
PALS	periarterial lymphatic sheaths
PCR	polymerase chain reaction
PI-PLC	phosphoinositol-specific phospholipase C
RAG	recombination activating gene
SCID	severe combined immunodeficiency
TAF	TATA-binding protein associated factor
TCF-1	T cell factor 1
TCR	T-cell receptor
Tdt	terminal deoxynucleotide transferase
TFIID	transcription factor IID
THAM	thymocyte-activating molecule
TNF	tumor necrosis factor

INTRODUCTION

The immune system in higher vertebrates consists of phagocytic granulocytes (neutrophils, basophils, and eosinophils) and monocytes/macrophages and lymphocytes that are highly specific for antigen recognition. The immune cells, together with erythrocytes and other blood cells, are produced continuously from bone marrow throughout the lifespan of an individual. This hematopoiesis process demands self-replication of hematopoietic stem cells (HSC), proliferative expansion of progenitor cells, and the progressive commitment of progenitors to single lineage differentiation. Self-replication and maintenance of the HSCs are supported by the hematopoietic microenvironment. Mice deficient for both c-kit and flk2/flt3 receptor tyrosine kinase exhibited a severe reduction of hematopoietic cell numbers, indicating that c-kit and flk/flt3 are important for the growth of the HSC (Mackarehtschian et al. 1995).

Differentiation of HSCs into each hematopoietic cell lineage is likely to be regulated by lineage specific transcription factors (Orkin 1995). Gene targeting studies have revealed the requirement for factors of several classes in single or multiple hemapoietic lineages. GATA-1, a member of GATA family, is essential for erythroid development (Pevny et al. 1991). PU.1, a member of the *ets* family, is expressed by B lymphocytes and myeloid cells (Hromas et al. 1993). PU.1 deficient embryos produced normal numbers of megakaryocytes and erythroid progenitors, but failed in the generation of progenitors for B and T lymphocytes,

monocytes, and granulocytes, indicating that PU.1 is required for lymphopoiesis and myelopoiesis (Scott et al. 1994). Mice with a homozygous mutation of the *Ikaros* gene, a member of the zinc finger family, have intact erythroid and myeloid lineages, but lack B, T, and natural killer cells and their earlier defined progenitors (Georgopoulos et al. 1994). These studies not only demonstrated the importance of these transcription factors in the lineage commitment, but also supported a hypothesis that the lineage commitment of HSC is composed of several steps, first into multipotent progenitors that can generate myeloid and lymphoid cells, and then into a common precursor for all lymphoid lineages. Transcription factors are also important in the further stepwise differentiation of lymphoid cells. BSAP/Pax5, a member of the *Pax* gene family expressed by B cells; E2A, a ubiquitous helix-loop-helix protein; and EBF, which contains a DNA-binding domain encompassing a novel type of zinc-coordination motif, are required for early B cell development at distinct stages (Neurath et al. 1995; Zhuang et al. 1994; Lin & Grosschedl 1996). TCF-1, an HMG-box-containing factor, is essential for early T lineage cell differentiation (Verbeek et al. 1995).

Lymphocyte development

The progenitor cells committed to B lineage start to rearrange *immunoglobulin* (Ig) genes, first in the heavy chain locus, and then in the κ or λ light chain locus. The gene rearrangement is mediated by the recombinase-activating genes *RAG1* and *RAG2*, and diversity is enhanced by terminal deoxynucleotide transferase (TdT), which are exclusively expressed by early lymphocytes (McBlane et al. 1995; Gilfillan et al. 1993; Komori et al. 1993). The rearranged μ heavy chain proteins are first expressed on precursor B (pre-B) cells and paired covalantly with surrogate light chains, V_{pre-B} and $\lambda 5$ gene products and

noncovalantly with Igα and Igβ molecules, encoded by the *mb-1* and *B29* genes (Melchers et al. 1995a; Lassoued et al. 1993). The pre-B cell receptor signals for allelic exclusion, which terminates heavy chain gene rearrangement on the other allele, and the cells undergo clonal expansion and start light chain rearrangement. Targeted inactivations of the transmembrane portion exon of the μ heavy chain gene, the *λ5* gene, or the *Igβ* gene, all of which disrupt the pre-B cell receptor formation, lead to a block in the pre-B cell development (Kitamura et al. 1992; Kitamura et al. 1991; Gong & Nussenzweig 1996). Expression of a productively rearranged transgenic μ chain inhibits the endogenous heavy chain gene rearrangements (Storb 1995). Since the μ heavy chain has a very short cytoplasmic tail with 3 amino acids, Igα and Igβ proteins are probably mediating signal transduction intracellularly. The cytoplasmic domains of Igα and Igβ proteins are reported to independently induce the pre-B cell transition and allelic exclusion (Papavasiliou et al. 1995). The protein tyrosine kinases *syk* may be involved in the downstream signal transduction, as pro-B to pre-B differentiation is blocked in mice deficient for *syk* (Harnett 1996).

Cells committed to T lineage migrate from bone marrow to thymus. The earliest thymocyte progenitors, which lack CD3, CD4, and CD8 cell surface expression, start to rearrange *TCRβ* genes. After successful recombination, TCRβ protein is expressed on the cell surface associated with the pre-Tα chain (pTα), a surrogate α chain, and CD3 molecules involved in signal transduction (Saint-Ruf et al. 1994). This pre-T cell receptor, comparable to the pre-B cell receptor, signals the cells to shutdown *β* gene rearrangement and commence *α* gene rearrangement. $CD4^-CD8^-CD3^-$ thymocytes in mice deficient for

TCRβ, pTα, or some CD3 components failed to develop efficiently into a stage with CD4, CD8, and CD3 coexpression (Malissen et al. 1995; Malissen et al. 1993; Ohno et al. 1993; Mombaerts et al. 1992; Fehling et al. 1995). However, in one study, pTα mutation had no effect on allelic exclusion in the β locus (Xu et al. 1996). A *src* family protein tyrosine kinase, *lck* plays an essential role in the pre-T cell receptor signal transduction (Molina et al. 1992; Anderson et al. 1993; Levin et al. 1993; Anderson et al. 1992; Mombaerts et al. 1994).

Early B or T lineage progenitors that fail in a successful V(D)J gene rearrangement and assembly of the pre-B or pre-T cell receptors undergo programmed cell death; those succeeding undergo clonal expansion and respond to growth factors provided by the lymphopoietic microenvironment. Interleukin 7 (IL-7) is the predominant growth factor for murine pre-B cells. In mice lacking the IL-7 receptor, pre-B cell development is severely decreased (Peschon et al. 1994). Deletion of the gene for the common γ chain, which transduces intracellular signals from the IL-2, IL-4, IL-7, and IL-15 receptors, or the gene for the Jak3 tyrosine kinase that associates with it, reduces both B and T cell maturation (DiSanto et al. 1995; Thomis et al. 1995; Nosaka et al. 1995). Interferon (IFN) α/β, produced in the bone marrow and thymic microenvironments, antagonizes the IL7-driven pre-B or pre-T cell proliferation (Wang et al. 1995; Su et al. 1996).

After the rearrangement of an Ig light chain gene or *TCRα chain* gene and the assembly of IgM or TCR on the cell surface, B and T cells enter a stage of clonal selection. IgM^+ immature B cells are selected by self-antigens. Experiments with *Ig* transgenic mice have shown that the strength of the IgM binding to the antigens determines the fate of the autoreactive B cells (Cornall et al. 1995; Goodnow et al. 1995). When a multivalent

membrane-bound antigen binds to immature B cells in the bone marrow, the cells are eliminated. Weaker crosslinking of sIgM by soluble antigens drives the B cell into an anergized state (Cornall et al. 1995). T cells with CD4 and CD8 molecules on their surface are selected positively and negatively by the cells in the thymic microenvironment that express MHC molecules (Fowlkes & Schweighoffer 1995). Positive selection favors the survival of T cells capable of cognate recognition and also involves selection of T cell to become CD8 or CD4 single positive, which are restricted in their MHC class I or II recognition, respectively. T cells recognizing peptide presented by MHC are deleted as a mechanism of eliminating self-reactive immune response.

Lymphocytes generated from primary lymphoid organs, bone marrow, or thymus, enter circulation between the blood stream and secondary lymphoid tissues, spleen, lymph nodes and Peyer's patches. Circulating lymphocytes pass into lymphoid organs by means of a specialized kind of blood vessel, the high endothelia venule. Immunohistological analysis of peripheral lymphoid tissues reveals a discrete segregation of cellular subsets. In the lymph nodes, B cells localize primarily in the cortex as nodules or follicles, while T cells tend to migrate to the peri-follicular spaces and to the medulla. Spleen is composed of an erythrocyte- and macrophage-rich red pulp and a lymphocyte-rich white pulp. The white pulp consists of densely packed cuffs of lymphocytes that surround the central arteries and are referred to as periarterial lymphatic sheaths (PALS). T cells reside in the areas proximal to the central arteries, while B cells tend to home to the follicles or accumulate in a corona or marginal zone around the outer edge of the PALS. B cell entry of the B-rich primary follicles is a competitive process (Cornall et al. 1995). Little is known about how the segregation of immune cells in peripheral lymphoid tissues is regulated. Lymphotoxin, also

designated as tumor necrosis factor (TNF) β, appears to play an important role. In mice deficient for lymphotoxin, there is failure of normal segregation of B and T cells in the spleen in addition to absence of lymph nodes and Peyer's patches (Togni et al. 1996). Immunization of these mice with sheep red blood cells failed to induce germinal center formation (Matsumoto et al. 1996).

After reaching the periphery, B cells start to express the IgD receptor in addition to IgM. The interaction of antigens with specific receptors initiates the chain of events leading to B-cell activation, including activation of several tyrosine kinases, *lyn*, *blk* and *lck* (Weiss & Littman 1994). These kinases induce the phosphorylation of intracellular proteins, including phosphatidylinositol (PI) 3-kinase, phospholipase C-γ, Ras GTPase-activating protein, Vav, and Shc (Weiss & Littman 1994). A second signal, ligation of CD40 molecule by its ligand gp39 on activated T cells is required for B cell differentiation. In mice deficient in CD40, B cells failed in their response to T-dependent antigens and differentiation (Castigli et al. 1994). Activated B cells undergo a second round of mutation of the *immunoglobulin* genes. Two events happen: IgH class switch recombination and somatic hypermutation of the *V* genes. In most mammals, there are five classes of Ig determined by the constant region portion of the heavy chain: IgM, IgD, IgG, IgE, and IgA. In mouse and human, the IgG class is further divided into four subclasses: IgG_1, IgG_{2b}, IgG_{2a} and IgG_3 for mouse and IgG_1, IgG_2, IgG_3 and IgG_4 for human. In the course of a humoral response, a progressive replacement of IgM antibodies with other Ig classes occurs (Zhang et al. 1996). Somatic mutation in the *IgV region* genes also occurs in the specialized germinal centers of secondary follicles in the peripheral lymphoid organs. These random mutations are selected by the antigen to increases the antibody affinity (Winter & Gearhart 1995).

Lymphocyte ectoenzymes

Generation of monoclonal antibodies against cell surface glycoproteins has been an invaluable tool in the study of the immune system development and function. These antibodies help define the cellular lineages and developmental stages of the immune cells. Study of the physiological roles of the antigen molecules benefits our understanding of how immune cells interact with each other and with their microenvironment. Biochemical and molecular analysis of the glycoproteins expressed on lymphoid and myeloid cells has revealed that some of them are membrane-associated ectoenzymes. These include CD10/neutral endopeptidase-24.11, CD13/microsomal alanyl aminopeptidase, BP-1/6C3 antigen/glutamyl aminopeptidase, CD26/dipeptidyl peptidase IV, Ly31/alkaline phophatase, CD73/ecto 5'-nucleotidase, and CD38/ADP-ribosyl cyclase. Although these ectoenzymes are usually found in many tissues, such as kidney and intestine, they are expressed by different haematopoietic cell types at unique stages of cell differentiation and activation, suggesting a function in a stage and lineage-specific fashion. These ectoenzymes possess not only diverse enzymatic activities, including peptidases, glycosidases, phosphatases, and nucleotidases, but also some other biological functions, such as cell/matrix adhesion and signal transduction.

CD10/endopeptidase 24-11. CD10, initially described as the common acute lymphocytic leukemia antigen (CALLA), is also expressed by normal B cell precursors and by activated, mature B cells (Greaves et al. 1983; Salles et al. 1992), and on the bone marrow stromal cells (Kee et al. 1992). Cloning and sequencing analysis revealed that CD10 was identical to the endopeptidase 24-11 (Letarte et al. 1988). CD10 cleaves a variety of biologically active peptides including angiotensins I and II, enkephalins, bradykinin,

neurotesin, oxytocin, and substance P (Kee et al. 1992). Inhibitors to CD10 can promote B cell reconstitution and maturation in spleen and IL-1 response by bone marrow stromal cells (Delikat et al. 1994; Salles et al. 1993; Kee et al. 1992).

CD13/Aminopeptidase N. CD13 is a type II glycoprotein with a large extracellular region and small intracellular NH_2-terminal segment that is expressed on myeloid cells and their precursors and on myeloid leukemias (Razak et al. 1994; Look et al. 1989). It is also present on small intestine and renal proximal tubule epithelial cells, on synaptic membranes of the central nervous system, and on fibroblasts and osteoclasts. In renal microvilli, it constitutes about 8% of the total membrane proteins (Look et al. 1989; Kenny et al. 1987). The predicted amino acid sequence of CD13 is identical to aminopeptidase N, a zinc-binding metalloprotease catalyzing the removal of N-terminal amino acids from peptides (Look et al. 1989). The APN has a broad specificity, although it prefers neutral amino acids (Kenny et al. 1987). It may be involved in the metabolism of regulatory peptides such as enkephalins and interleukin 8 by diverse cell types and has been postulated to function in the hydrolytic inactivation of these peptides (Look et al. 1989; Kanayama et al. 1995). On myeloid cells, CD13 has been proposed to regulate peptide-mediated signals by removing key NH_2-terminal residues from active peptides, or by converting inactive peptides to active forms (Ashmun & Look 1990). Cross-linking of the CD13 antigen leads to calcium mobilization and superoxide production (MacIntyre et al. 1989). Interestingly, APN is reported to serve as a receptor for human coronavirus 229E (Delmas et al. 1992) and probably mediates human cytomegalovirus infection (Soderberg et al. 1993). CD13 gene expression has been found to be regulated by *Ets* and *Myb* families of transcription factors (Shapiro, 1995).

CD26/Dipeptidyl peptidase IV (DPP IV). CD26 is a highly glycosylated type II membrane sialoglycoprotein comprising two identical subunits of approximately 110 kDa (Fleischer 1994). CD26 is constitutively expressed on a variety of different cell types, particularly on epithelial cells of the intestine, prostate gland, and kidney proximal tubules. By contrast, the expression of CD26 on T cells is regulated stringently and accompanies T-cell activation (Fleischer 1994). It is also expressed on mature thymocytes in the medulla (Fleischer 1994). The *CD26* gene, which encompasses more than 90 kb, is composed of 26 exons and located on human chromosome 2q23 (Bernard et al. 1994; Darmoul et al. 1994; Mathew et al. 1994; Abbott et al. 1994).

CD26/DPP IV is a proteinase with a unique specificity: It cleaves dipeptides from the N-terminus of polypeptides if proline is at the penultimate position. Peptides are also cleaved if alanine or hydroxyproline occupies the next (p1) position, although with 100-1000-fold lower efficiency (Fleischer 1994). The enzymatic activity of DPP IV plays an obligatory role in the renal transport and intestinal digestion of proline-containing polypeptides. Indeed, CD26 comprises 4% of the kidney brush-border membrane protein (Fleischer 1994). Furthermore, rats of the Fischer 344 strain lack functional DPP IV, owing to a missense mutation in the active site, and suffer from severe disturbance in the renal absorption of proline-containing peptides (Fleischer 1994).

Mab against human CD26 induced redirected lysis in human cytotoxic T-lymphocyte clones, and IL-2 secretion and proliferation in human pre-activated $CD4^+$ and $CD8^+$ T cells (Fleischer 1994). Similarly, the mouse thymocyte-activating molecule (THAM) was found to be the same as mouse DPP IV (Marguet et al. 1992). Stimulation of CD26 leads to the activation of all functional programs of the T cell, including cytotoxicity and granule

exocytosis (Fleischer 1994). The molecular mechanism of CD26 signal transduction is not clear. DPP IV enzymatic activity may be important, but not absolutely required, for the costimulatory activity of CD26 (Steeg et al. 1995; Hegen et al. 1993). CD26 stimulation, which enhances CD3ζ phosphorylation and increases p56lck activity, appears to require TCR/CD3 expression (Mittrucker et al. 1995). Interestingly, CD45 co-modulates with CD26 and co-precipitates with anti-CD26 in T-cell lysate (Fleischer 1994).

CD26 is also associated with adenosine deaminase (ADA) protein, the deficiency of which results in severe immunodeficiency in humans and mice (Martin et al. 1995; Dong et al. 1996b). The extracellular domains of CD26 bind directly to ADA and thereby transport it to the cell surface in T cells and other cells. The enzymatic activity of CD26 is not required for this interaction because mutant CD26 molecules can still bind and transport ADA (Fleischer 1994). CD26 can bind to components of the extracellular matrix, such as fibronectin and collagen (Bauvois 1988; Piazza et al. 1989). The binding is of low affinity and, in the case of fibronectin, appears not to require the enzymatic activity (Fleischer 1994).

CD26 has been suggested to be a co-receptor for human immunodeficiency virus (HIV) in addition to CD4 (Callebaut et al. 1993). Co-expression of CD4 and CD26 in mouse fibroblasts rendered them permissive to HIV infection, whereas transfection of CD4 alone was not sufficient. Moreover, peptides with the X-pro motif that are competitive inhibitors of DPP IV enzymatic activity block HIV entry into cells. However, these data have been criticized (Lazaro et al. 1994). The HIV Tat protein, which has a potent inhibitory effect on human T-cell responses to soluble antigens and to anti-CD3 antibodies in vitro, was shown

to bind specifically to the Ta1 epitope of CD26 and to partially inhibit DPP IV enzymatic activity (Gutheil et al. 1994).

CD38/ADP-ribosyl cyclase. The CD38 molecule is a type II transmembrane glycoprotein of 42 kDa with an unusual pattern of discontinuous expression during lymphocyte differentiation (Malavasi et al. 1994; Lund et al. 1995a). In humans, CD38 is expressed by early B cell progenitors and germinal center B cells, by all human thymocytes, and by a large proportion of human peripheral T cells (Malavasi et al. 1994). Mouse CD38 is expressed abundantly by all murine B-lineage cells, with the exception of the weakly expressing germinal center B cells and barely on T lineage cells (Lund et al. 1995a). CD38 expression can be induced on human myeloid cells by retinoic acid (Drach et al. 1994).

CD38 is likely to be an important immunoregulatory molecule on lymphocytes. Agonistic antibodies to CD38 can induce the proliferation of B and T lymphocytes and protect B cells from apoptosis (Funaro et al. 1990; Santos-Argumedo et al. 1993; Zupo et al. 1994). The human CD38 molecule has been reported to associate with the antigen receptor complexes (Funaro et al. 1993), and tolerized B cells isolated from the spleens of double-transgenic mice become unresponsive to anti-CD38 and anti-IgM treatment (Lund et al. 1995b). CD38 ligation induces the influx of extracellular calcium, apparently independent of the IP_3 pathway (Santos-Argumedo et al. 1993). Protein tyrosine kinases are involved in CD38 signal transduction (Silvennoinen et al. 1996; Kontani et al. 1996), which is impaired in *xid* mice, in which Bruton's tyrosine kinase (BTK) is mutated (Lund et al. 1995b). Anti-CD38 antibodies inhibited B lymphopoiesis in a stroma-supported culture system of human B cell progenitors (Kumagai et al. 1995). CD38 is also involved in lymphocyte adherence to

endothelium (Malavasi et al. 1994). Recently, a human CD38 ligand has been identified as a 120 kDa protein that is expressed predominantly on endothelial cells (Deaglio et al. 1996).

