The Identification of a Nonclassical Cadherin Expressed during B Cell Development and Its Interaction with Surrogate Light Chain*

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A 130-kDa glycoprotein (p130) has been found to be associated with surrogate light chain on pro- and pre-B I cells. Using peptide sequences obtained from purified p130 we have cloned its gene. The gene encodes a typical cadherin type 1 membrane protein with six extracellular cadherin domains (one pseudo domain) but lacking the catenin-binding site in its cytoplasmic part. Even without this catenin-binding site, p130 mediates Ca2+-dependent homotypic adhesion of cells. The interaction of p130 with surrogate light chain is confirmed by cotransfection and co-immunoprecipitation experiments. The expression of p130 is biphasic during the B cell development. Reverse transcriptase-polymerase chain reaction and flow cytometric analyses revealed that it is expressed on B220+c-Kit+ pro-B and pre-B-I cells as well as on B220+c-Kit+IgM+ immature and mature B cells but not on B220+c-Kit+ pre-B-II cells. It is also expressed in fetal liver, at low levels in myeloid cells, and strongly in intestinal epithelial cells. In the spleen, p130-expressing cells are mainly localized in the marginal zone. We call this B lineage-, intestine-, liver- and leukocyte-expressed gene BILL-cadherin. The possible functions of BILL-cadherin in B cell development are discussed.

In adult mice, antibody-producing B cells develop from hematopoietic stem cells in bone marrow. The early development of B cell progenitors, the timing and the order of the Ig gene rearrangements, and the subsequent positive and negative selection of the newly emerging B cells are regulated by a programmed pattern of gene expression and by ordered interactions of different stages of B lineage cells with the bone marrow microenvironment (see reviews in Refs. 1–5).

Several key molecules play crucial roles in this cellular development. The development of early precursor B cells (pro/pre-B-I) in bone marrow requires attachment to stromal cells, which involves interactions with Flk-2 and c-Kit on B lineage precursors and their corresponding ligands on stroma cells (6). Interaction of progenitor B cells with the stromal cells induces the production of IL-7 by stromal cells (7). The IL-7 acts via IL-7 receptor on pro- and pre-B cells to allow their survival and proliferation, in concert with other interactions between the two lineage of cells (8–10). In the adhesive pro/pre-B cell-stromal cell interactions, CD44 (Pgp-1), a receptor for hyaluronate, and the integrin VLA-4 (CD49d/CD29) are involved (11). In the bone marrow, B cell precursors migrate from the peripheral region near the surrounding bone to the central sinusoids as they mature and differentiate (4, 12). The chemokine stroma-cell-derived factor-1 might play a role in this migration (13). During this movement, differentiating B cells are thought to interact with various types of stromal cells. However, most of the molecular mechanisms responsible for these vectorial and sequential interactions remain to be elucidated.

Other migrations occur during the generation of the peripheral B cell compartments. Immature B cells exit the bone marrow and enter the spleen through the central artery, the arteriole, and finally the marginal sinuses to become localized in the marginal zone and follicular areas (14–16). Again, almost nothing is known about the genes and the molecules that control these cellular migrations and selections.

A 130-kDa glycoprotein, p130, has been found associated with surrogate light chain (SL chain) on pro-B and pre-B-I cells, which either have all Ig gene loci in germline configuration or are DμH rearranged and hence do not yet express μ heavy chains. The SL chain is composed of the Vpre-B1 and λ5 proteins. Together with μ heavy chains, it can form pre-B cell receptors that become expressed in and on large VμH-DμH rearranged pre-B cells (17). Pre-B cell receptors signal clonal expansion of these so-called large pre-B-II cells and are thought to be also involved in allelic exclusion of the H chain locus (9, 17–20).

In pro-B, pre-B-I, and large pre-B-II cells, the majority of SL chain molecules are localized in the cytoplasm. However, small amounts are also found on the surface (21, 22). Because pro-B and pre-B-I cells do not yet express μ heavy chains, other molecules might be associated with SL chain in these early B lineage cells to allow surface deposition (22). A complex of glycoproteins (of 200, 130, and 105 kDa and several proteins between 35 and 65 kDa) have been found associated with SL chain on the surface of mouse pro-B and pre-B-I cell lines (23, 24). A similar complex is also found on human pro-B cells in which the major component is a 125-kDa protein (25).