The predicted amino acid sequence of mouse and human CD38 shares homology with ADP-ribosyl cyclase isolated from the sea mollusc *Aplysia californica*, which catabolizes the conversion of NAD to cyclic ADP-ribose (cADPR) (Glick et al. 1991; Lee & Aarhus 1991). CD38 has then been demonstrated not only to convert NAD to cADPR, but also to hydrolyze cADPR into ADPR (Howard et al. 1993). An extra pair of cysteine residues in CD38, which is not found in the molluscan ADP-ribosyl cyclase, plays a role in this additional cADPR hydrolase activity (Tohgo et al. 1994). cADPR is emerging as a novel endogenous calcium mobilizing agent, which induces a calcium flux from an intracellular store (Lee 1994a; Lee 1994b; Galione 1994). It acts independently of 1,4,5-phosphoinositol triphosphate (IP_3), probably through ryanodine receptors, although cADPR and IP_3 synergize with each other in the calcium mobilization (Lee et al. 1994). Little is known about whether cADPR plays a role in the immune system, except that exogenous cADPR could enhance B cell proliferation in response to CD38 and IL4 (Howard et al. 1993). Recently, a ryanodine receptor has been identified in a T lymphoma cell as a potent target of cADPR (Bourguignon et al. 1995). However, how cADPR generated by CD38 outside a cell acts intracellularly remains an enigma. CD38 may have additional enzymatic activities, including ADP-ribose transferase (Grimaldi et al. 1995), and it can generate another novel calcium-release agent, nicotinic acid adenine dinucleotide phosphate (NAADP), from NADP at low pH in the presence of nicotinic acid (Aarhus et al. 1995).

CD38 is also expressed by a variety of nonlymphoid tissues, including pancreatic beta cells where cADPR synthesis by CD38 plays a role in glucose regulation of insulin secretion

(Kato et al. 1995). CD38 expression is impaired in pancreatic islets of the Goto-Kakizaki (GK) rat, a rodent model of spontaneously occurring non-insulin dependent diabetes mellitus that exhibits impaired glucose-stimulated insulin secretion (Matsuoka et al. 1995).

CD73/ecto 5'-nucleotidase. CD73 is a 69-kDa glycoprotein that is attached to the external plasma membrane of human lymphocytes via a GPI linkage (Thompson et al. 1990). It is expressed on subsets of lymphocytes: Approximately one-third of adult peripheral T cells and three-fourths of adult peripheral B cells express CD73 (Thompson et al. 1990). The enzyme activity in both thymocytes and fetal spleen or cord blood B cells is much lower than in the corresponding adult peripheral lymphocytes. This suggests that CD73 serves as a maturation marker for developing B and T cells.

CD73 functions in the purine salvage pathway by catalyzing the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to the corresponding nucleosides, which are then able to enter the cell via facilitated diffusion (Thompson et al. 1990). Deficiencies in two other purine salvage enzymes, adenosine deaminase and purine nucleoside phosphorylase, are known to cause SCID and T cell immunodeficiency, respectively. Patients with a variety of immunodeficiency diseases have reduced, or in some cases virtually absent, levels of CD73 expression (Thompson et al. 1990). Such disorders include SCID, Wiskott-Aldrich syndrome, common variable immunodeficiency, congenital IgA deficiency, hypogammaglobulinea, and AIDS. Proper purine salvage enzyme activity is vital to normal lymphocyte differentiation and function.

CD73 appears to function in T cell activation, as Mabs to it have a profound effect on T cell proliferation and synergize with anti-CD3 treatment in inducing IL-2 secretion and IL-2 receptor expression (Massaia et al. 1990). CD73 enzyme activity and GPI anchorage are not

required for this action (Gutensohn et al. 1995; Resta et al. 1994). CD73 could also mediate the adhesion of lymphocytes to endothelium (Airas et al. 1995).

BP-1/6C3 antigen/Glutamyl aminopeptidase. Glutamyl aminopeptidase (EAP) was identified in the immune system as BP-1, a cell surface differentiation antigen expressed by murine pre-B cells (Cooper et al. 1986). It is a type II integral membrane glycoprotein composed of two 140 kDa disulfide-linked subunits that have multiple N-linked glycosylation sites in their extracellular domains (Cooper et al. 1986). BP-1 antigen is phophorylated at tyrosine residue(s) (Wu et al. 1989; Wang and Cooper unpublished results). An antigen previously identified on leukemia pre-B cells by the 6C3 antibody was found to be the same molecule (Wu et al. 1989). The BP-1/6C3 cDNAs predict a protein of 965 amino acids, which is homologous to CD13/APN (Wu et al. 1990). Subsequent biochemical analysis confirmed that it is an aminopeptidase that selectively cleaves the acidic amino terminus of short peptides (Wu et al. 1991). EAP contains a zinc-binding motif, shared by many enzymes and proteins, mutation of which leads to the loss of its enzymatic activity (Wang & Cooper 1993). The murine *EAP* gene has recently been isolated. It spans more than 100 kb and contains 20 exons (Wang et al. 1996). It is located in a distal region of mouse chromosome 3 in a region homologous to human chromosome 4q25 (Wang et al. 1996).

The *EAP* gene is evolutionarily conserved and expressed in a wide variety of tissues (Li et al. 1993). Abundant EAP is found on the intestinal brush border of the small intestine, renal glomeruli, proximal renal tubules, and vascular endothelium in many organs (Li et al. 1993). Other tissues expressing the EAP include stromal cells in the thymus cortex, bile canaliculi in liver, gall bladder epithelium, pulmonary alveolar cells, interlobular ducts in the

pancreas, the ovarian theca interna, basement membrane of the epididymis, and the splanchnopleure in placenta (Li et al. 1993).

Among haematopoietic cells, however, EAP expression is restricted to pre-B cells and is rapidly turned off in IgM^+ immature B cells (Cooper et al. 1986). Elevated BP-1 expression is found on virus-transformed pre-B cells and on pre-B cells generated in long-term bone marrow cultures. Interleukin 7 treatment in vitro and in vivo induces both the proliferation of pre-B cells and expression of the BP-1/6C3 antigen (Morrissey et al. 1991; Welch et al. 1990; Sherwood & Weissman 1990; Dong et al. 1994b). IFNα/β, while inhibiting pre-B cell growth, also upregulated BP-1 expression (Wang et al. 1996). This is likely to be through an interferon responsive element located in the promoter region of the *EAP* gene (Wang et al. 1996). Also in this region, there are putative DNA-binding motifs for Ikaros, BSAP, PU.1, and octamer binding proteins, transcription factors known to regulate gene expression in B lineage cells (Wang et al. 1996).

Restricted expression of EAP on pre-B cells suggests a role in the proliferation and differentiation of these cells. ASD4, an antagonistic antibody against EAP, was found to suppress pre-B cell growth in long-term bone marrow culture and IL-7-treated neonatal mice in vivo (Wang and Cooper unpublished; Dong et al. 1994b). ASD4 treatment did not abolish pre-B cell differentiation into B cells, suggesting that EAP only plays an accessory role in the pre-B cell proliferation. In an effort searching for a EAP substrate at haematopoietic tissues, the transcripts of type I receptors for angiotensin II, a substrate for EAP in renal-brain system, were identified in bone marrow stromal cells (Lin et al. 1994). The role of angiotensin and its receptor in early B cell development needs to be addressed. ASD4 suppression could alternatively be explained by the idea that artificial ASD4

engagement may transduce a negative signal inside the pre-B cells. In support of this view, *EAP* gene deficient mouse has no apparent defect in the development of B and other lineage cells (Lin et al. 1995). Other ectoaminopeptidases may compensate for the EAP deficiency in these mice.

Identification of the murine BP-3 molecule

In order to study how pre-B cell growth and differentiation is regulated and to identify molecules that mediate the interaction between these cells and their microenvironment, a panel of monoclonal alloantibodies were produced by immunizing an outbred wild mouse with a Abelson virus-transformed murine pre-B cell line, 18.81 (McNagny et al. 1988). One of the antibodies, BP-3, recognizes a glycosyl-phosphatidylinositiol-linked molecule of 38-48 kDa (McNagny et al. 1988). The BP-3 antigen is variably glycosylated, and the pattern of glycosylation is cell-type specific (McNagny et al. 1988). BP-3 marks all B cell precursors in bone marrow (Fig. 1). When these cells mature, migrate to the peripheral lymphoid tissues, and start to express IgD coreceptor, BP-3 expression is downregulated (Fig. 1; McNagny et al. 1988). In contrast to its expression on the B lineage cells, the level of BP-3 expression increases as a function of maturation on myeloid cells, being expressed at much higher levels on mature macrophages and granulocytes than on myeloid precursors in the bone marrow (Fig. 2; McNagny et al. 1988). The BP-3 antigen was found on a subpopulation of reticular cells in the spleen, lymph nodes, and Peyer's patches, but not on those in bone marrow and thymus (McNagny et al. 1991). The expression of the BP-3 antigen by splenic reticular cells is restricted to the white pulp area where lymphocytes are located and is coincident with the seeding of peripheral lymphoid tissues with lymphocytes, suggesting that it may play a role in the generation of a receptive microenvironment (McNagny et al.

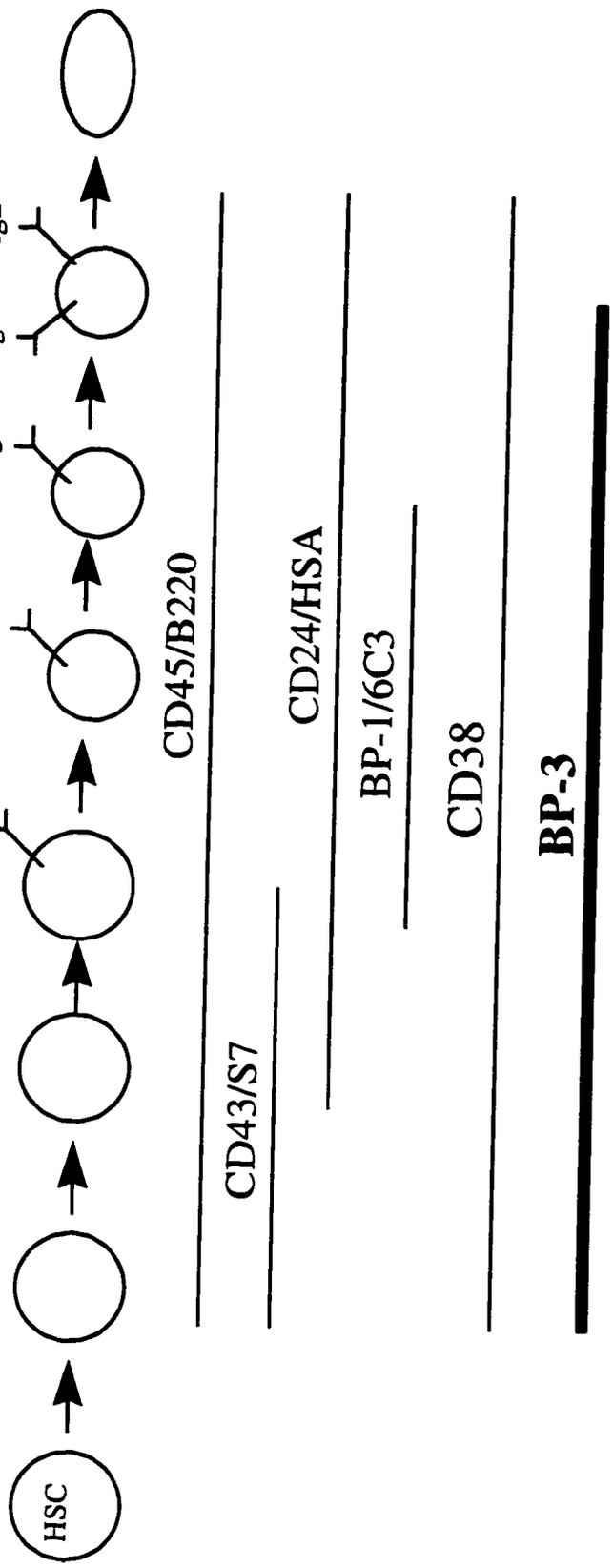


Fig. 1. Expression of the BP-3 antigen in B lineage cell development.

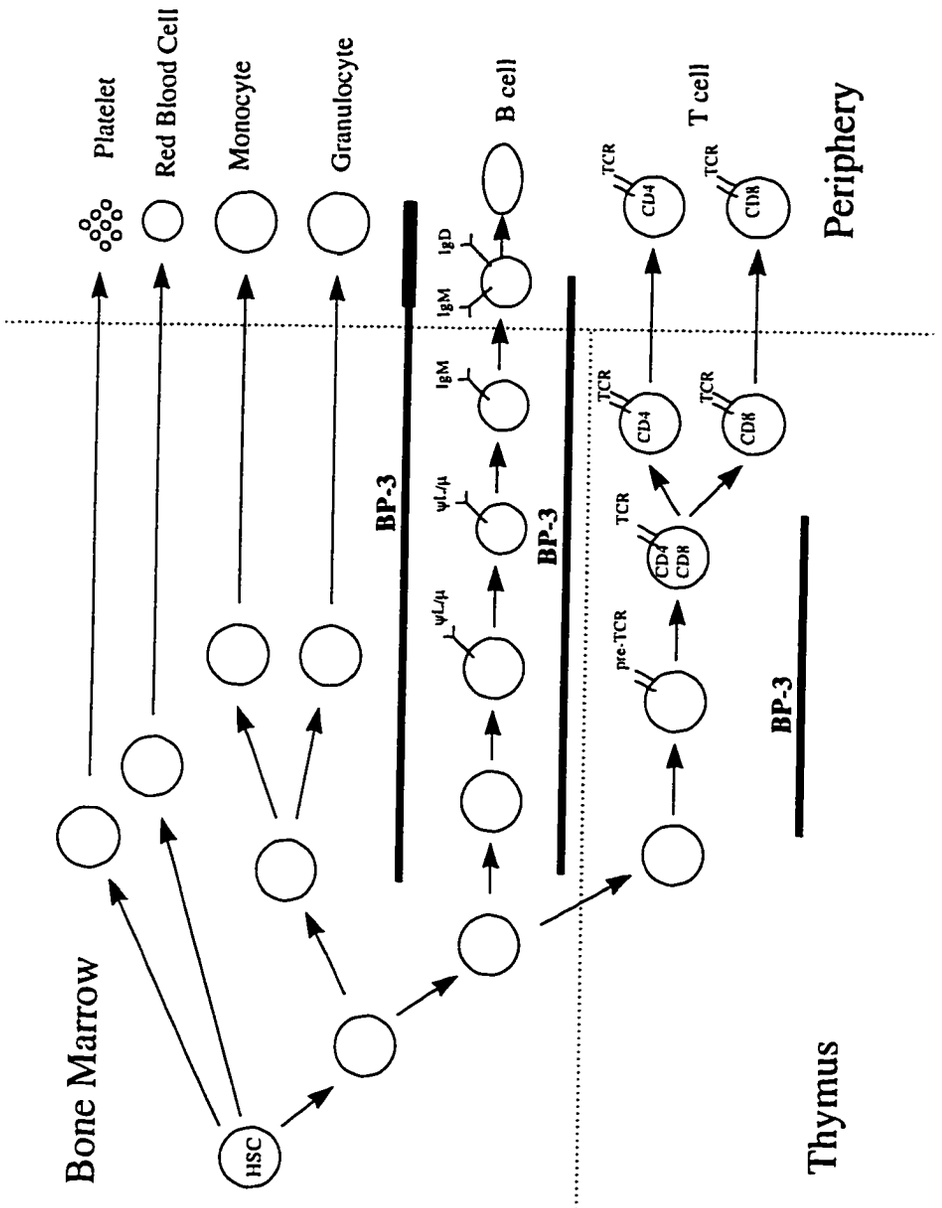


Fig. 2. Expression of the BP-3 antigen in the immune system development.

1991). In nonlymphoid tissues, the BP-3 molecule was detected at high levels on the brush borders of intestinal epithelial cells and within the collecting tubules of the kidney (McNagny et al. 1991), suggesting that the BP-3 molecule may be an ectoenzyme.

The scope of this dissertation

The objectives of this study were to understand the structure of the murine BP-3 gene, and its function and regulation in the development of immune system. The following sections contain (a) cloning and analysis of BP-3 cDNA; (b) genomic structure and chromosomal location of the BP-3 gene; (c) biochemical analysis of BP-3 enzymatic activity; (d) determination of the transcriptional start sites of the BP-3 gene and its upstream regulatory sequences; and (e) construction of a BP-3 gene deficient mouse model in order to gain insight into its functional roles.

THE MURINE BP-3 GENE ENCODES A RELATIVE OF THE CD38/NAD GLYCOHYDROLASE FAMILY

CHEN DONG*, JIYANG WANG*‡, PETER NEAME†, AND MAX D. COOPER*‡

Division of Developmental and Clinical Immunology*, and Departments of Medicine, Microbiology, and Pediatrics, University of Alabama at Birmingham, and the Howard Hughes Medical Institute‡, Birmingham, AL 35294 and the Shriners Hospital for Crippled Children†, Tampa, FL 33612

Transmitting Editor: Takeshi Watanabe, M.D.

Published in *International Immunology*. 6:1353-1360, 1994

Abstract

The murine BP-3 antigen is a variably glycosylated glycosyl-phosphatidylinositol (GPI)-linked molecule that is selectively expressed by early B and T lineage cells and a discrete subpopulation of reticular cells in the peripheral lymphoid organs. It is also expressed on the brush border of intestinal epithelial cells, the lumenal surface of renal collecting tubules and mature myeloid cells. To further explore the nature of the BP-3 antigen, we purified the protein, obtained peptide sequences and used these to isolate cDNA clones. Two BP-3 cDNA clones were found to share the same open reading frame, but to utilize different polyadenylation sites. Expression of a full-length cDNA clone confirmed that it encodes the BP-3 antigen. Northern blot analysis with this cDNA probe revealed BP-3 transcripts of 1.3 and 2.3 kb in various tissues and cell lines representing myeloid, B and T cell lineages, while a probe containing the most 3' untranslated region of the longer cDNA clone hybridized only with the 2.3 kb RNA species. Analysis of the BP-3 cDNA sequence indicates that it represents a previously undescribed gene that shares significant homology with genes encoding nicotinamide adenine dinucleotide (NAD) glycohydrolase of *Aplysia californica* and the CD38 antigens in mouse and human. However, cells expressing the recombinant BP-3 protein did not exhibit NADase activity, suggesting that it may be a distant relative of NAD hydrolase with different function.

Introduction

The murine antigen recognized by the monoclonal BP-3 alloantibody is a variably glycosylated cell surface protein of Mr 38-48 kDa that is attached to the plasma membrane via glycosyl-phosphatidylinositol (GPI) linkage (1-2). Initially found on early lymphoid cells and mature myeloid cells (1), this variably glycosylated core protein of 32 kDa is also

expressed on the brush border of intestinal epithelial cells and collecting tubules of the kidney, thus raising the possiblity that it could be an ectoenzyme (2).

BP-3 antigen expression by B lineage cells extends from the earliest detectable progenitors in the bone marrow to immature B cells in the periphery (1). A discrete subpopulation of reticular cells in the peripheral lymphoid organs also expresses the BP-3 antigen (2). The splenic BP-3$^+$ reticular cells are confined to the white pulp areas and their expression of the BP-3 antigen begins when the neonatal spleen becomes a secondary lymphoid organ, thus raising the possibility that the BP-3 molecule could be involved in lymphocyte-stromal interactions (2). Like immature B lineage cells, the immature thymocytes also express the BP-3 antigen, but the expression is down-regulated with the onset of TCR expression (our unpublished results). In contrast to the pattern of BP-3 antigen expression by lymphoid cell lineages, BP-3 antigen expression on myeloid cells increases as a function of maturation (1).

As a first step toward determining the physiological role(s) of the BP-3 antigen, we have obtained peptide sequences and identified cDNA clones, one of which was expressed and shown to encode the BP-3 antigen. The deduced amino acid sequence of the BP-3 cDNA was found to have significant homology with NAD glycohydrolase and CD38 antigens in humans and mice, suggesting that the BP-3 molecule may be a relative of this nucleotidase family.

Methods

Protein purification and sequencing. GPI-linked glycoproteins were obtained from the 1H6A pre-B cell line, which expresses high levels of the BP-3 antigen, by treatment of cells (3×10^{10}) with phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma, St. Louis, MO; 10^8 cells in 1 ml Hank's balanced salt solution containing 0.25 enzyme unit) for

1 h at 37°C. The treated cells were pelleted, and the supernatant was pooled and precleared by incubation with Affigel 10 beads (Bio-Rad, Hercules, CA) coupled with a nonspecific mouse IgG antibody, followed by passage over a BP-3 antibody-conjugated Affigel 10 column. The affinity column was washed extensively with PBS and bound materials were eluted with 0.125 M acetic acid. The protein eluate was concentrated by a Speed-vac (Savant Instruments, Inc., Farmingdale, NY), electrophoresed on a 10% SDS-PAGE gel, blotted onto an Immobilon membrane (Millipore, Bedford, MA) and subjected to N-terminal protein sequencing performed by the protein sequencing facility of the Howard Hughes Medical Institute in St. Louis. Another preparation of the protein was digested with Endoprotease Lys-C (Boehringer Manheim, Indianapolis, IN) and sequences of internal peptide fragments were determined as described (3).

Construction and screening of a cDNA library and DNA sequencing. Total RNA was extracted from 1H6A cells by guanidium isothiocyanate-cesium chloride gradient (4), and the poly(A) RNA was isolated using an oligo-d(T) cellulose spin-column (5'-3', Inc., Boulder, CO). cDNA was then synthesized using an oligo-d(T) primer and cloned into the Uni-ZAP vector (Stratagene, La Jolla, CA). A 43-mer oligonucleotide probe (GIGGIGAIGGIACIACICCICAIC[T]TICAITC[AG]IATITTIC[T]TIGG) corresponding to the BP-3 N-terminal amino acid sequence was labeled with ^{32}P by T_4 kinase and used to screen the library. The prehybridization was carried out in 6 x SSC/5 x Denhardt's/0.05% sodium pyrophosphate/0.05% SDS/100 μg /ml boiled salmon sperm DNA at 42°C for 2-3 h, and the hybridization were performed at 42°C in 6 x SSC/2 x Denhardt's/0.05% sodium pyrophosphate/100μg/ml yeast tRNA overnight, after which the filters were washed three times at room temperature and once at 48°C with 6 x SSC/0.05% sodium pyrophosphate (4). DNA from the hybridized clones was excised into pBluescript plasmid and the inserts

sequenced by use of Sequenase 2.0 (USB, Cleveland, OH). The 5' < 400-bp *SmaI-Hinc*II fragment of clone 74 was used to re-screen the library for clones with full-length open reading frames, which were processed in the same fashion. The nucleotide and deduced protein sequences were analyzed by use of Genetics Computer Group package from the University of Wisconsin.

BP-3 cDNA expression and analysis. The insert of clone 104 was subcloned into a pMKITneo vector provided by Dr. Kazuo Maruyama, Tokyo Medical and Dental University. Plasmid DNA (10 μg) was transfected into COS-7 cells by the DEAE-Dextran method (5). At 48 h after transfection, cells were harvested and stained with biotinylated BP-3 antibody, the binding of which was revealed by streptavidin - phycoerythrin and FACScan analysis. An aliquot of the cells was labeled for 7 h with tran^{35}S-Label™ (ICN Biochemicals, Inc., Irvine, CA) in Dulbecco's modified Eagle's medium without methionine and cysteine but with 10% dialyzed FCS. Washed cells were lysed, and the BP-3 antigen in the cell lysate was purified on BP-3-coated Affigel beads. *N*-linked sugars were released from one aliquot of the BP-3 antigen sample by treatment with an *N*-glycanase (PNGase F; New England Biolabs, Beverly, MA), and the treated and non-treated BP-3 antigen aliquots resolved by SDS-PAGE analysis. For stable transfection, the clone 104 insert was subcloned into pCDNA3 (Invitrogen, San Diego, CA), which was linearized by *ScaI* and transfected into mouse Ltk$^-$ cells by use of lipofectin (Gibco BRL, Gaithersburg, MA) as described (5).

Northern blot analysis. Total RNA was isolated from mouse cell lines and fresh tissues, and 10-20 μg RNA samples were electrophoresed in 1.2% agarose/MOPS-formaldehyde gels, then transferred onto nylon (Bio-Rad) or nytran (Schleicher & Schuell, Keene, NH) membranes. The RNA blots were then hybridized with the 1.2 kb cDNA

insert of clone 74 or ~ 600 bp most 3' *PstI/XhoI* fragment of clone 78 labeled with ^{32}P by use of Prime-It RT Kit (Stratagene) (4, 6).

NADase assay. The NADase assay was performed on 1H6A pre-B cells and COS-7 and Ltk$^-$ transfectants by use of the NAD analog 1,N^6-ethano-NAD (ε-NAD) (Sigma) as described (7), except that the enzyme reaction was carried out for 30 min instead of 10 min. Fluorescence emission was measured at 410 nm by a SLM-8000 fluoremeter (SLM Instrument) with an excitation wavelength of 300 nm.

Results

Purification and partial amino acid sequencing of the BP-3 antigen protein. The BP-3 protein released by PI-PLC treatment from 1H6A cells was isolated on an antibody affinity column. N-terminal analysis of the purified protein yielded a sequence of the first 21 amino acids, and additional sequences of 13 and 14 amino acids were obtained from two endoprotease Lys-C proteolytic peptide fragments (Fig. 1b). Analysis of protein sequences by the BLASTNCBI program of University of Wisconsin Genetics Computer Group (GCG) package did not reveal a protein which contains these sequences.

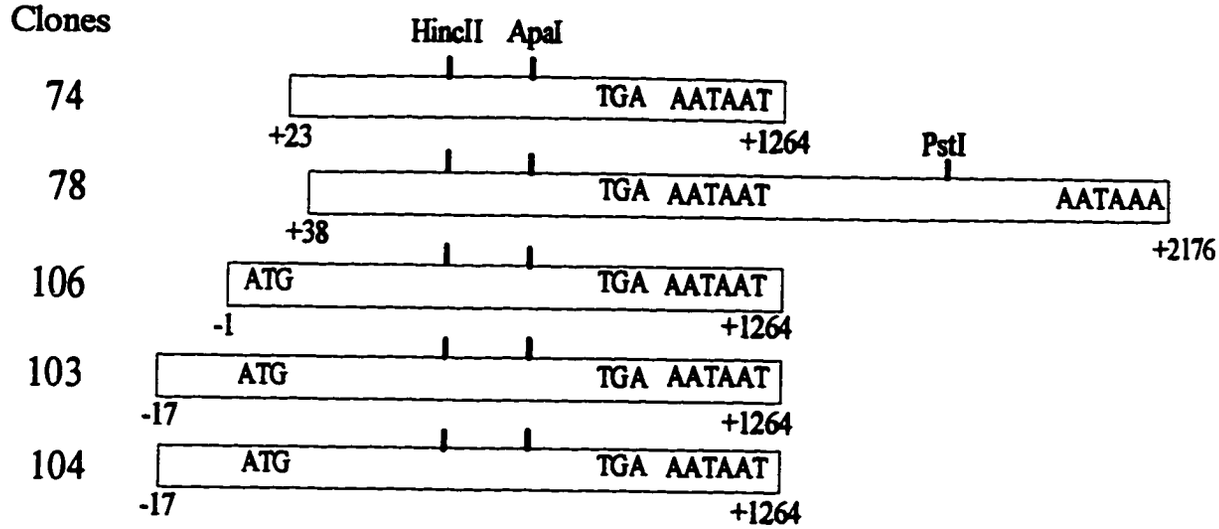
Isolation and sequencing of the BP-3 gene. To isolate BP-3 cDNA clones, 43-mer oligonucleotides with degeneracy of 8 were synthesized according to the sequence of amino acids 7 - 20 in the N-terminal protein sequence. These oligonucleotides were used to identify two clones, designated 74 and 78, by screening 3 x 10^5 recombinants in a Uni-ZAP cDNA library prepared from 1H6A cells that express the BP-3 antigen at relatively high levels. The inserts of < 1.2 (clone 74) and 2.2 kb (clone 78) were sequenced (Fig. 1a), and the protein sequences predicted in one of the three frames of both clones were found to contain the N-terminal protein sequence. No upstream ATG was found in the same frame of either clone. Two polyadenylation signal sites were noted in clone 78. The first of these

was shared by clone 74, in which poly(A) was added at nucleotide C, 19 bp after this sequence (Fig. 1a). Upstream of the initial AATAAT sequence, clone 78 shares the same sequence with clone 74. These two cDNA clones thus appear to reflect RNA transcripts derived from the same gene as a result of differential poly(A) addition. We also noted that clone 74 has two copies of ATTTA sequences in the 3' untranslated region, while clone 78 has two more in its unique region. This consensus AU sequence has been found in the 3' untranslated region of several cytokine genes and oncogenes, and is believed to mediate mRNA stability (20). To identify clones with a full-length open reading frame, a probe containing the most 5' ~ 400 bp region of clone 74 was used to re-screen the library. Of six clones thus identified, clone 103 and 104 are the longest; these contain an ATG and a 17 bp upstream region, and they share identical sequences (Fig. 1a). Comparison of their restriction enzyme mapping patterns indicates that they share the same 3' end with clone 74.

The complete nucleotide sequence of the BP-3 clones contains a single open reading frame encoding 311 amino acids which include the N-terminal and the two internal amino acid sequences that we obtained by protein analysis (Fig. 1b). The N-terminus of the predicted protein has 24 hydrophobic amino acids, suggestive of a leader peptide sequence, next to which are the residues indicated by the N-terminal amino acid sequence of the BP-3 antigen protein. Because these 24 hydrophobic amino acids are long enough for a functional leader peptide, there is unlikely to be another ATG translation initiation codon upstream in the same reading frame. The C-terminus of the protein contains a relatively hydrophobic region of 19 amino acids (Fig. 1b), which is short for transmembrane anchoring, and there is no possible cytoplasmic tail. These features are typical of GPI-linked molecules (8-10), which we know from previous studies is the mechanism for BP-3 attachment to the cell

Figure 1. (a) Schematic representation of BP-3 cDNA clones designated as 74, 78, 106, 103 and 104. Clones 74 and 78 begin 23 and 38 bp 3' to the translational initiation codon ATG respectively, while clones 106, 103 and 104 begin 1, 17 and 17 bp 5' to the ATG respectively. The positions for the TGA stop codon and several restriction sites are also shown. (b) Nucleotide and deduced amino acid sequences of the BP-3 gene. The ATG start codon, TGA stop codon and two polyadenylation signal sites are in bold print. The AU consensus sequences in the 3' untranslated region are underlined. The potential N-linked glycosylation sites (Asn) are marked in boldface. The N- and C-terminal hydrophobic regions are indicated by boldface. The amino acids determined by N-terminal and internal peptide sequencing are underlined. The suspected cleavage site for GPI attachment is also marked in boldface print.

a.



b.

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      AGGAATCAAAGTTCTTGATGGCTGTCCAGGGAGGCCTGTTGTCTCTGTGGCTGTGGCTATGGCTGTCCTTGCTG   74
                      MetAlaValGlnGlyGlyLeuLeuSerLeuTrpLeuTrpLeuTrpLeuSerLeuLeu    19

ACAGTGCTGCTGGGGGCAAGAGCGCGGTGGAGAGGGGAGGGTACCACTCCTCACCTCCAGAGCATCTTCCTGGGCCGCTGC  155
ThrValLeuLeuGlyAlaArgAlaArgTrpArgGlyGluGlyThrThrProHisLeuGlnSerIlePheLeuGlyArgCys   46

GCAGAGTACACCACGCTGCTGAGCCTGGGGAACAAAAACTGCACAGCCATCTGGGAGGCCTTCAAGGGGGTGCTGGACAAG  236
AlaGluTyrThrThrLeuLeuSerLeuGlyAsnLysAsnCysThrAlaIleTrpGluAlaPheLysGlyValLeuAspLys   73

GACCCCTGCTCTGTGCTCCCCTCAGACTATGATCTCTTCATTAACCTCTCCAGGCACCCCATTCCTAGGGACAAGTCACTG  317
AspProCysSerValLeuProSerAspTyrAspLeuPheIleAsnLeuSerArgHisProIleProArgAspLysSerLeu  100

TTCTGGGAGAATAACCACCTGCTCGTTATGAGCTATGGGGAGAACACCCGCCGCCTCGTGGCCCTGTGCGACGTGCTGTAT  398
PheTrpGluAsnAsnHisLeuLeuValMetSerTyrGlyGluAsnThrArgArgLeuValAlaLeuCysAspValLeuTyr  127

GGCAAAGTTGGCGATTTCTTGAGCTGGTGTCGACAGGAAAATGCCTCTGGACTTGACTACCAGTCCTGCCCCACATCAGAA  479
GlyLysValGlyAspPheLeuSerTrpCysArgGlnGluAsnAlaSerGlyLeuAspTyrGlnSerCysProThrSerGlu  154

GACTGTGAAAACAACGCCGTGGACTCCTATTGGAAAAGTGCATCCATGCAGTATTCAAGAGACAGTTCAGGGGTGATCAAC  560
AspCysGluAsnAsnAlaValAspSerTyrTrpLysSerAlaSerMetGlnTyrSerArgAspSerSerGlyValIleAsn  181

GTCATGCTGAATGGCTCGGAGCCAAAAGGAGCCTATCCCACGAGAGGGTTTTTTGCAGATTTTGAAATCCCGTACTTACAA  641
ValMetLeuAsnGlySerGluProLysGlyAlaTyrProThrArgGlyPhePheAlaAspPheGluIleProTyrLeuGln  208

AAGGATAAAGTCACAAGAATAGAGATCTGGGTTATGCATGACGTTGGAGGGCCCAATGTGGAATCCTGTGGCGAAGGCAGT  722
LysAspLysValThrArgIleGluIleTrpValMetHisAspValGlyGlyProAsnValGluSerCysGlyGluGlySer  235

GTGAAAATCCTGGAAGACAGATTGGAGGCCCTGGGGTTCCAGCACAGCTGTATCAATGACTACCGACCAGTGAAGTTCTTA  803
ValLysIleLeuGluAspArgLeuGluAlaLeuGlyPheGlnHisSerCysIleAsnAspTyrArgProValLysPheLeu  262

ATGTGTGTGGACCACAGCACTCACCCCGACTGTATCATGAACTCAGCCTCAGCATCTATGCGGAGGGAGAGCGCATCTCTT  884
MetCysValAspHisSerThrHisProAspCysIleMetAsnSerAlaSerAlaSerMetArgArgGluSerAlaSerLeu  289

CATGCAATAGGAGACGCCAGCCTTCTCATTTCTCTCTTAGTAGCTTTGGCTTCAAGTTCTCAGGCGTGACTAGAAAATCCC  965
HisAlaIleGlyAspAlaSerLeuLeuIleSerLeuLeuValAlaLeuAlaSerSerSerGlnAlaTER              311

CACAGGGTTCTAACAGAAGCCAGCCTTGCAGCAACCCTTGAGTAGTCCACTGTCCTGTGTAAACTAAGGAAACCCACTGTC 1046
TCAAGACAAACCCTTCTAGAAAAACAATGTAACCAGACGGTAATCCAGTTGTATTTACACTCATGATTCAAGAAACAAGGC 1127
ATTCATCTAGTCCTGCTTAGCAGACAGAAGTTGCTACTGTGGAGTTAAAGTTATTATCTCTTATTTGATAATGGTACAAAG 1208
CACTGAGACCTGGAGAATAGTTTAATCTTAAATAATTAAATGTATTTATGGAAGCATCTGTATTTTTATTTTCTGTATTTC 1298
TTGTGTTTCAATGATACACACGTGTTTGTTAAGAGCTTCAATTACTGTACAGCATATAAAGGTATACATATATAGATGTTA 1370
TGCAAAGATGTATATCTAAAGATGTCTATTTAATGGCTACTTATGATAAGGAAATAACCCAAAATGTCAAGCACACACAAG 1451
CACACACAGGTTTGGAACTGCTACATAAGGTTTTGTATACCCATAGGATGGAACGTTCATTGTTAAGTAAATGTCATGTGG 1532
TCACCCTGACTGGGTGAACAGTTAAGTCAACTGTAGTGCATTCTGTAACTGCAGTGCATTCTGTACTGGGCACTACTGCTC 1613
AGCCATCAGCAGGAACTAGTATTGATGTATAGTCATATGAATGGCAATTCCAGTGCATTGTGCTATGGGAAAGAAGCCTGA 1694
CCAAATGGCTACACACTGCAGATAAAAATACAGAGTTCTGCAAAGGGCCCGAAGCTATGGCGCAAGAGGAATGCGGTGGAT 1775
TCCAGGATGGGTGCCCGGGGTGCAGAGGCTTGAGGGGGGGAGGGGCTAAGTTAAAGGAGAAGTGAGAAACACCAAGGTGGC 1856
ATCTCACATAAACTTCTCAAAACTCTCAAAGCAGAGCATCAGAAGGAGTAAATCTCACAGGATGCAAATTTAACATCTTTG 1937
TCTTAGGAACATTCATCACATAAGAAAATGTTAACATGTGTTAGAGATGGGATATAAAACTACTCACAATATGATGCAAGT 2018
GTTATGAAATATAATATGAATGTAAGAAATACTATCACATTCCTTATGTGTGTTTATAGCATATGCATACACTGTAAATGA 2099
ATAAAAGAAAATAACCCCAAGGAGAGTGCAGTTCTCTCTGAATAGGGTCTTCACAGCTAAAAAAAAAAAAAAAAAAAA   2176
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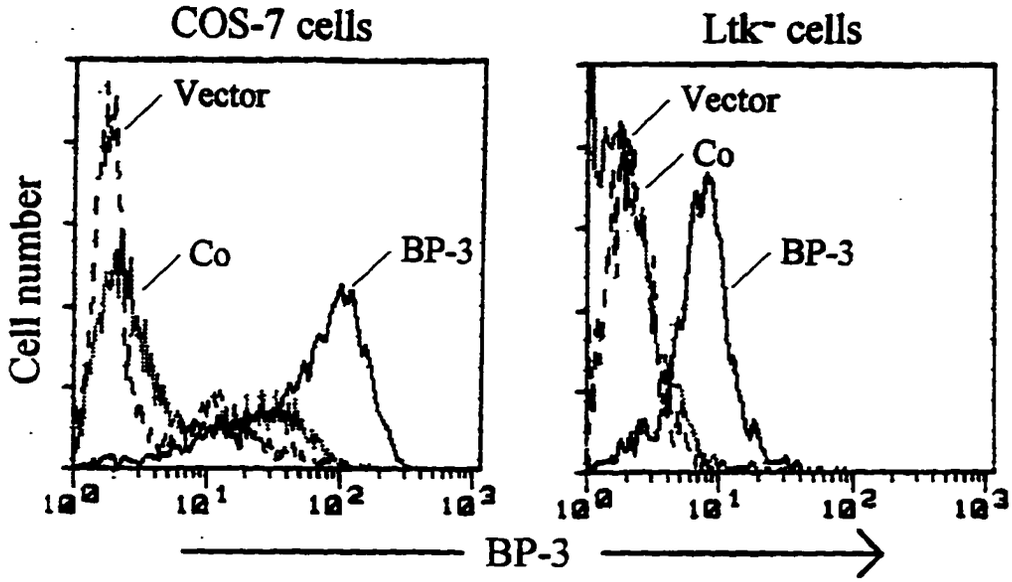
membrane (2). Referring to the pattern of known GPI-linked proteins, we suspect that the protein may be cleaved between amino acids 279 and 280 before the GPI attachment (8-10). Four possible *N*-linked glycosylation sites are indicated by the Asn-X-Ser/Thr sequence, a result that agrees with the biochemical characteristics of the BP-3 antigen (1).