Received for publication, July 5, 2000

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Published, JBC Papers in Press, July 20, 2000, DOI 10.1074/jbc.M005901200

1 The abbreviations used are: SL chain, surrogate light chain; RT, reverse transcription; PCR, polymerase chain reaction; NEPHGE, nonequilibrium isoelectric focusing; PAGE, polyacrylamide gel electrophoresis; MALD-MS, matrix-assisted laser desorption mass spectrometry; BILL-cadherin, B cell, intestine, liver, and leukocyte cadherin; mAb, monoclonal antibody; RACE, rapid amplification of cDNA ends; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; HPLC, high pressure liquid chromatography; HPRT, hypoxanthine phosphoribosyltransferase; HPT-1, human intestinal peptide transporter 1; CNR, cadherin-related neuronal receptor; FITC, fluorescein isothiocyanate; PE, phycoerythrin; IL, interleukin.
Here we report the purification and partial amino acid sequence determination of the p130 molecule associated with SL chain. This, in turn, then allows the cloning of the gene encoding p130, which is found to be a new member of the cadherin family. We show that the expression of this nonclassic cadherin molecule is biphasic during the B cell ontogeny and that the expression in SL chain-producing cells allows the surface disposition of SL chain-p130 complexes.

EXPERIMENTAL PROCEDURES

Animals and Cell Lines—Female BALB/c and C57BL/6 mice (3–5-weeks old) were purchased from Biological Research Laboratories, Ltd. (Fuellinsdorf, Switzerland). 38B9 mouse pro-B cell line and most of the cell lines used here were described previously (23). In addition, murine leukemia virus transformed μ− cell lines from BALB/c mice IgG3 (29), the immature T cell lines SCFET27F, and B6.2.16.BU (27) were used. All cell lines were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal calf serum, 10 units/ml of penicillin-streptomycin, 2 mM l-glutamine, and 5 × 10−5 M 2-mercaptoethanol.

Antibodies—Rat monoclonal antibodies were used LM34 and LM33 specific for mouse λ5 (23). MaI specific for murine μ heavy chain (28), 9EG7 (anti-β1-integrin, kindly provided by Dr. Beat Imhof), and M1/9.3.4 (anti-B220 (29)). All other monoclonal antibodies were purchased from Pharmigen. The polyclonal anti-β1-integrin was kindly provided by B. Imhof.

Immunoprecipitation Analysis—Isotope labeling of the cells, lysis, and subsequent immunoprecipitations were as described previously (30). Briefly, 12C1-labeled cells (5 × 109) were lysed with 1 ml of ice-cold lysis buffer containing 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 10 mM iodoacetamide, and protease inhibitors. After a centrifugation, the supernatant was preclarified with rabbit normal Ig-Sepharose conjugate and then immunoprecipitated with 20 μg of each antibody immobilized to Sepharose 4B. Samples were then washed three times and analyzed on the one-dimensional and/or two-dimensional electrophoresis as described previously.

Purification of p130—About 100 pmol (10 μg) of p130 was purified from 5 × 1011 of 38B9 (pro-B) cells by several batches of the purification steps as described below. 38B9 (2.5 × 1010) cells were lysed with 250 ml of Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide, 20 mM e-aminocaproic acid, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 50 μg/ml aprotinin, and 1 μg/ml pepstatin) on ice for 10 min. As the tracer for λ5-associated p130, anti-λ5 immunoprecipitate from 12C1-labeled 38B9 cells were added to the final batch of the purification. The lysate was then cleared by centrifugation (105,000 × g). The supernatant was incubated with 1-mg gel of RCA30-Sepharose (Ricinus communis agglutinin type I, Pharmacia LKB) at 4°C overnight. The RCA-Sepharose bound samples were washed three times with Nonidet P-40 lysis buffer containing 0.5 M NaCl and eluted with 0.5 M galactose/2 mM NaCl in Nonidet-P-40 lysis buffer at 4°C for 4 h. The eluate was stored at −20°C as RCA30 fraction.