Expression of the BP-3 cDNA. The cDNA insert from clone 104 was subcloned into the pMKITneo vector under the control of a SRα promoter. When this construct (SRαBP-3) was used to transfect COS-7 cells, the transfected cells could be stained specifically with BP-3 antibody (Fig. 2a), and the BP-3 staining was reduced by 70% after the treatment of PI-PLC (data not shown). When these cells were labeled with ^{35}S and the BP-3-reactive proteins examined by SDS-PAGE analysis, transiently transfected COS cells were found to express 36-42 kDa proteins. The size variation observed for the recombinant BP-3 molecules, like the native BP-3 antigen, proved to be due to variable glycosylation of a 32 kb core protein (Fig. 2b). The insert of clone 104 was also subcloned into pCDNA3 vector (pCDNA3-BP3) which was linearized and transfected into mouse Ltk$^-$ cells. When limited dilution was used to isolate multiple clones, most of these clones expressed BP-3 antigen at varying levels (Fig. 2a). These results confirm that we have identified the BP-3 cDNA.

The BP-3 protein shares significant sequence homology to NADase, but may lack this enzyme activity. A search of the sequence data bases using the BLASTNCBI and TFASTA programs of the GCG package did not reveal a gene with identical sequence, indicating that BP-3 represents a novel gene. However, the predicted BP-3 protein sequence has ~ 30% identity and 50% similarity with NAD glycohydrolase of *Aplysia californica* (11) and the CD38 antigens of mice and humans [Fig. 3 (12-13)]. Sequence alignment of these proteins revealed that 46 amino acids are highly conserved, including 10 cysteine residues (Fig. 3). This degree of homology is suggestive of common structural features.

Figure 2. Expression of the BP-3 gene. (a) COS-7 cells and Ltk$^-$ cells transfected with BP-3 expression constructs were analyzed by flow cytometry. The BP-3 transfectants were stained with BP-3 antibody (BP-3) or an isotype-matched control antibody (control). The control COS-7 transfectants or Ltk$^-$ cells were also analyzed by BP-3 staining (vector). (b) Immunoprecipitation of BP-3 antigen from ^{35}S-labeled COS-7 cells. SRαBP-3- or control-transfected COS-7 cells were labeled with ^{35}S and precipitated with BP-3 coated beads. The reactive material, with or without N-glycanase treatment, was electrophoresed on a 10% SDS-PAGE gel. The positions of molecular mass markers in kDa are shown on the left.

a.



b.

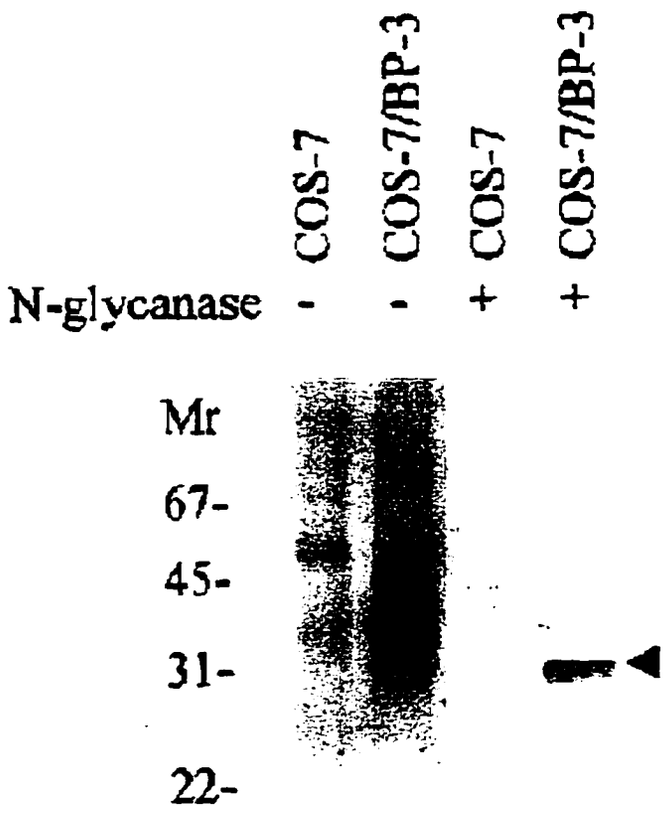


Figure. 3. Sequences of the mature chains of CD38, NADase and BP-3 antigen aligned with each other. The upper sequence is human CD38 and is used as a reference. The three lines below this are the residues that differ between human CD38 and mouse CD38, human CD38 and BP-3 antigen reported here, and between human CD38 and NADase. To obtain the alignments, mouse and human CD38 sequences were aligned to each other, and then BP-3 antigen and NADase were aligned to the consensus sequence. The four lower lines are the consensus sequences of all four proteins and of CD38 with BP-3 antigen, of CD38 with NADase and of BP-3 antigen with NADase.

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VPRWRQTWSGPGTTKRFPETVLARCVKYTEI-----HPEMRHVDCQSVWDA-FKGAFISKHPCNITEEDYQPLMKLGT--   CD38hum
R  SLLV T EP   H SDIF G  LI  Q ----LR    DQN  EILST-     V  N     R   A  V  V --   CD38mus
RA   ---- E   PHLQSIF G  AE  ------TLLSLGNKN TAI E -    -VLD D  SVLPS  DLFINLSRHP   BP3 antigen
VP---------- RELENVF G  KD -EITRYLDILPRVRS  SAL KDF  -AFSF N  DLDLGS KDFFTSAQ--   NADASE
             T       L RC  Y                C       FK     K PC      Y               consensus
 P           T       L RC  Y  I             C       FK AF  K PC      Y               CD38 + NADASE
             T       L RC  Y T              C       FK     K PC      Y               CD38 + BP3
             T  L   FLGRC  Y         L      C A W   FK     K PC      Y  F            BP3 + NADASE

QTVPCNKILLWSRIKDLAHQFTQVQRDMFTLEDTLLGYLADDLTWCGEFNTSKINYQSCPDWRKDCSNNPVSVFWKTVSR   CD38hum
  I    T F  KS H    Y WI GK           I    R   DPS  DM  V   H SEN P   ITM   VI Q   CD38mus
  I RD S F ENNHL VMSYGENT RLVA C V Y KVG F S  RQE A GLD Q   T-SED E  AVDSY  SA M   BP3 antigen
 QL K  VMF  GVY E  DYANTG KYI      P  MLNS V   QRANPGF EKV  D-FKT PVQARES  GMA S   NADASE
Q  P  K   W                    L D L G    L WC            CP     C        W   S   consensus
Q  P  K   WS     AH            LEDTL GY    L WCG        N   CP     C        FW  S   CD38 + NADASE
QT P NK L W     L             TL D L G   D L WC         Y SCP     C NN     W   S   CD38 + BP3
Q  P  K  FW         Y    R    L D L G    L WC Q    G     CP     C   A  S W  AS      BP3 + NADASE

RFAEAACDVVHVMLNGSRSK-IFDKNSTFGS-VEVHNLQPEKVQTLEAWVIHGGREDSRDLCQDPTIKELESIISKRNIQ   CD38hum
K   D  G  Q      LRE-P Y       -L  FS D N  HK Q   M DIEGA SNA SSSSLN  KM VQ   MI   CD38mus
QYSRDSSG IN      EPK-GAYPTRG FADF IPY QKD  TRIEI  M DVGGPNVES GEGSV I  DRLEALGF    BP3 antigen
SYAHSAEGE TY VD  NPKVPAYRPDS FGKY LPN -TN  TRVKVI L RLGEKIIEK GAGSLLD  KLVKAKHFA   NADASE
           M  GS           F    E   L   KV      V H         C       L              consensus
           M  GS           F    E   L   KV      V H         C       L              CD38 + NADASE
        V  VMLNGS          F    E   L   KV      V H         C       L              CD38 + BP3
 Y     G V  M  GS PK  AY    FF   E P L   KVTR    V H  G    E CG GS   LE    A  F    BP3 + NADASE

FSCKNIYRPDKFLQCVKNPEDSSCTSEI
 A VDN   AR          HP  RLNT                                                       CD38hum
H  I D   V   M  DHSTHPD IMNSASASMRRESASLHAIGDASLLISLLVALASSSQA                     CD38mus
FD VENP AVL  L SD  NARE RLAKRFYRIA                                                 BP3 antigen
  C    R   FL C        C                                                          NADASE
  C    R   FL C  NP    C                                                          consensus
  C   YRP  FL CV       C                                                          CD38 + NADASE
  C    R V FL C D      C                                                          CD38 + BP3
                                                                                  BP3 + NADASE
```

The possibility that the BP-3 antigen is an ecto-NADase was tested by use of the NAD analog ε-NAD. The 1H6A pre-B cells, which express both BP-3 antigen and CD38 on the cell surface (data not shown), could hydrolyze the ε-NAD substrate as indicated by a 5-fold enhancement of the fluorescence intensity at 410 nm. However, NAD hydrolase activity could not be demonstrated as a result of transfection of either COS-7 or Ltk$^-$ cells with BP-3 expression vectors (Table 1). Thus the BP-3 antigen does not appear to possess the same enzymatic activity as its NADase and CD38 relatives.

Table 1. Analysis of ecto-NADase activity in 1H6A pre-B cell line and BP-3 transfectants

Cells	Fluorescence$_4$ emission(x10)
None	1.1
1H6A	5.0
COS-7	2.6
COS-7/BP-3	2.8
Ltk$^-$	1.1
Ltk$^-$/BP-3	1.2

Cells (5×10^4) were incubated with ε-NAD, and the fluorescence emission was measured by a fluorometer.

Northern blot analysis reveals two BP-3 transcripts in multiple cell types and tissues. When RNA from mouse cell lines and tissues was analyzed in Northern blots with the cDNA insert of clone 74, a major 1.3 band and a minor 2.3 kb band were detected in mouse cell lines representing pre-B, immature B, pre-T and probably T stages, but not in a mature B cell (Table 2). BP-3 messages were also expressed by two myeloid lines, but were not detected in bone marrow or thymic stromal cell lines (Table 2). The distribution of BP-3 transcripts in these cells correlates well with reported profiles of surface expression of BP-3 antigen (1). Among the eight tissues examined, heart, lung, intestine, kidney, spleen, and thymus expressed BP-3 transcripts, whearas brain and liver did not (Fig. 4a). This result

Table 2. Northern blot analysis of BP-3 transcription in murine cell lines

Cell lines	Cell type	BP-3 transcripts
1H6A	pre-B	+
38B9	pre-B	+
WEHI-231	immature B	+
WEHI-279	mature B	-
YAC-1	thymoma	+
EL-4	mature T	(+)
HTX-1	myeloid	+
WEHI-3	myeloid	+
BHM	BM stroma	-
TEPI	thymic stroma	-

RNA (10-20 μg) from cell lines were electrophoresed in a 1.2% agarose/ MOPS-formaldehyde gel, blotted onto nylon membrane, and probed with the 1.2 kb cDNA insert of clone 74.

is consistent with earlier immunohistological studies (2), except that the BP-3 antigen was not seen previously in heart and lung. As in the cell lines, transcripts of two sizes were present in the tissues, although longer exposure of the Northern blot was needed to show the bands in the thymus (Fig. 4a). To determine whether these two kinds of mRNA might result from differential polyadenylation, we re-probed the blot with an ~ 600 bp *Pst*I-*Xho*I fragment of clone 78, which represents the most 3' region that is not present in the 1.2 kb clones. Only the 2.3 kb RNA band hybridized with this probe, indicating that it represents RNA transcripts using the second poly(A) addition site (Fig. 4b).

Discussion

In this study, cDNA clones encoding the murine BP-3 antigen have been isolated. When these were used as probes in Northern blot analyses, BP-3 expression was observed in B, T and myeloid lineage cells and in a variety of tissues. The protein predicted by the BP-3 cDNA sequence shares significant homology with NAD glycohydrolase and the CD38 antigen in humans and mice.

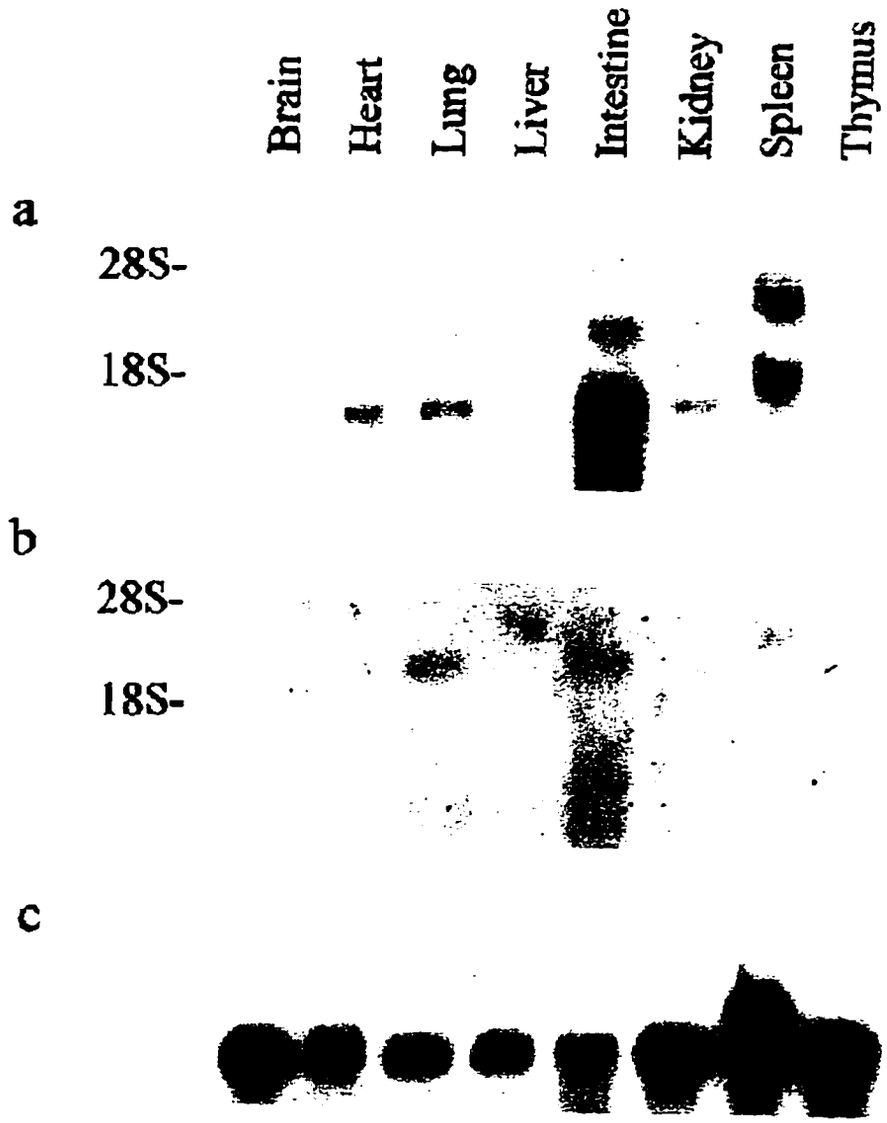

Figure 4. Northern blot analysis of tissue distribution of BP-3 expression. (a)Total RNA (20 µg) from brain, heart, lung, liver, intestine, kidney, spleen and thymus of a adult mouse was electrophoresed, blotted, and probed with the clone 74 insert. The positions of 28S and 18S RNA are indicated on the left. The blot was striped and rehybridized with (b) the most 3' ~ 600 bp *Pst*I/*Xho*I fragment of clone 78 or (c) a cyclophilin probe.

Both the cDNA clone sequence and the Northern blot analysis provided evidence for two forms of BP-3 gene transcripts. Sequencing of one cDNA clone, 78, revealed two possible polyadenylation signals. The first polyadenylation site has the sequence AATAAT, the terminal nucleotide of which does not match the highly conserved AATAAA sequence of the most frequently utilized polyadenylation site. This could explain why a portion of BP-3 transcripts use the second polyadenylation site. This type of differential polyadenylation site utilization has been reported for other genes (14-19), for some of which tissue-specific distribution of the different transcripts may be observed (17, 19). Transcripts of different lengths may also vary in stability and translatability (16, 18). Judging from our Northern blot analysis, the two BP-3 gene transcripts did not appear to be tissue-specific or cell developmental stage-specific. The unique 3' untranslated region in the longer clone contains two additional copies of the AU consensus sequences reported to mediate mRNA degradation (20). This implies that the longer transcript may be less stable than the shorter one.

A search of sequence data bases with the BP-3 nucleotide and protein sequences indicates that the BP-3 cDNA represents a novel gene which has significant homology with NAD glycohydrolase from *A. californica* (11) and the mouse and human CD38 antigens (12, 13) at the protein level. Unlike the CD38 molecule, which has an N-terminal transmembrane sequence, the BP-3 antigen has a GPI anchor at the C-terminus. Forty six amino acids, including 10 cysteine residues, are conserved, which suggests that the proteins could share common features in their tertiary structure. However, CD38 molecules have an additional pair of cysteines, and BP-3 has one unpaired cysteine at position 123, suggesting potential structural differences. NADase of the mollusca *A. californica* and murine CD38 are enzymes that can catalyze the hydrolysis of the nicotinamide-ribose bond of NAD and

synthesize cyclic-ADP-ribose (cADPR) (21, 22). They are considered to be ADP-ribosyl cyclases because they mediate the synthesis of cADPR, a Ca^{2+} mobilizing agent that acts independently of inositol 1,4,5-triphosphate (23).

Based on amino acid sequence homology, an attractive hypothesis is that BP-3 is also an ecto-NADase. However, while a CD38-bearing pre-B cell was able to hydrolyze the NAD analog, ε-NAD, this enzymatic activity could not be shown for BP-3 cDNA transfected cells that expressed the recombinant BP-3 antigen on the cell surface. This was true for both transient and permanent transfectants that expressed the recombinant BP-3 protein. There are several possible explanations for this negative result. One possibility is that the COS-7 and Ltk^- transfectants might produce inhibitive factors. A second possibility is that NADase activity of BP-3 requires the presence of accessory molecules that are not present in the transfected cells. On the other hand, it has been reported that recombinant murine CD38 alone possesses enzyme activity (22). A third possibility, which we favor currently, is that the BP-3 protein does not have the same enzymatic activity of CD38. In this regard, it is noteworthy that the region of the BP-3 antigen that is most similar to CD38 is not the same as the region of CD38 which is most similar to NAD hydrolase (Fig. 3). This may explain the apparent lack of NADase activity for the recombinant BP-3 antigen. BP-3 and CD38 are expressed together on pre-B cells; it would seem an unnecessary redundency for both to have the same function. Further analysis of the enzymatic potential of BP-3 is currently being pursued to resolve this issue.

The function of the BP-3 antigen is thus unclear at the present time. Cross-linkage of CD38 molecules can enhance B lymphocyte proliferation in response to IL-4 and lipopolysaccharide (24, 25). The BP-3 molecule, a distant relative of CD38, may conceivably have similar function, thereby acting in concert with CD38. Alternatively, BP-3 and CD38

may have opposing functions in order to regulate cell signaling and growth. In the long term bone marrow culture system of Whitlock-Witte (26), our preliminary studies indicate enhanced growth of early B lineage cells following incubation with the BP-3 antibody (J. Wang, unpublished observations). The widespread tissue distribution of the BP-3 antigen, as revealed by immunohistochemical studies and by Northern blot analysis, suggests that this molecule may function in a variety of non-hematopoietic cell types as well as in cells of the immune system. The molecular cloning of the cDNA reported here, together with the BP-3 monoclonal antibody, should greatly facilitate study of its physiological roles in lymphocytes, myeloid cells, specialized reticular cells and enterocytes.

Acknowledgments

J. W. is a research associate of the Howard Hughes Medical Institute. M. D. C is an investigator of the Howard Hughes Medical Institute. We thank Dr David McCourt of the Howard Hughs Medical Institute in St Louis for performing N-terminal protein sequencing, Dr Kazuo Maruyama at Tokyo Medical and Dental University for providing the pMKITneo vector, Mr Zheng Gong and Dr Herbert Cheung in the Biophysics Department of the University of Alabama at Birmingham for their invaluable help with the fluorometric assay of NAD glycohydrolase acitvity, and Ms Ann Brookshire for excellent secretary assistance. This work has been supported in part by NIH grants AI 30879 and CA 13148.

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GENOMIC ORGANIZATION AND CHROMOSOMAL LOCALIZATION OF THE MURINE *Bp3* GENE, A MEMBER OF THE CD38/ADP-RIBOSYL CYCLASE FAMILY

CHEN DONG, DENNIS WILLERFORD,
FREDERICK W. ALT AND MAX D. COOPER

Division of Developmental and Clinical Immunology, Departments of Medicine, Pediatrics and Microbiology, University of Alabama at Birmingham, and the Howard Hughes Medical Institute, Birmingham, AL and Children's Hospital, Boston, MA

Immunogenetics, in press

Abstract

The murine *BP-3* antigen is a variably glycosylated phosphatidylinositol-linked cell surface glycoprotein that is expressed on early B and T lineage cells, myeloid cells, intestinal epithelial cells and a discrete population of reticular cells in peripheral lymphoid tissues. The deduced amino acid sequence of *BP-3* cDNA shares significant homology with human and mouse CD38 and molluscan ADP-ribosyl cyclase, enzymes that generate the calcium-mobilizing agent cyclic ADP-ribose from NAD. In this study, we have cloned and characterized the *BP-3* gene. The gene consists of 9 exons and spans approximately 27 kb. The overall exon organization is very similar to that reported for the ADP-ribosyl cyclase gene in the mollusc, *Aplysia kurodai*. The *BP-3* gene is located on mouse chromosome 5 very near the gene for CD38, suggesting that this family arose by gene duplication. The major transcriptional start site of the *BP-3* gene in a pro-B cell line (-17 from the ATG start codon) contains a weak initiator sequence. The upstream region lacks a TATA box, but contains consensus recognition sequences for the PU.1, Ikaros/LyF-1, E2A and TCF-1 transcriptional factors that regulate gene expression in lymphoid and myeloid cells. Consensus motifs for cytokine responsive factors, NF-IL6/C-EBP, H-APF-1/APRF and AP-1 are also present in the flanking region, and interleukin 6 treatment enhances expression of the BP-3 antigen by a myeloblastoid cell line.

Introduction

The murine BP-3 cell surface antigen is a variably glycosylated glycosyl-phosphatidylinositol (GPI)-linked molecule that is selectively expressed by early B and T lineage cells and a discrete subpopulation of reticular cells in the peripheral lymphoid tissues (McNagny et al., 1988; McNagny et al., 1991). It is also expressed on mature myeloid cells, the brush border of intestinal epithelial cells and the lumenal surface of renal collecting

tubules (McNagny et al., 1988; McNagny et al., 1991). As a first step toward understanding the structure and function of the BP-3 molecule, we have isolated, sequenced and expressed BP-3 cDNA clones (Dong et al., 1994). Kaisho et al. independently isolated the related human *BST-1* gene that encodes an antigen expressed in relatively high levels on bone marrow stromal cells from rheumatoid arthritis patients (Kaisho et al., 1994). The deduced amino acid sequences of these two genes show 73% identity and 85% similarity. Consistent with the suggestion that these represent mouse and human homologues, the mouse *BST-1* gene cloned on the basis of this homology proved to be identical to the *BP-3* gene (Itoh et al., 1994).

The deduced amino acid sequences of *BP-3/BST-1* cDNAs show significant homology with the CD38 molecule and molluscan ADP-ribosyl cyclase (Koguma et al., 1994; Harada et al., 1993; Jackson and Bell, 1990; Glick et al., 1991). These enzymes hydrolyze NAD and generate a novel calcium-mobilizing messenger, cyclic ADP-ribose, which acts independently of inositol 1,4,5-triphosphate (IP3) (Takasawa et al., 1993; Summerhill et al., 1993; Zocchi et al., 1993; Howard et al., 1993; Lee and Aarhus., 1991; Hellmich and Strumwasser, 1991; Galione et al., 1991; Meszarous et al., 1993). Although we failed to detect NAD hydrolase activity of the recombinant BP-3 molecule at neutral pH (Dong et al., 1994), Hirata et al. found that the recombinant human BST-1 protein displays ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activity at low pH, which is enhanced by the presence of zinc (Hirata et al., 1994). We have since observed that the recombinant mouse BP-3 molecule has low ADP-ribosyl cyclase activity under similar acidic conditions (C.D. and H. C. Lee, et al, unpublished data).

The CD38 molecule is a type II transmembrane glycoprotein with an unusual pattern of discontinuous expression during lymphocyte differentiation (Malavasi et al., 1994; Lund et

al., 1995; Tedder et al., 1984). Agonistic antibodies to CD38 can induce the proliferation of B and T lymphocytes (Malavasi et al., 1994; Lund et al., 1995; Funaro et al., 1990; Santos-Argumedo et al., 1993), protect B cells from apoptosis (Santos-Argumedo et al., 1993; Zupo et al., 1994), and inhibit B lymphopoeisis (Kumagai et al., 1995). The human CD38 molecule has been reported to associate with the antigen receptor complexes (Funaro et al., 1993). CD38 ligation induces the influx of extracellular calcium, apparently independent of the IP_3 pathway (Santos-Argumedo et al., 1993), and protein kinases may be involved in CD38 signal transduction (Kirkham et al., 1994; Santos-Argumedo et al., 1995). Recently, a human CD38 ligand has been identified as a 120 kDa protein that is expressed predominantly on endothelial cells (Deaglio et al., 1996).

The differentially regulated expression of the CD38/BP-3/BST-1 family of proteins during lymphoid and myeloid development predicts restricted functional roles. While the function of BP–3/BST-1 is still unclear, it has been reported that stromal cell or fibroblast cell lines that express human BST-1 may enhance pre-B cell growth (Kaisho et al., 1994). In unpublished studies, we (J. Wang and M.D.C.) observed that treatment of murine bone marrow cells with the BP-3 antibody enhanced the growth of early B-lineage cells under both short-term and long-term culture condition. An agonistic antibody against the mouse BP-3/BST-1 molecule also enhanced pre-T cell proliferation and differentiation (Vicari et al., 1996). BP-3/BST-1 thus could play a role in early B and T lineage development. As a first step toward defining the function of the *BP-3/BST-1* gene, we have determined its genomic structure, compared its exon configuration with another representative of the CD38/ADP-ribosyl cyclase gene family, ADP-ribosyl cyclase of mollusc, *Aplysia kurodai*, and mapped the gene to chromosome 5 near the mouse gene for CD38.