The RCA30 fractions from 5 × 1011 cells were pooled, and the proteins were precipitated by the method of Wessel and Flugge (31). The proteins were then solubilized with the buffer A (8 M urea, 50 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% CHAPS, 0.5 mM dithiothreitol, and 2 mM EDTA). After a passage through a 0.2-μm filter, the sample was loaded on Mono-Q column (Amersham Pharmacia Biotech) and eluted in a gradient of NaCl (0–1.0 M NaCl in buffer A). As the fractions corresponding to 0.5–0.6 M NaCl contained p130, these fractions were pooled, dialyzed against 10 mM NH4HCO3, and lyophilized.

The lyophilized sample was dissolved with the sample buffer of NEPHGE, cleared by a centrifugation, and subjected to NEPHGE/SDS-PAGE as described previously (30). After the electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, which separated as above, was rendered to the digestion with lysine-C. The digestion, separation on HPLC, MALD-MS of the HPLC fractions, and N-terminal amino acid sequencing of the peptides were performed by Dr. William S. Lane’s Laboratory (Harvard microchemistry facility) and the facility in our Institute.

PCR and cDNA Library Screening—The design of the gene-specific oligonucleotide PCR primers (p130pk37sens, 5′-AARATYGTBCARGGNGA-3′; p130pk37anti, 5′-GCRTTIIYGGTDGRTCGNTG-3′) was based on the N-terminal six amino acids and seven C-terminal amino acids of peak-37 peptide (KVIVGQDXMXELLYTDPTIN) using a preferred linker in the rabbit. The PCR products that hybridized to the 5′-end-labeled internal, gene-specific oligonucleotide probes p130pk37 m5′-ACNGARIYIIYGTIGRGTIC-3′) were identified and subcloned into the pcRT3II vector using the TA Cloning system (InVitrogen Corp.). From the deduced sequence, the primers p130pk37-3′ (5′-AAGATTGACCCGAGGAGA-3′) and p130pk37-5′ (5′-TGAGGATCTGTTGACA-3′) were designed for Marathon (34) cDNA amplification method (CLONTech Laboratories, Inc., Palo Alto, California), and RACE-PCR (32) was performed according to the protocol from the company. The amplified products were size fractionated on a 1.2% agarose gel and subcloned into the pcRT3II vector. To obtain longer fragment, p130RACE-5′ (5′-AATAATCTACAGTGCGGCTCCTGAGAT-3′) and p130RACE-3′ (5′-GACGACCTGCTCCTGCAGAAGATC-3′) were designed from p130pk37-5′ and p130pk37-3′, respectively, and ExpandTM High Fidelity (Roche Molecular Biochemicals) was used. The amplified fragments were cloned into pCR-ScriptSM (Stratagene, La Jolla, CA) and sequenced as described below.

In parallel, the ρgt10 38B9 cDNA library was screened by the plaque lift method with a 32P-labeled 0.2-kilobase PCR product (Hpk37) amplified using Hpk37 (5′-GACGACCTGCTCCTGCAGAAGATC-3′) and Hpk37 (5′-TGAGGATCTGTTGACA-3′) as the primers after the method described in Ref. 33. cDNA clones were recovered from positive ρgt10 plaques and cloned into pBluescript II SK+. DNA sequence analysis of cDNA inserts were performed by the Taq DyeDeoxyTM Terminator Cycle sequencing method using an automated sequencer (model 373A, Applied Biosystems, Inc., Foster City, CA). A Northern Blot and RT-PCR Analysis—Total RNA was isolated from various mouse tissues and from various cell lines by a modification of phenol and guanidine isothiocyanate method described by Chomczynski and Sacchi (34). Total RNA (20 μg) was electrophoresed through 1% agarose gels containing 0.67% formaldehyde, transferred to nylon filters (Zeta-Probe membrane, Bio-Rad). PCR products were hybridized with 0.2 μg/ml of cDNA, prehybridized for 1 h at 37°C and then 24 h at 58°C in 2× SSC containing 0.01% SDS for 30 min at room temperature and then 0.1× SSC containing 0.1% SDS for 40 min at 50°C. The filters were then autoradiographed on a film or analyzed by a PhosphoImager (Molecular Dynamics).