Materials and Methods

Isolation and characterization of the BP-3/BST-1 genomic clones. A λFixII mouse (strain 129) genomic library (Stratagene, La Jolla, CA) was screened with a 1-kb BP-3 cDNA probe radiolabeled by a Prime-It RT kit (Stratagene). The phage DNA of the positive clones was prepared by use of Wizard Lambda Prep (Promega, Madison, WI) or Qiagen Lambda Midi kits (Qiagen, Chatsworth, CA) and subjected to restriction enzyme mapping analysis. *Apa* I and *Xba* I (Boehringer Mannheim, Indianapolis, IN) fragments of BP-3 cDNA clone 78 and oligonucleotide probes were used to determine the positions of the exons.

Sequence analysis. *Eco*R I-digested genomic fragments were subcloned into pBluescript SK (Stratagene). Oligonucleotides derived from the cDNA sequence or from flanking intron sequences were used for DNA sequencing (Sequenase 2.0, USB, Cleveland, OH) to determine the exon-intron boundaries. Polymerase chain reaction (PCR) was employed to determine the approximate intron sizes. The 5' flanking region was also sequenced, and putative transcription factor binding sites were identified by visual inspection or by use of the TFD SITES database of transcription factors (Faisst and Meyer, 1992; Ghosh, 1990).

Backcross panel mapping of the BP-3/BST-1 gene chromosomal location.. Genomic DNA samples from the Jackson Laboratory (Bar Harbor, ME) BSS interspecific backcross DNA panel of 94 N2 segregants from the cross (C57B/6JEi x SPRET/Ei) x SPRET/Ei (Rowe et al., 1994) (10 μg each) were digested with *Bgl* I (Boehringer), resolved on 0.6% agrose-TBE gels, and blotted onto Nytran Plus membranes (Schleicher & Schull, Keene, NH). The blots were then probed with a 0.8 kb *Hind* III genomic fragment in the 5' flanking region. The resulting allele distribution was compared to those of the 1400 loci previously mapped in the cross, and the map position was determined by the method of minimizing double recombinants.

Mapping of transcription start sites by RNase protection assay. A genomic fragment consisting of 97 bp coding and 187 bp 5' flanking sequences was amplified from DNA of a BALB/C mouse, subcloned into pBluescript SK between *BamH* I and *Kpn* I sites and confirmed by sequencing analysis. The plasmid containing this fragment was linearized with *BamH* I, and an antisense RNA probe was generated by T7 RNA polymerase (Promega) using α-^{32}P-UTP, and purified by a G50 spin column (Boehringer) and a 6% standard-size denaturing polyacrylamide gel. An aliquot of the purified RNA probe (2×10^5 cpm) was incubated with 30 μg of total RNA of the 38B9 pro-B cell line or control yeast tRNA at 42°C overnight and then subjected to RNaseA+T1 digestion at room temperature for 30 min. RNase activity was inactivated by proteinase K treatment, and the RNA was precipitated and analyzed by denaturing electrophoresis.

Interleukin 6 treatment of myeloblastoid cells. The M1 myeloblastoid cell line, obtained from American Type Culture Collection (ATCC, Rockville, MD), was cultured in RPMI-1640 medium containing 10% fetal calf serum with or without interleukin 6 (100 U/ml). Cells harvested from the cultures were incubated with biotinylated BP-3 antibody or an isotype-matched control antibody, and reactive cells were revealed by streptavidin-PE staining (Southern Biotechnology Association, Birmingham, AL) analyzed with a FACScan instrument (Becton Dickinson, Braintree, MA).

Results

Genomic structure of the mouse BP-3/BST-1 gene. A 1.0 kb BP-3 cDNA probe containing the entire coding sequence, the 5' untranslated region (UTR) and part of the 3' UTR was used to screen a λFixII genomic DNA library of the 129 mouse strain. Twenty positive phage clones were obtained, and then DNA was digested with restriction enzymes and probed with three *Apa* I/*Xba* I cDNA fragments representing different regions of the

BP-3 cDNA. This assessment indicated that some clones were identical and focused the analysis to 4 overlapping clones, named 10.2, 4.2, 10.1 and 1.1 (Fig. 1). A restriction map of these clones was then constructed using the *EcoR* I, *Bgl* I and *Sal* I enzymes (Fig. 1).

The *EcoR* I fragments of phage 10.2, 10.1 and 1.1 were subcloned and sequenced. The primers used in this analysis are shown in Fig. 1. PCR was employed to estimate the distance between exons or from exons to the next *EcoR* I sites. This analysis indicated that the *BP-3/BST-1* gene consists of 9 exons and spans approximately 27 kb (Fig. 1; Table 1). The first exon of 184 bp contains all of the 5' UTR, the leader peptide coding sequence (24 amino acids) and the N-terminal 32 amino acids (Table 1). The 5' UTR has a two-nucleotide insertion not seen in the reported cDNA sequence, possibly attributable to a strain polymorphism between BALB/c and 129 mice (data not shown). The first 4 exons are clustered within approximately 5 kb and these encode 171 of the 311 BP-3/BST-1 amino acids. Exons 5 through 8 are relatively small, 83, 77, 93, and 60 bp respectively, and these are dispersed over an approximately 15 kb region (Table 1; Fig. 1). Exon 9, which is the largest, contains the terminal coding sequence and all of the 3' UTR (Table 1). Each of the mouse BP-3/BST-1 splice junction sequences conforms to the eukaryotic splice consensus sequence (AG/GT). The first amino acid encoded in exon 9 is located at the position predicted for the phosphatidylinositol linkage (Dong et al., 1994). The amino acids encoded by this exon are thus not present in the mature cell surface protein. Comparison of the murine and human BP-3/BST-1 sequences indicates that the amino acids in this region show the least homology. Another coding region of low homology is the leader peptide (not shown).

The genomic structure of the ADP-ribosyl cyclase gene of the mollusk, *Aplysia kurodai* was previously described by Nata et al (Nata et al., 1995). It consists of 8 exons: the first

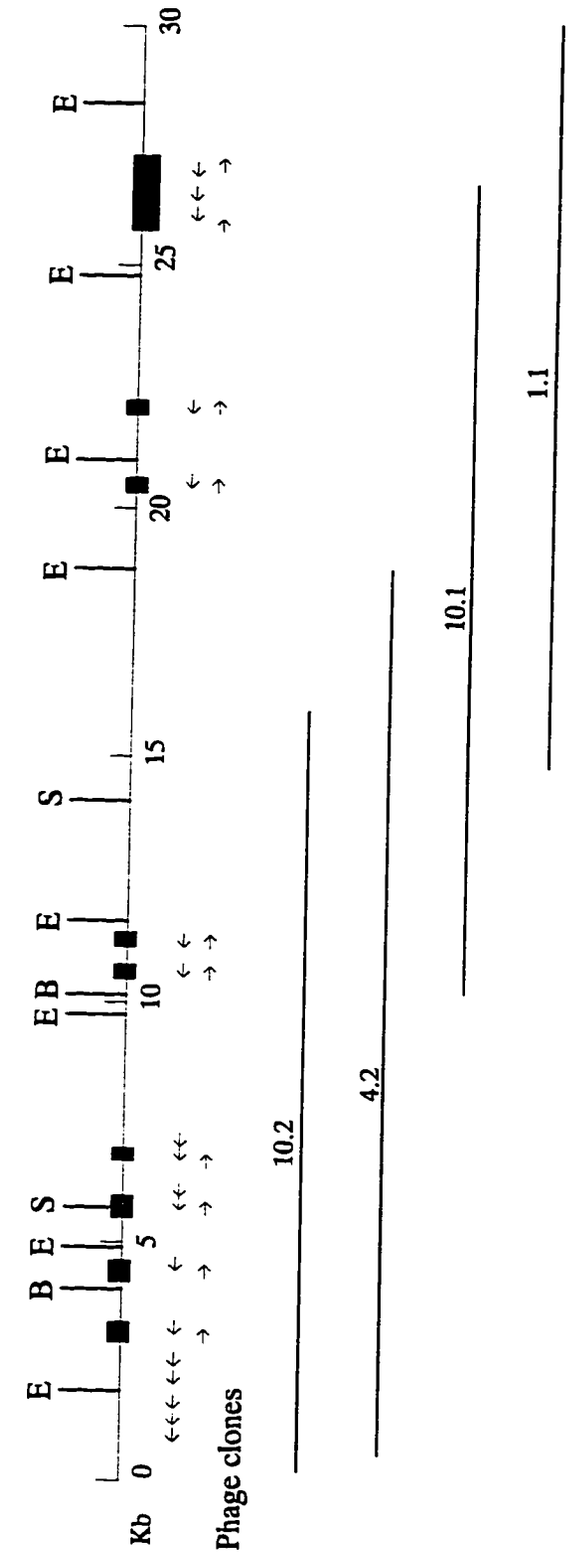


Figure 1. Schematic model of the *BP-3/BST-1* gene. The gene spans approximately 27 kb as determined by analysis of 4 overlapping phage clones, 10.2, 4.2, 10.1 and 1.1. The 9 exons are shown as filled boxes. Recognition sites for restriction enzymes *EcoR* I (E), *Bgl* I (B) and *Sal* I (S) are indicated. Small arrows represent the location of oligonucleotide primers used for sequencing.

Table 1. Intron-exon junction sequences of the *BP-3/BST-1* gene

Exon #	Exon size (bp)	5′ flanking sequence	Exon and amino acid sequences	3′ flanking sequence
1	184	CACGGGCTAG	1 AGGAATCAAG...GAGCCTGGG 184 SerLeuGly 56	GTAAGTGCTA
2	127	TTTTGCACAG	185 GAACAAAAAC...TAGGGACAAG 311 57 AsnLysAsn....ArgAspLys 97	GTAAGGACAG
3	136	TTCTCATCAG	312 TCACTGTTCT...AATGCCTCTG 447 98 SerLeuPhe....AsnAlaSer 143	GTAAGACTTT
4	83	TTTCTCCTAG	448 GACTTGACT...ATCCATGCAG 530 144 GlyLeuAsp.....SerMetGln 171	GTAATCCTGG
5	77	CTTCCTGCAG	531 TATTCAAGAG.. CCACGAGAGG 607 172 TyrSerArg...ProThrArgGly 197	GTAAGGACAC
6	93	TGTCTCCCAG	608 GTTTTTTGCA...GGCCCAATGT 700 198 PhePheAla.....ProAsnVal 228	GTAAGTTTTG
7	87	TATGTTTTAG	701 GGAATCCTGT TGACTACCG 787 229 GluSerCys AspTyrArg 257	GTAGTGGCGC
8	60	TCTTCTGTAG	788 ACCAGTGAAG...CATGAACTC 847 258 ProValLys....MetAsnSer 277	GTAAGTAAAT
9	1311	TTCTCTTTAG	849 AGCCTCAGCA...CTTCACAGCT 2159 278 AlaSerAla.....	ATTCTGTTTT

exon contains all of the 5' untranslated region, and the latter seven have coding sequences (Nata et al., 1995). Comparison of the *BP-3/BST-1* gene with this molluscan gene reveals considerable similarity in exon organization (Fig. 2). When the two protein sequences were aligned together, most exons share boundaries at the same positions Interestingly, the fourth exon of the Aplysia gene is represented by two exons in the *BP-3/BST-1* gene, indicating that they split during evolutionary from the more primitive gene. The *BP-3/BST-1* gene has one additional exon, exon 9, which encodes a hydrophobic region that signals for PI-linkage (Fig. 2).

The BP-3/BST-1 gene is located near the gene encoding CD38 on mouse Chromosome 5. The allo-anti-BP-3 antibody was generated by immunizing an outbred M. spretus mouse with the transformed 18.81 pre-B cell line derived from the BALB/C inbred laboratory strain (McNagny et al., 1988). We employed a (C57BL/6JEi x SPRET/Ei) x SPRET/Ei backcross panel to determine the chromosomal location of the *BP-3/BST-1* gene (Rowe et al., 1994). Significant polymorphism was observed between the C57BL/6 and *M. spretus* genomes with a variety of restriction enzymes including the *Eco*R I, *Hind* III, *Pst* I and *Bgl* I (data not shown). When probed with a genomic probe 0.8 kb upstream of the first *Eco*R I site, the *Bgl* I-digested genomic DNA of the C57BL/6 strain yielded a single band of 4 kb, and for *M. spretus* a 3.5 kb band. The 94 backcross mice from the Jackson BSS panel were typed for this polymorphism, and the allele pattern was found to match that of the *Pmv5* proviral locus on chromosome 5. (Fig. 3a). The locus is designated as *Bp3* by the Mouse Locus Nomenclature Committee. Use of the Mouse Genome Database (MGD) World Wide Web and Encyclopedia of the Mouse Genome 3.0 software indicated that *Pmv5* is located 27 cM from the centromere on chromosome 5. The mouse CD38-encoding gene,

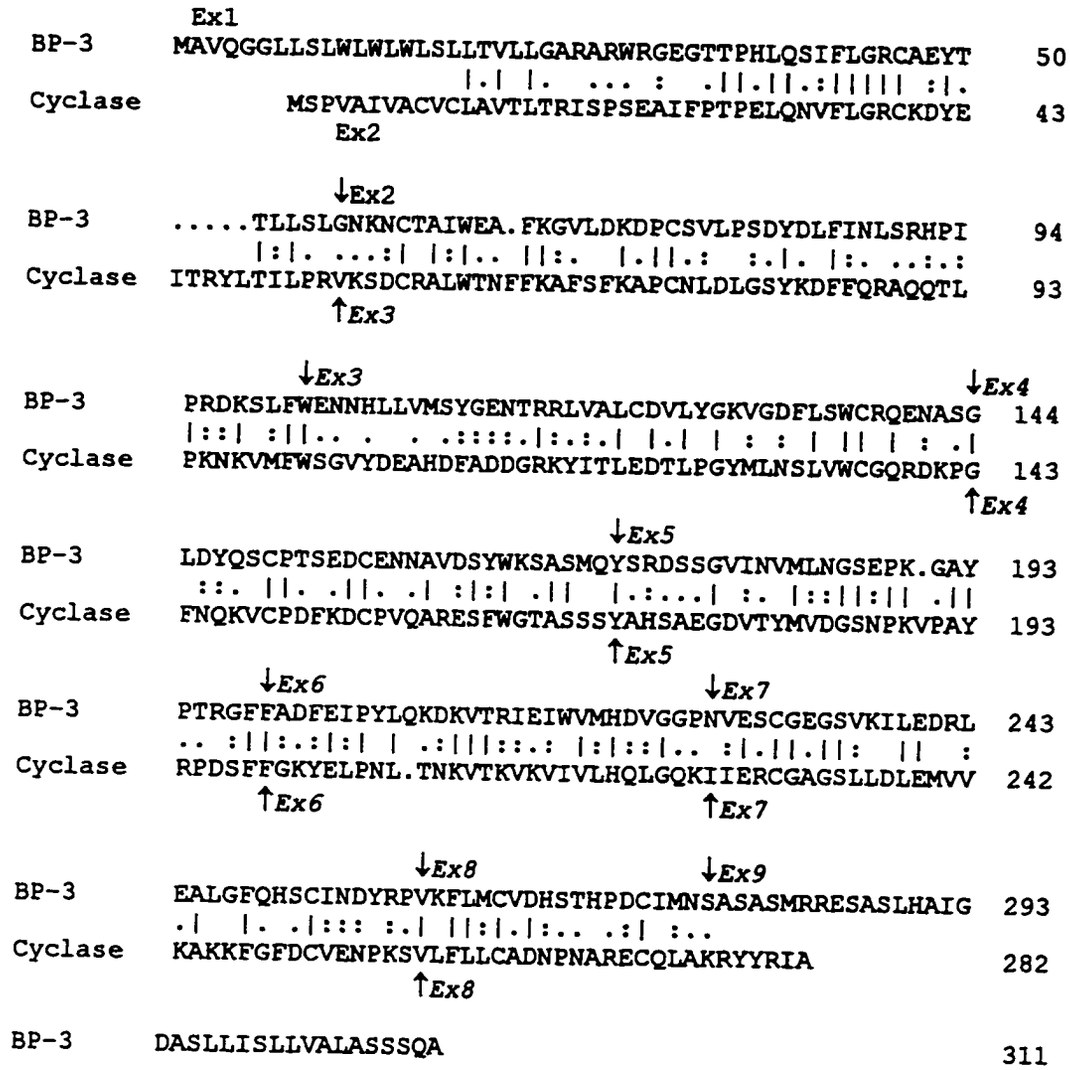


Figure 2. The exon organization of the murine *BP-3/BST-1* gene shares conserved features with that of the *ADP-ribosyl cyclase* gene in *Aplysia kurodai*. The amino acids of *the BP-3/BST-1* (BP-3) and *Aplysia ADP-ribosyl cyclase* (cyclase) genes are aligned using the Bestfit program of the Genetic Computer Group package. Identical amino acids are linked by short lines, and similar ones by one or two dots depending on the extent of similarity. Arrows indicate the beginning of an exon.

a.

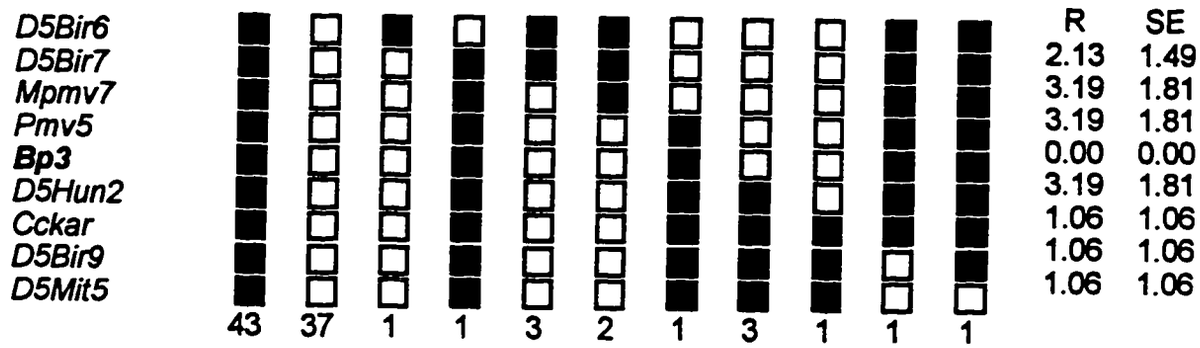
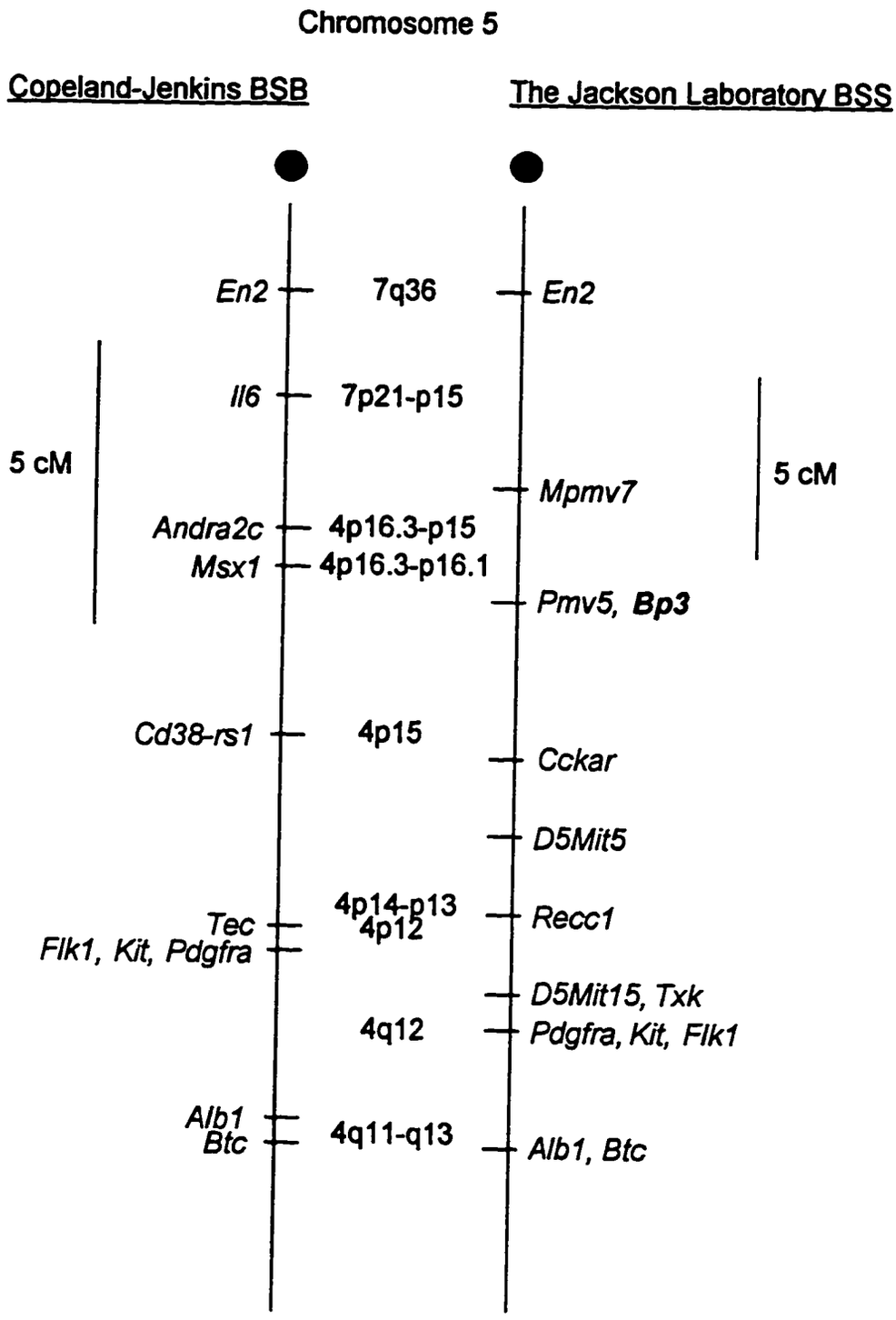


Figure 3. Location of the *Bp3* gene on mouse chromosome 5. (a) Haplotype figure from the Jackson BSS backcross showing a part of chromosome 5 with loci linked to *Bp3*. Loci are ordered with the ones most proximal to centromere at the top. The black boxes represent the C57BL/6JEi allele and the white boxes the SPRET/Ei allele. The number of animals with each haplotype is indicated below each column of boxes. The percent recombination (R) between adjacent loci is indicated on the right side of the figure together with the standard error (SE). Missing typings were inferred from surrounding data where assignment was unambiguous. Complete public data from this cross is available on the World Wide Web at address *http://www.jax.org/resources/documents/cmdata*. (b) The map resulting from the data from the Jackson Laboratory BSS cross in which *Bp3* was mapped is compared with the map derived from the Copeland-Jenkins BSB cross in which mouse CD38-encoding gene (*cd38-rs1* locus) was mapped. Raw data were obtained from the mouse Genome Database with the address *http://www.informatics.jax.org/crossdata.html*. Partial maps of the relevant region of chromosome 5 are depicted with the centromere toward the top. The two maps are aligned at the ends of the relevant region by adjusting the scales (5 cM scale bars are given separately for each cross). Known human homogies are listed between the maps aligned with the locus mapped in both species.

b.



reported as the *Cd38-rs1* locus, is located 28 cM from the centromere (Fig. 3b; Harada et al., 1993).

Mouse mutants that map near the *Cd38-rs1/Bp3* locus are *tlt* (tilted), *tht* (thick tail) and *lx* (luxate), respectively located 28, 26 and 22 cM from the centromere. The corresponding human locus would be 4p15-16, where human CD38 is located, although the human *BST-1* gene has been reported to map to 14q32.3 near the immunoglobulin heavy chain gene cluster (Kaisho et al., 1994).

Transcription start sites and the 5' flanking region of the Bp3 gene. The BP-3 cDNA that we isolated previously contained a very short 17-bp 5' UTR (Dong et al., 1994), and Itoh et al. employed a PCR-based 5' RACE technique to locate the 5' terminus of the mouse BST-1 mRNA 15 bp from the initiation codon ATG (Itoh et al., 1994). These independent analyses suggest that t ranscription of the murine *Bp3* gene is initiated very near the translation start site. To test this inference, we performed RNase protection assays. When an antisense RNA probe containing 97 bp coding and the 187 bp upstream region was incubated with RNA from a mouse pro-B cell line, 38B9, expressing BP-3 at high levels (Dong et al., 1994), and analyzed with single strand-specific RNases, a major fragment of approximately 114 nucleotides was protected. This fragment corresponded to the size estimated from our cDNA clones (Fig. 4). A minor band of 120 nucleotides was also seen (Fig. 4). The major start site contains a weak transcription initiator sequence, $CTA_{+1}GAGG$, that resembles the consensus sequence, $PyPyA_{+1}NT/APyPy$ (Ernst and Smale, 1995a). The initiator sequence is thought to be recognized by a TAF within the TFIID complex and to direct the formation of a preinitiation complex (Ernst and Smale, 1995a), but the function of this weak initiator sequence in regulating BP-3 expression remains to be resolved experimentally.

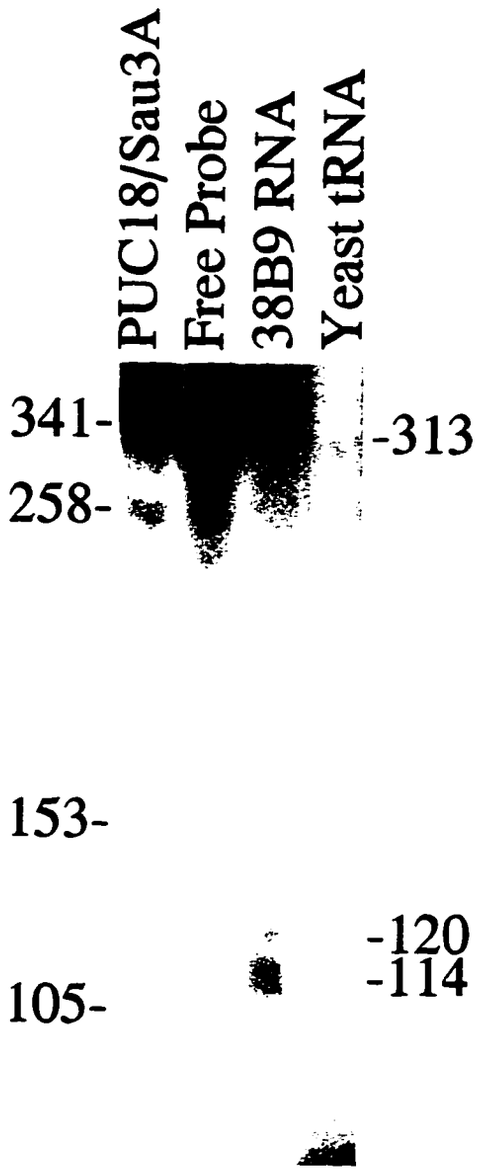


Figure 4. Mapping of transcription start sites of the *Bp3* gene by an RNase protection assay. A 313 ^{32}P-labeled nucleotide RNA probe was generated, incubated with 30 μg total RNA of 38B9 pro-B cells or yeast tRNA as a control. The RNA samples were then digested with RNases and analyzed on a 6% denaturing polyacrylamide gel. PUC18 plasmid *Sau 3A* fragments were end-labeled by T4 kinase and used as molecular weight markers. The nucleotide length is shown for each band.

Table 2. Consensus sequences for transcriptional factors in the 5' flanking region of the *Bp3* gene

Factors	Sequence	Position	Mismatch	Cell specificity/function	Reference
PU.1	AGAGGAACT	- 55	0	B and myeloid cells	34-43
Lyf-1	ACTGGGAGG	- 49	1	early lymphocytes	44-47
TCF-1	TACAAAG	- 607	0	TCR/CD3 gene expression and T cell development	51-55
	GACAAAG	- 570	1		
E2A	GCAGGTG	- 506	0	immunoglobulin gene expression and B cell development	48-49
NF-E1/YY1/delta	ACAATCTT	-1057	0	multiple genes including immunoglobulin	70-72
NF-IL6 (C/EBP)	TTTTGCAAT	-1664	0	response to IL6 and some other cytokines	62-64
	TGTCGTAAT	- 134	0		
H-APF-1/APRF	CTGGAAA	-1469	0	synergize with NF-IL6 in response to IL6	60-61, 64
	CTGGAAA	- 312	0		
AP-1	ATGAGTCAG	-1788	0	primary response gene	68
		-1231	0		
PEA3	AGGAAG	-1268	0	embryoinic, epithelial and fibroblastic cells and response to stimuli, like TPA, etc	69
	AGGAAG	- 466	0		
p53	AAAACAAGCC C	- 537	0	tumor suppressor gene	74
engrailed (En-1)	ACTATTAAA	-1615	0	regulate embryoinic and neural development	73
poly(dT-dG)	$(TG)_{13}$	- 240	0	may form Z-DNA and enhance transcription	52-54

A search of the 5' immediate flanking region failed to identify TATA- and CAAT-like promoter sequences (not shown). To identify elements potentially involved in regulating the gene expression, we examined the sequence of approximately 1.8 kb of the 5' flanking region. Recognition motifs for transcription factors were sought visually and by the use of the TFD SITES database of transcription factors (Faisst and Meyer, 1992; Ghosh, 1990). PU.1 and LyF-1 sites are located 37 and 30 bp upstream from the cDNA start site, and these overlap with each other (Table 2). PU.1, expressed by B and myeloid cells, has been shown to be essential for lymphopoiesis and myelopoiesis (Rosmarin et al., 1995; Schwarzenbach et al., 1995; Moreau-Gachelin, 1994; Voso et al., 1994; Scott et al., 1994; Eichbaum et al., 1994; Zhang et al., 1994; Hromas et al., 1993; Eisenbeis et al., 1993; Shin and Koshland, 1993) . The LyF-1/Ikaros site was identified first in the Tdt promoter, and the LyF-1 transcription factors are predominantly expressed in early lymphocytes (Hahm et al., 1994; Omori and Wall, 1993; Ernst et al., 1993; Lo et al., 1991). The PU.1 and LyF-1 transcription factors have been reported to activate transcription of genes lacking TATA promoters (Eichbaum et al., 1994; Lo et al., 1991).

The 5' upstream region also contains recognition sequences for E2A, which regulates immunoglobulin expression and is required for B cell development (Zhuang et al., 1994; Ernst and Smale, 1995b), and TCF-1, which regulates T cell receptor gene expression and is essential for T cell development (Verbeek et al., 1995; Carlsson et al., 1993; Leiden, 1993; Oosterwegel et al., 1991a; Oosterwegel et al., 1991b; van de Wetering et al., 1991). This region also contains a stretch of TG repeats, which tends to adopt a left-handed conformation (Z-DNA) and is reported to have enhancer-like activity (Rich et al., 1984; Hamada et al., 1984; Hamada et al., 1982). Other identified recognition sites (Table 2) include NF-E1/YY-1, PEA3, p53 and engrailed-1 (Xin et al., 1992; Becker et al., 1994;

Safrany and Perry, 1993; Park and Atchison, 1991; Wurst et al., 1994; Zambetti and Levine, 1993).

Interestingly, some of the consensus sequences observed in the upstream region of the *Bp3* gene implicate transcription factors that respond to extracellular stimuli (Table 2). The recognition sites for AP-1, a primary response factor, were identified as well as those for NF-IL6/C-EBP and H-APF-1/APRF, which respond synergistically to interleukin 6 (IL-6) (Hill and Treisman, 1995; Akira et al., 1994; Wegenka et al., 1993; Akira, 1992; Natsuka et al., 1992; Majello et al., 1990; Melamed et al., 1993). This observation suggested that expression of the *Bp3* gene could be regulated by cytokines, such as IL-6.

Interleukin 6 treatment upregulates BP-3 antigen expression in myeloblastoid cells. To test the possibility that BP-3 may be regulated by IL-6, we treated the M1 mouse myeloblastoid cell with IL-6. IL-6 has been shown to induce the differentiation of M1 cells by upregulating NF-IL6 activity (Akira, 1992). BP-3 antigen expression was significantly enhanced by the IL-6 treatment. The enhanced expression was observed by day 1 and reached maximal levels on day 3 (Fig. 5). This finding is concordant with the observation that BP-3 expression increases as a function of myeloid cell maturation (McNagny et al., 1988). We also found that PMA, PMSF, and LPS, inducing agents that do not drive M1 differentiation, had little or no effect on BP-3 expression (data not shown). The upregulation of BP-3 expression thus correlates with IL-6 induced progression of myeloid differentiation.

Discussion

The BP-3/BST-1 protein sequences share approximately 30% identity with CD38 and molluscan ADP-ribosyl cyclases, and they are functionally related molecules that probably are derived from a common ancestor. In support of this interpretation, we found that the genomic structures of the ADP-ribosyl cyclase gene in *Aplysia kurodai* and the *Bp3* gene are

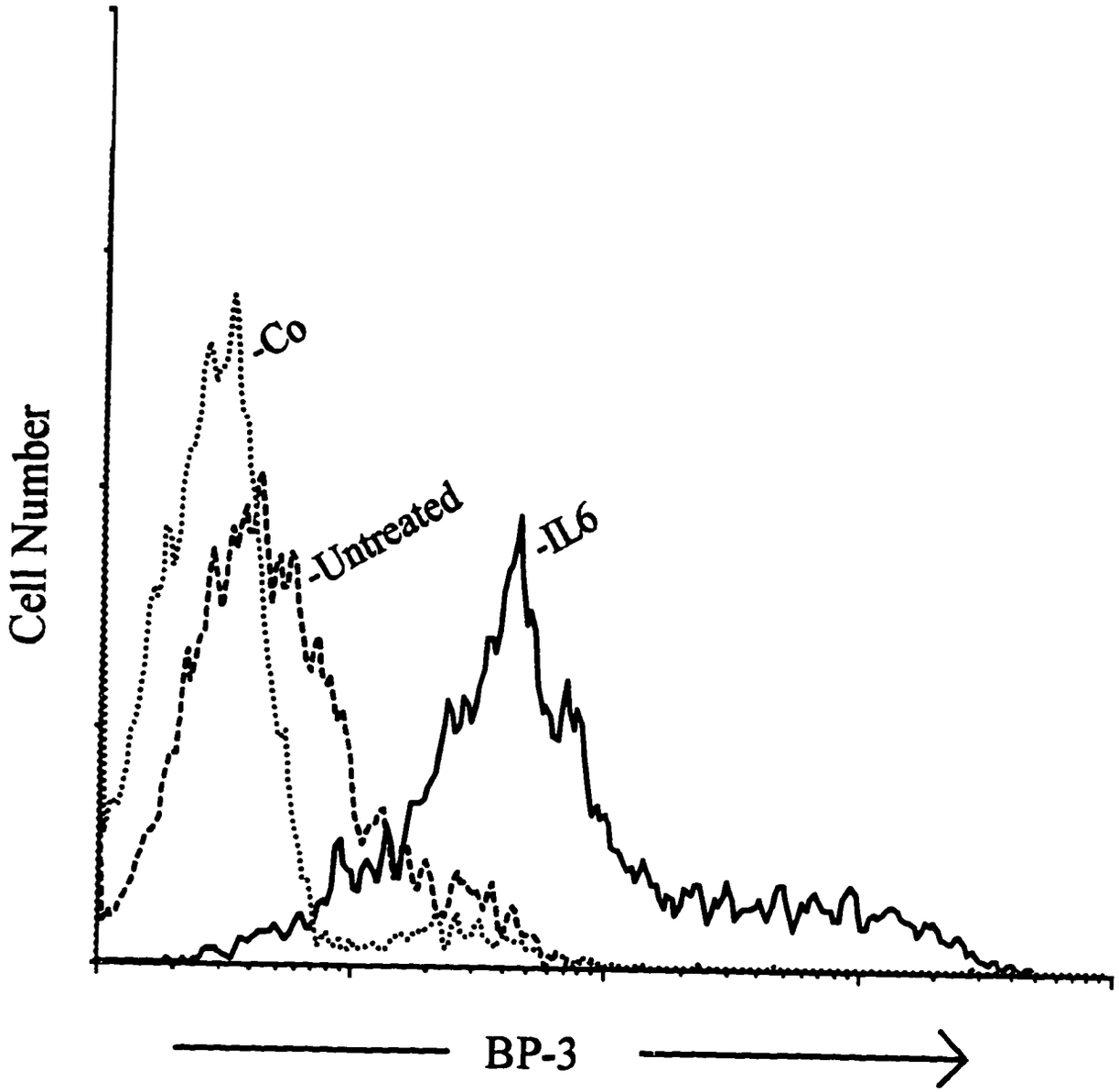


Figure 5. IL6 treatment enhances BP-3 antigen expression by M1 mouse myeloblastoid cells. M1 cells were cultured with or without IL6 (100 u/ml) for 3 days, stained with BP-3 or a control (Co.) antibody and analyzed by a flow cytometry.

very similar (Nata et al., 1995). Our analysis indicates that the exons 3 and 4 of the *Bp3* gene probably evolved by bifurcation of a primordial exon 4 as represented in *Aplysia kurodai*. The *CD38* gene may prove to have a similar conserved exon organization, although when the ancestoral ADP-ribosyl cyclase evolved and for what physiological role is presently unknown. The molluscan enzyme is a soluble factor produced in the ovotestis (Glick et al., 1991; Nata et al., 1995), while the BP-3/BST-1 and CD38 molecules are expressed on the surface of many cell types in rodents and humans (McNagny et al., 1988; McNagny et al., 1991; Dong et al., 1994; Kaisho et al., 1994; Itoh et al., 1994; Malavasi et al., 1994; Lund et al., 1995).

The cell surface topologies of the BP-3/BST-1 and CD38 glycoproteins differ in that BP-3/BST-1 is GPI-linked to the cell surface and CD38 is a type II transmembrane protein (McNagny et al., 1991; Kaisho et al., 1994; Malavasi et al., 1994; Lund et al., 1995;). Mapping of the *Bp3* gene near the CD38-encoding gene (*cd38-rs1 locus*) on mouse chromosome 5 reinforces the idea that these genes arose from a common ancestor through gene duplication. Because these two genes were mapped using different markers (Harada et al., 1993), their proximity has not been precisely defined. It is also possible that the *CD38* and *Bp3* genes are members of a larger gene family, and that the establishment of a physical map of the *Cd38-rs1/Bp3* locus would allow a search for other homologous genes in the region. The *CD38* and the *Bp3* genes are both expressed in lymphoid cells (McNagny et al., 1988; McNagny et al., 1991; Dong et al., 1994; Kaisho et al., 1994; Itoh et al., 1994; Malavasi et al., 1994; Lund et al., 1995), raising the possibility of common transcriptional factors that result in the expression of the two related genes with similar function by the same cell, such as the pre-B cell (McNagny et al., 1988; Lund et al., 1995). However, the recombinant BP-3/BST-1 proteins do not possess comparable ADP-ribosyl cylase activity comparable to

that of CD38 and are inactive at physiological pH (Dong et al., 1994; Hirata et al., 1994; C.D., H.C. Lee et al, unpublished). The BP-3/BST-1 molecule may thus have a very different function than the CD38 molecule.

The genes and genetic markers flanking the murine *CD38-rs1/Bp3* locus map to chromosome 4p15-16 in humans where the *CD38* gene is also located (Nakagawara et al., 1995). However, the human *BST-1* gene has been reported to be located at 14q32.3 near the immunoglobulin heavy chain gene cluster (Kaisho et al., 1994). The basis for this discordance in mouse and human chromosomal locations is unknown, and further study is needed to resolve this issue.

The expression of the *Bp3* gene is highly regulated as a function of lymphoid and myeloid cell differentiation (McNagny et al., 1988; Dong et al., 1994), and our sequence analysis of the 5' upstream region of the gene provides clues to the regulatory mechanisms involved. Like some other genes transcribed in early lymphocytes (Ernst and Smale, 1995a), the *Bp3* gene has no TATA or CAAT box, and the major transcriptional start site has a weak initiator consensus sequence. Overlapping PU.1 and LyF-1 sites were identified within 40 bp of the transcription start site. PU.1 has been reported to activate Fcγ receptor 1b gene transcription in myeloid cells, and LyF-1 can activate Tdt transcription in T and B cell progenitors (Eichbaum et al., 1994; Lo et al., 1991). Both the *FcγR1b* and *Tdt* genes lack a TATA box, and it has been suggested that PU.1 and LyF-1 may serve to recruit the basic transcription machinery (Ernst and Smale, 1995a; Eichbaum et al., 1994). The two factors could jointly contribute to *Bp3* gene expression in lymphoid and myeloid cell lineages. E2A and TCF-1, transcriptional factors that can regulate immunoglobulin or T-cell receptor gene transcription, may also influence BP-3 expression in a lineage specific fashion (Table 2; Zhuang et al., 1994; Ernst and Smale, 1995b; Verbeek et al., 1995; Carlsson et al., 1993;

Leiden, 1993; Oosterwegel et al., 1991a; Oosterwegel et al., 1991b; van de Wetering et al., 1991; Hagman and Grosschedl, 1994), but few clues exist to suggest how transcription might be regulated in other cell types. The human BST-1 cDNA isolated from bone marrow stromal cells has a longer 5' untranslated region than the mouse BP-3 cDNA (Dong et al., 1994; Kaisho et al., 1994), so it is possible that these genes may utilize a different promoter for expression in different cell types.

The presence of recognition motifs for NF-IL6/C-EBP, H-APF-1/APRF and AP-1 in the 5' flanking region of the *Bp3* gene suggests the possibility of cytokine mediated gene regulation. Since NF-IL6/C-EBP has been shown to synergize with H-APF-1/ARPF in response to IL-6 (Akira, 1992; Majello et al., 1990), we treated the M1 mouse myeloblastoid cell line with IL-6 and found that BP-3 antigen expression was enhanced significantly. IL-6 has also been shown to induce the differentiation of M1 cells, and this effect may correlate with NF-IL6 and AP-1 activation (Natsuka et al., 1992; Melamed et al., 1993). In this regard, BP-3 expression increases as a function of myeloid cell differentiation (McNagny et al., 1988). Agents that do not induce M1 differentiation, such as PMSF, PMA and LPS, failed to enhance BP-3 expression, further supporting the linkage between induction of myeloid differentiation and BP-3 upregulation. Alternatively, the upregulation of the BP-3 antigen could be an indirect effect of the IL-6 treatment that accompanies myeloid differentiation.

In conclusion, analysis of the genomic structure of the murine *Bp3* gene indicates an exon organization similar to that of the ADP-ribosyl cyclase in *Aplysia Kurodai*. Located near the *CD38*-encoding gene on mouse chromosome 5, *Bp3* gene is structurally related to the members of the CD38/ADP-ribosyl cyclase family and may have evolved from a common ancestor.

Acknowledgments

We thank Ms. Lucy Rowe and Mary Barter at the Jackson Laboratory for their help in backcross panel mapping, Dr. Etty Benveniste and her colleagues for their help in Rnase protection assay, Ms. Lucy Rowe and Dr. Peter Burrows at the University of Alabama at Birmingham for critical reading of the manuscript, and Ms. Ann Brookshire for secretarial assistance. This work was supported in part by a USPHS grant AI39816. Dr. Max D. Cooper and Dr. Frederick W. Alt are Howard Hughes Medical Institute Investigators.

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RECOMBINANT BP-3 PROTEIN POSSESSES RELATIVELY LOW ADP-RIBOSYL CYCLASE ACTIVITY AT pH 4

CHEN DONG AND MAX D. COOPER

Department of Microbiology, Medicine and Pediatrics, University of Alabama at Birmingham, and the Howard Hughes Medical institute, Birmingham, AL 35294

Abstract

The murine BP-3 molecule is a new member of the CD38/ADP-ribosyl cyclase family. We have examined whether the BP-3 molecule has ADP-ribosyl cyclase activity by use of recombinant BP-3 protein produced by the COS cells transfected with a BP-3 expression vector. The recombinant BP-3 molecule displays a low ADP-ribosyl cyclase activity only at pH 4. This assay suggests a distinct function for the BP-3 molecule.

The predicted amino acid sequence of the BP-3 cDNA shares significant homology with CD38 and ADP-ribosyl cyclase in *Aplysia californica*, enzymes that hydrolyze NAD and synthesize cyclic ADP-ribose (cADPR), a novel calcium mobilizing agent (1). Our previous experiment failed to detect any NAD glycohydrolase activity of recombinant BP-3 protein (1). Independently, Kaisho et al. have cloned *BST-1* gene, the human homologue of BP-3, from bone marrow stromal cells derived from rheumatoid arthritis patients (2). The recombinant BST-1 molecule was shown to have low ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activity only at acid pH, which could be enhanced by Zn^{2+} and Mn^{2+} (2).

In this study, we address whether the recombinant BP-3 protein has ADP-ribosyl cyclase activity under the similarly low pH conditions. A BP-3 expression vector was transfected into COS-7 cells as previously described (1). Forty-eight hours after transfection, the COS cells were harvested and washed three times with PBS containing no calcium, after which the cell pellets were quickly frozen and stored at -70°C. An aliquot of transfected cells was analyzed by immunofluorescence to confirm the BP-3 expression. To measure the enzyme activity, the cell pellets were reconstituted with 25 μl of pH 4 buffer and subjected to 4 freeze/thaw cycles. The reactions were started at 37°C with the addition

of NAD at 1 mM final concentration. About once per hour up to 5 h, aliquots of 2 μl were added to 200 μl sea urchin egg homogenate, and cADPR production was measured by a calcium release assay.

A representative result is shown in Fig. 1. The BP-3 transfected COS cells constantly displayed several-fold increases of cADPR production over the control transfectants. Such an enhancement of cADPR production was not seen at neutral pH or by an less sensitive assay using HPLC (data not shown).

We conclude that like its human homologue, BP-3 protein has a relatively low ADP-ribosyl cyclase activity, measurable only at pH 4. This finding suggests that the BP-3 molecule may have a distinct function from other members of the CD38/ADP-ribosyl cyclase family.

Acknowledgments

We appreciate the contribution of Dr Hon Cheung Lee and Mr Richard Graff at the University of Minnesota in this part of work. This work has been supported in part by NIH grants AI 30879 and CA 13148. M. D. C. is an investigator of the Howard Hughes Medical Institute.

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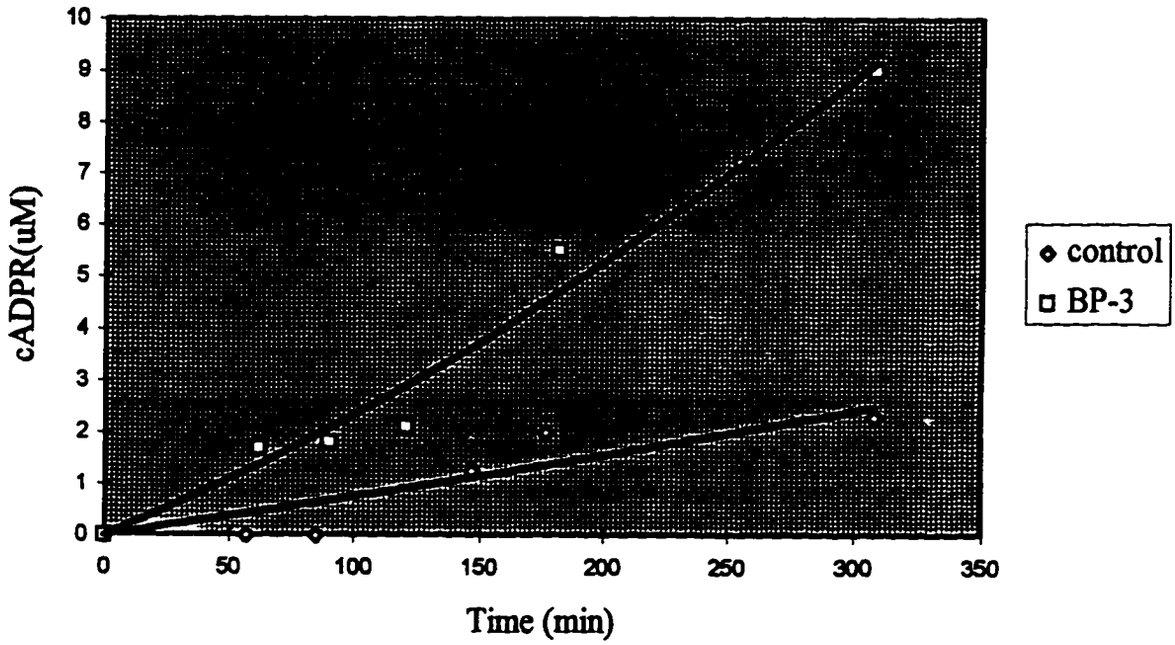

Fig. 1. Recombinant BP-3 protein displays low ADP-ribosyl cyclase activity at pH 4. The COS cells transfected with or without a BP-3 expression construct were incubated with 1 mM NAD under pH 4 at 37°C. Every hour, 2 μl of the reaction was added to 200 μl sea urchin egg homogenate and calcium release was measured. The amount of cADPR production was calculated on the basis of the calcium release.

GENERATION OF A *BP3* GENE DEFICIENT MOUSE MODEL

CHEN DONG AND MAX D. COOPER

Department of Microbiology, Medicine and Pediatrics, University of Alabama at Birmingham, and the Howard Hughes Medical institute, Birmingham, AL 35294

Abstract

In order to examine the function role of the BP-3 molecule in early development of the immune system, we have constructed a vector for Bp3 gene targeting in mouse embryonic stem (ES) cells. The ES cells were screened by Southern blot analysis, and those carrying a knockout allele were injected into blastocytes to generate Bp3 gene deficient mice. We have obtained three mice heterozygous for the Bp3 gene deficiency. They are being bred to create the homozygous Bp3 gene knockout mice, which will provide as a valuable tool for the study of the BP-3 molecule.

The BP-3 molecule is a novel member of the CD38/ADP-ribosyl cyclase family that is expressed by early B and lymphocytes and myeloid cells (1-4). To further study its physiological roles in the immune system development, we constructed a Bp3 knockout vector and used it for gene targeting in embryonic stem (ES) cells to create a Bp3 gene deficient mouse model.

The Bp3 knockout vector is shown in Fig. 1. A 5-kb genomic region of the Bp3 gene containing 1-kb 5' flanking region, exon 1, 2 and part of exon 3, with the first 44% of the coding sequence, was replaced by neomycin-resistant gene. The 5' and 3' arm homologous sequences are 1.6 and 8.6 kb respectively. The vector was linearized with *Not*I enzyme and transfected into D3 and J1 ES cell lines by electroporation. The transfected ES cells were selected by neomycin-containing G418 medium. Cells surviving the selection were subcloned, and more than 200 D3 and more than 100 J1 transfectants were obtained.

DNA from these ES clones was extracted, digested with *Xba*I, blotted onto Nytran Plus membranes and hybridized with a 300-bp genomic fragment upstream of the 5'

homologous region of the construct. Hybridization of this probe should give a 9-kb band from the germline allele and a 21-kb fragment after homologous recombination, because of an *XbaI* site deletion. A representative blot is shown in Fig. 2. Several clones have a knockout allele, as well as one in germline configuration. To confirm these results, the blots were further hybridized with a neo probe and a cDNA probe consisting of the deleted sequence. The neo probe hybridized with a band the same size as the knockout allele, while the cDNA probe reacted only with the wild type allele. This analysis demonstrated that we achieved gene targeting in ES cells, which disrupted the BP-3 gene on one allele.