Semi-quantitative RT-PCR on cell lines and sorted cells was performed basically as described previously (35). Briefly, total RNA was isolated from sorted 3 × 106 cells reverse transcribed using SuperScript II reverse transcriptase (Life Technologies, Inc.). RT-PCR assays were carried out with the series of template dilutions to obtain a linear correlation between input template and PCR product. The primer pairs used are as follows: p130, 5′-GACATCTTACCATCATCGTCA-3′ and 5′-GCTAACACATCAACAGATTTTG-3′; HPRT, 5′-GCTGGTGAAA-AGGAATCGTAA-3′ and 5′-GACGACCGACTACATGTC-3′; λ5, 5′-GAGCATCTACGCTCCTGAAGCC-3′ and 5′-CTTGGCGCTGACC-TAGGATT-3′. The PCR products are examined by Southern blot using the probe as described above (p130) and previously (HPRT, λ5 (35)). The hybridization signals were quantitated by the PhosphoImager equipped with ImageQuant software (Molecular Dynamics).

The relative contents of the messages in each cell and tissue were normalized by HPRT and estimated from the linear region of the template dilution versus PCR product plot.

Isolation of p130 Transfected Cell Lines—X63.Ag8.653 cell line transfected with Vpre-B1 and λ5 cDNA, X63/Vpre-B1/λ5, was described previously (36) and maintained in 1 mg/ml hygromycin B (Roche Molecular Biochemicals). Supertransfection of X63.Ag8.653 was carried out using DOTAP transfection reagent (Boehringer Mannheim, Germany). Full-length X63.Ag8.653 cell line was transfected with the retrovirus vector, LXSX. LXSX is a derivative of LXSN (37), in which puromycin resistant gene was introduced in place of neomycin resistant gene. LXSXP130 was linearized by PvuI cut and transfected into packaging cell line, gp+E+ (38) by DOTAP method (M[1-2,3-dioleoyloxy]propyl)-N,N,N,N-tetramethylammonium methylsulfate, Roche Molecular Biochemicals). After the selection of LXSXP130 transfected gp+E+86 cells with 3 μg/ml puromycin, the cells were cultured to

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confluent monolayer, γ-irradiated with 3000R and the X63/Vpre-B1/A5 cells were overlaid for overnight without addition of puromycin. After the selection with 3–6 μg/ml puromycin, the transfectants were cloned by limiting dilution. Culture, transfection, and cloning of Drosophila Schneider cells (SL-3) were performed according to Ref. 39. Briefly, p130 cDNA was cloned into pRmHa-3 (40) under the control of metallothionein promoter and transfected into SL-3 cells with phls-pha-puro (containing the puromycin resistance gene under the heat shock promoter; a kind gift of K. Karjalainen, Basel Institute for Immunology) using Lipofectin (Life Technologies, Inc.). After the selection with 6 μg/ml puromycin, cells were tested for the aggregation according to Berndorff et al. (41).

RESULTS

Purification and Amino Acid Sequencing of p130—The surrogate light chain is expressed on the cell surface of pro-B cells in mouse bone marrow as well as pro-B cell lines such as 38B9 and 63-12 in the absence of μ heavy chains (23). Cell surface iodination and subsequent immunoprecipitation with anti-α5 or anti-Vpre-B1 antibody revealed that 130- and 35–65-kDa molecules are the main components that appear associated in a complex with SL chain (Fig. 1A). The two-dimensional gel patterns show that p130 is a slightly acidic protein (pI = 5.8), whereas the 33-65-kDa molecules are relatively neutral (pI = 5–7). The 130- and 35–65-kDa molecules are glycoproteins. Hence, deglycosylation of the complex with PNGase F gave rise to a 100-kDa protein and several 30–45-kDa proteins of slightly different neutral pIs. The differential SDS-PAGE analysis under nonreducing versus reducing conditions revealed no differences in migration of the 100- and 30–45-kDa deglycosylated components, indicating that they are not disulfide-linked (23). The association of the glycosylated 35–65-kDa molecules appears to be more ubiquitous because they are also co-precipitated, for example, in complexes with anti-B220 mAb in which SL chain and p130 are not found (Fig. 1B). Therefore, we chose the 130-kDa molecule (subsequently referred to as p130) as the most probable protein partner of SL chain on pro-B and pre-B1 cells.