Several clones of ES cells were then implanted into C57BL/6 blastocytes to create chimeric animals. Three male chimeras were mated with C57BL/6 female mice for germline transmission. We have obtained 7 mice with the ES-cell derived allele, judging from their coat color. The tail DNA from these mice was extracted and subject to a PCR analysis, which amplifies a >700-bp fragment from the knockout allele and 500 bp from the germline. Three out of seven mice carry the Bp3 gene knockout allele. These heterozygous mice are currently being bred to generate mice with a homozygous mutation.

Acknowledgments

We acknowledge the contribution from Drs Dennis Willerford and Fred Alt at Howard Hughes Medical Institute, Children's Hospital, Boston, in this work. This project has been supported in part by NIH grants AI 30879 and CA 13148. M. D. C. is an investigator of the Howard Hughes Medical Institute.

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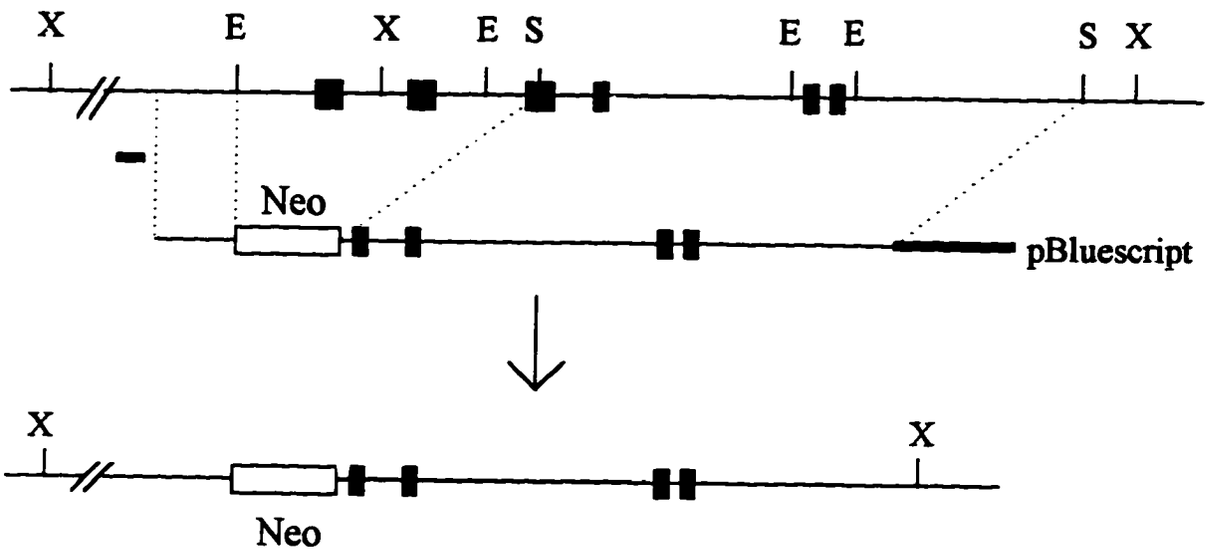

Fig. 1. Bp3 gene targeting vector for homologous recombination in ES cells. A 5-kb genomic fragment containing the first two and part of the third exon (exons shown as boxes) was replaced by neomycin-resistant gene (neo). Recognition sites for restriction enzyme *Xba*I, *Eco*RI and *Sal*I were shown as X, E and S respectively. The 5' genomic probe used for a southern blot analysis is shown as a small bar.

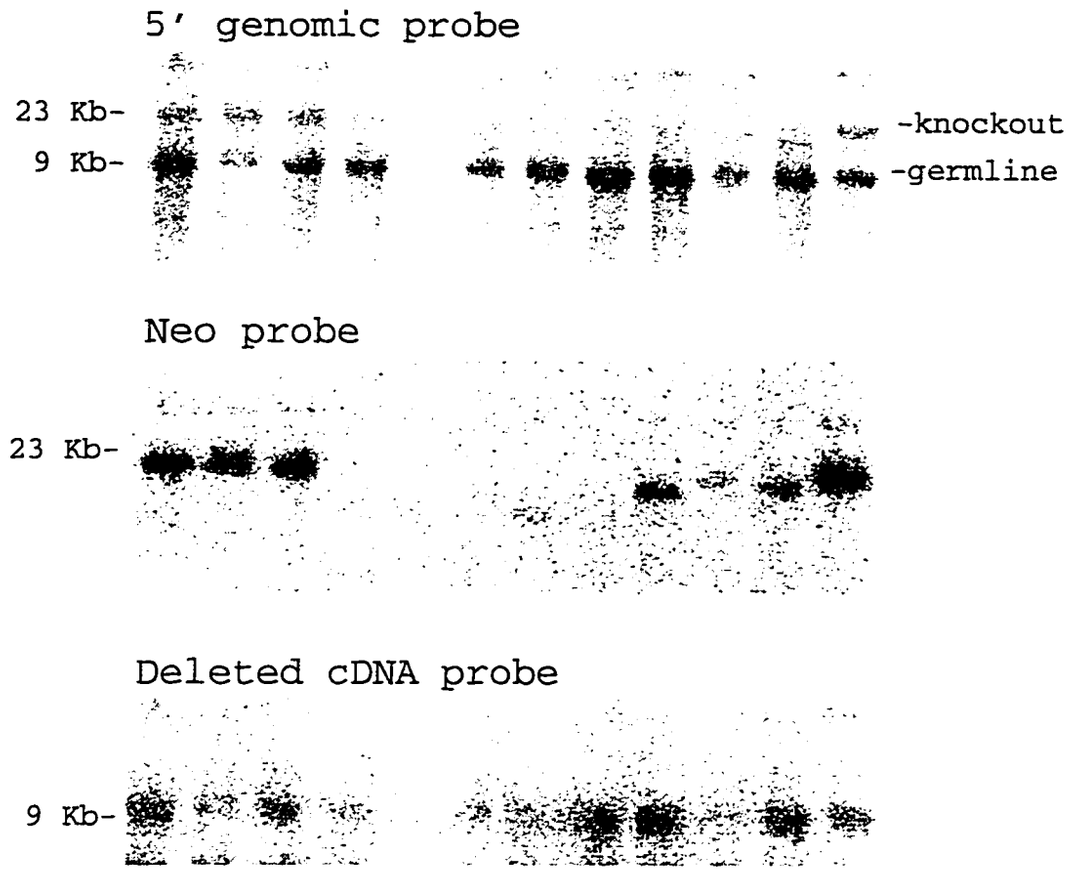

Fig. 2. Southern blot analysis of ES cells transfected with the Bp3 gene targeting construct. Genomic DNA from ES cells surviving neomycin selection was digested with *Xba*I and blotted. The blot was first hybridized with a 5' genomic probe shown in Fig.1, which was further confirmed by reprobing with a neomycin-resistant gene probe and a cDNA probe consisting of the deleted sequence.

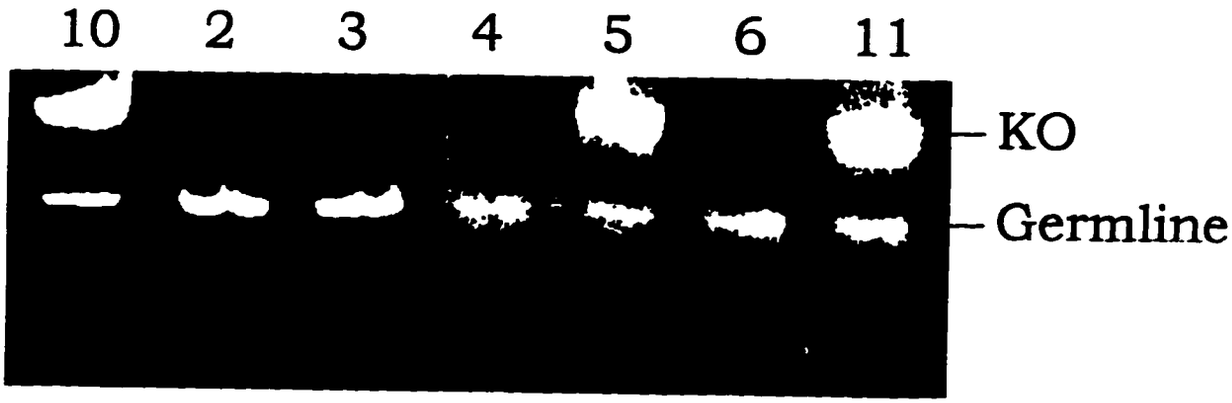

Fig. 3. PCR analysis of the tail DNA from mice with germline transmission. Chimeric mice were bred with C57BL/6 mice and tail DNA from the littmates with coat color of 129 strain was extracted and subject to PCR analysis. Three primers were used in a single reaction, which amplifies a fragment of 500 bp from germline allele and a >700 from knockout allele. Mice #10, 5 and 11 were identified to be heterozygous for the knockout allele.

GENERAL DISCUSSION

As a first step to determine the physiological role of BP-3 in the development of immune system, the BP-3 cDNA was isolated, sequenced and expressed. The predicted BP-3 amino acid sequence shares homology with CD38 and molluscan ADP-ribosyl cyclase, and the recombinant BP-3 had minimal ADP-ribosyl cyclase activity at pH 4. The *Bp3* gene had similar exon organization with *ADP-ribosyl cyclase* gene in *Aplysia kurodai*, and is located on mouse chromosome 5 very near the CD38-encoding gene, supporting the notion that BP-3 is a novel member of the CD38/ADP-ribosyl cyclase gene family. I have determined the transcriptional start sites of the *Bp3* gene in a pro-B cell and their upstream sequence, and identified recognition motifs for transcription factors known to regulate gene expression in lymphoid and myeloid lineages. A *Bp3* gene deficient mouse model is being created in order to study the physiological role of the BP-3 molecule in the immune system development.

Monoclonal antibodies have been successful in identifying novel cell surface molecules mediating lymphocyte development and function. Notably, the protocol to generate BP alloantibodies, immunizing outbred *M. spretus* mice with a pre-B cell line derived from the BALB/c strain, enriches for antibodies against ectoenzymes. So far, all characterized BP antigens are ectoenzymes: BP-1 is glutamyl aminopeptidase (Wu et al. 1991), BP-3 is ADP-ribosyl cyclase (Dong et al. 1994a), BP-4 is alkaline phosphates, and BP-6 is CD26/dipeptidyl peptidase IV (M. D. Cooper et al. unpublished). The basis for this phenomenon is not clear. One clue may be that the genes encoding BP-1, BP-3 and BP-

6/CD26 are relatively large with a lot of intron sequence and may have a long evolutionary history (Wang et al. 1996; Dong et al. 1996a; Bernard et al. 1994; Abbott et al. 1994). Since the separation of the inbred laboratory and outbred *M. spretus* strains, a large gene is likely to have more mutations, and the *Bp3* gene was found to be very polymorphoic between C57BL/6 and *M. spretus* mice (data not shown). Gene duplication during evolution generates enzymes related to these molecules, which may provide backup and allow for the mutations and a greater allotypic difference. Whatever the reason, this approach could be useful for the study of ectoenzymes.

Structural analysis of the *Bp3* gene revealed that it is a novel member of the CD38/ADP-ribosyl cyclase family. The predicted amino acid sequence of the BP-3 cDNA shares approximately 30% identity and 50% similarity with CD38 and ADP-ribosyl cyclase, and 10 cysteine residues in the BP-3 protein are conserved among them, suggesting a structural similarity (Dong et al. 1994a). The exon organization of the *Bp3* gene is very similar with that reported for the ADP-ribosyl cyclase in *Aplysia kurodai*, revealing that the genes of this family are conserved during evolution (Dong et al. 1996a). Furthermore, the *Bp3* gene is located very close to the *CD38* gene, suggesting that these two genes are derived by a gene duplication (Dong et al. 1996a). Since there are presently only three members, it is still impossible to construct an evolutionary tree of this gene family. These molecules could share a common origin in evolution, but when and for what physiological role this ancestral enzyme came to exist is not clear. A clue would be gained to probe genomic DNA from a variety of species with BP-3 or CD38 cDNA probes to search for homologous sequences. Since the *Bp3* and *CD38* genes are located 27-28cM from the centromere on mouse chromosome 5, an exploration in this chromosome region may

identify other genes encoding for related enzymes. The homology of primary sequence among these proteins can provide the basis for future mutagenisis studies to address the structure and function relationship. Resolution of crystal structure of a member should benefit the study of these enzymes and reveal the structural and functional differences of these molecules.

Despite its homology with CD38 and molluscan ADP-ribosyl cyclase, the BP-3 molecule appears not to have a comparable enzymatic activity: The recombinant protein displayed a minimal activity only at pH 4. It is possible that the BP-3 protein requires a cofactor or membrane microenvironment for its enzymatic activity, but this seems unlikely since a mouse myeloid cell line expressing BP-3 but not CD38 displayed similar enzymatic features as the COS cells transfected with a BP-3 expression construct (data not shown). Second, the BP-3 molecule may exert its enzymatic activity only in certain cellular structures with low pH, such as endosomes. Future experiments will address whether the BP-3 molecule can be endocytosed into the endosomal compartment. The third possibility is that, unlike CD38, which is constitutively active, BP-3 requires an activation signal for its enzymatic function. A fourth hypothesis is that BP-3 has a different substrate or different function. To support this, several amino acid residues, which are conserved between the CD38 and ADP-ribosyl cyclase molecules, are not conserved in the BP-3/BST-1 molecules (not shown), which could provide a basis for the functional differences. Coexistence of the CD38 and BP-3 molecules and sometimes even on the same type of cells, such as pre-B cells, may seem rather redundant if they have the same function and regulation. In addition to ADP-ribosyl cyclase and cyclic ADP-ribose hydrolase activities, CD38 was reported to have a ADP-ribose transferase activity (Grimaldi et al. 1995). Considering that a glutamic

acid residue essential for transferring ADP-ribose by CD38 is missing in BP-3, BP-3 may not have such activity. CD38 could also generate nicotinic acid adenine dinucleotide phosphate (NAADP), a novel calcium-mobilizing agent which induces calcium release from an IP_3- and cADPR-insensitive store, from NADP (Aarhus et al. 1995). Generation of NAADP by CD38 requires a low pH (Aarhus et al. 1995). An experiment is being undertaken to find out if BP-3 has a similar activity and under what condition. Further biochemical analysis is surely needed to elucidate the BP-3 enzymatic activities.

It is not very clear either what role BP-3 plays in the development of the immune system. BST-1, the human homologue of BP-3, was isolated from bone marrow stromal cells derived from rheumatoid arthritis patients (Kaisho et al. 1994). Overexpression of this molecule on these bone marrow stromal cells or by a fibroblast line facilitates the growth of an IL7-dependent mouse pre-B cell line (Kaisho et al. 1994). This pre-B cell line was found to lack BP-3 expression (Itoh et al. 1994). Thus, existence of the BP-3 molecule in this culture system seems to enhance the cell growth. However, addition of a soluble form of the recombinant BP-3 protein to this pre-B cell did not enhance the growth (data not shown). My colleagues have discovered that antibody cross-linking of the BP-3 molecule enhanced pre-B cell growth in short-term and long-term cultures (J. Wang and M. D. Cooper, unpublished). The growth enhancement by the BP-3 antibody requires the presence of IL-7 (data not shown). Interestingly, Vacari et al. reported that ligation of the BP-3 molecule by a different agonistic antibody synergized with the CD3 cross-linking in promoting pre-T cell growth in vitro and could also accelerate pre-T cell differentiation in a fetal organ culture system (Vicari, 1996). Thus, BP-3 emerges as a possible costimulatory

growth factor for early B and T lineage cells. To rule out the possibility that these results are artifacts of the antibody cross-linking, a ligand for BP-3 should be identified.

It is intriguing how BP-3, a GPI-anchored protein, could transduce an intracellular signal after antibody cross-linking. A number of GPI-linked glycoproteins have been shown to have such a capacity and they may associate with certain transmembrane proteins or a cell membrane microenvironment, caveolae (Lisanti et al. 1995; Anderson 1993a; Anderson, 1993b). Caveolae are invagination of the membrane enriched for GPI-linked glycoproteins which also contain *src* family kinases (Lisanti et al. 1995; Anderson 1993a; Anderson 1993b). The glycoproteins in caveolae are often not soluble in Triton detergent. BP-3 was found previously in the insoluble fraction of Triton X-114 extracts, raising a possibility that it is localized in the caveolae (McNagny et al. 1988). Further studies are needed to confirm this hypothesis and to identify the protein(s) associated with the BP-3 molecule and may mediate its signal transduction. The downstream signal transduction pathway after the BP-3 ligation is not understood either. We and others failed to detect a calcium influx following BP-3 antibody ligation (C. Dong and M. D. Cooper, unpublished; Vicari et al. 1996). Whether or not this ligation induces other intracellular changes, such as protein phosphorylation/dephosphorylation and phosphoinositol turnover, will be tested in the near future.

The BP-3 function will be better elucidated with the *Bp3* gene deficient mouse model available very soon. The homozygous Bp3 knockout mice will be analyzed for their early lymphoid and myeloid lineage cell development. The cells in the bone marrow and thymus of these mice will be phenotypically analyzed using flow cytometry and tested for their response to various cytokines that influence early lymphocyte and myeloid cells. The

lymphocyte homing in the peripheral lymphoid organs of the BP-3 knocout mice will be analyzed by immunohistological studies. The function of immune cells will be tested in immunization assays. This analysis should provide a clue whether or not the BP-3 molecule plays an essential role in the immune system development and function.

To understand how the *Bp3* gene is regulated in the development of immune system, we determined its transcription start sites and upstream sequence. Like some other genes expressed by early lymphocytes, the *Bp3* gene lacks a TATA box, and the major transcription start site resembles an initiator sequence (Dong et al. 1996a). The 5' flanking region of the *Bp3* gene contains putative binding sites for PU.1, Ikaros/lyf-1, E2A and TCF-1, transcriptional factors known to regulate gene expression in lymphoid and myeloid cells (Dong et al. 1996a). Gene targeting studies of these factors have demonstrated their essential role in the early lymphopoiesis and myelopoiesis (Scott et al. 1994; Zhuang et al. 1994; Verbeek et al. 1995; Georgopoulos et al. 1994). However, a functional study is needed to establish whether they play a role in regulating the *Bp3* gene expression in different lineage cells. The genomic fragment containing the transcription start sites and the recognition sites for the transcription factors mentioned above will be subcloned into a luciferase reporter vector. This construct will be transfected into the mouse cell lines representing different hematopoietic lineages and distinct developmental stages of the B and T lymphocytes, and the promoter/enhancer activities will be assayed. Further mutation of the putative transcription factor binding sites will provide evidences on the role of these molecules in the Bp3 gene regulation. Identification of AP-1, NF-IL6 and H-APF-1 binding sites in the upstream region of the Bp3 gene leads to a hypothesis that the *Bp3* gene expression could be regulated by certain cytokines, such as IL-6. IL-6 was previously found

to induce differentiation of M1 myeloblastoid cells into monocyte/macrophage lineage, which could be blocked by overexpression of c-myc and c-myb genes, which are downregulated during M1 differentiation (Hoffman et al. 1996). IL-6 was found, in the present studies, to enhance BP-3 expression by M1 cells (Dong et al. 1996a). Agents that did not induce M1 differentiation had little effect on BP-3 expression (data not shown). The correlation between M1 differentiation and BP-3 upregulation fits with previous findings that BP-3 expression increases as a function of myeloid cell differentiation. However, BP-3 upregulation may just be an indirect effect of IL-6-driven M1 differentiation. Future studies will address if the BP-3 upregulation occurs at the transcription level. Functional dissection of the promoter/enhancer region of the *Bp3* gene will provide direct evidence if IL-6 upregulation of BP-3 expression is directed by IL-6 responsive transcriptional factors.

In this study, as a first step to understand the functional role of the BP-3 differentiation antigen in the development of the immune system, I have studied the structure of the *Bp3* gene and demonstrated that it represents a novel member of the CD38/ADP-ribosyl cyclase gene family. I have undertaken preliminary characterization of its enzymatic activity and obtained clues on the regulatory mechanism of the Bp3 gene transcription. Further studies on the BP-3 deficient mice that being developed should provide more information on the physiological roles of the molecule. Overall, the work presented here lays the ground work toward understanding the structure, function and regulation of the BP-3 molecule.

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GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Chen Dong

Major Subject Microbiology

Title of Dissertation BP-3, A Novel Member Of The CD38/ADP-Ribosyl Cyclase Family: Its Function And Regulation In Immune System Development

Dissertation Committee:

Dr. Max Cooper , **Chairman**

Dr. Peter Burrows

Dr. John Volanakis

Dr. Etty Benveniste

Dr. Eric Hunter

Director of Graduate Program

Dean, UAB Graduate School

Date 9/11/96

PS-1428