To purify sufficient quantities of p130 that could be entered into an amino acid sequence analysis, 5 × 10^11 38B9 pro-B cells were lysed with Nonidet P-40 lysis buffer and absorbed with ricin-lectin (RCA120-agarose). Bound material was eluted with 0.5 M galactose, then loaded on a Mono-Q column, and eluted with an NaCl gradient. p130 was eluted around 0.5 M NaCl (for details, see "Experimental Procedures"). The purity of the eluted fraction containing p130 was estimated to be about 50%. As revealed by a comparison of the two-dimensional NEPHGE/SDS-PAGE gel of the fraction where protein was detected by silver staining (Fig. 1C), with the spot corresponding to p130 as it could be identified by co-electrophoresed [125I]-labeled p130 tracer protein, which is immunoprecipitated with anti-α5 from the iodinated same cell line. The p130 spot appeared fairly isolated from other spots on the gel. Consequently it was blotted to a polyvinylidene difluoride membrane, subjected to lysine-C digestion, and the resulting peptides were separated on reverse phase HPLC (Fig. 1D). The amino acid sequences of some of the isolated peaks were determined (Table I). It became evident from this analysis that the p130 spot represented a mixture of β1-integrin and an unknown, p130 protein.

Because β1-integrin is a glycoprotein with an apparent molecular mass of 130 kDa that can be deglycosylated to a 100-kDa core protein, we tested its capacity to associate with SL chain. However, a mAb specific for β1-integrin co-precipitated neither Vpre-B1, α5 nor the 35–65-kDa molecules from the [125I]-labeled pro-B cell line (Fig. 1B). Furthermore, a mAb specific for α5-integrin (which is associated with β1-integrin on the pro-B cell line), proved also unable to co-precipitate Vpre-B1 and α5. Moreover, the 130-kDa spot of β1-integrin on two-dimensional gel exhibited a slightly different mobility on two-dimensional gel when compared with the SL chain-associated p130 spot (data not shown). These facts were taken as an indication that it was not β1-integrin but the unknown p130 protein that was capable of association with SL chain. Five of six unknown sequences with no sequence relationship to β1-integrin showed significant homologies to cadherin molecules (Table I). Cloning and DNA Sequence Analysis of p130 cDNA—One of the amino acid sequences obtained from the unknown, cadherin-like p130 material, IVQGDTEXRLVVTDDPTTNA from peak 37 (Fig. 1, D and E), was long enough to design PCR primers with which the cloning of p130 cDNA could be attempted using the RACE technique (see “Experimental Procedures”). By repeating the RACE-PCR with a 38B9 cell cDNA library, we were able to obtain a full-length cDNA sequence of p130 (Fig. 2).

The cDNA sequence has one long open reading frame encoding 827 amino acids. This is in agreement with the finding that the deglycosylated core protein of p130 is about 100 kDa. The FASTA search of this amino acid sequence revealed a high degree of homology to the superfamily of cadherin molecules, especially to rat Li-cadherin (90% homology) and human HPT-1 (79% homology). At the nucleotide level, the homology of p130 cDNA to rat Li-cadherin and human HPT-1 is 91.6 and 80.8%, respectively. The p130 sequence contains consensus cadherin signatures such as LDRE, DXDND, and DXD as well as four Cys residues in a membrane-proximal region (one in EC4 and three in premembrane domains; Fig. 2). The organization of cadherin domains in p130 resembles nonclassic rather than classic cadherins, because p130 lacks the “precursor segment” and is composed of five cadherin domains and one pseudo-domain. In addition, the HAV motif, which is conserved in EC1 of classic cadherins, and which is thought to be important in the counterpart homotypic recognition, is replaced in p130 by AAL, as it is in Li-cadherin and HPT-1. However, the most striking difference from the classic cadherins is a lack of the “catenin-binding motif” in the cytoplasmic domain. The cytoplasmic domain of p130 consists of 18 amino acids in which, by homology search, no significant consensus motifs are found that go beyond those for LI-cadherin and HPT-1.

The Association of p130 with α5/Vpre-B1—Because p130 was identified in biochemical studies as a SL chain-associated molecule, this interaction between p130 and α5/Vpre-B1 was probed by co-expression of the Vpre-B1, α5, and p130 genes in a suitable cell line, followed by co-immunoprecipitation. The myeloma cell line X63.Ag8.653 does not express p130 or SL chain. It was first transfected with mouse Vpre-B1 and α5 cDNAs. A stable transflectant, X63/Vpre-B1/A5, was further transfected with p130 cDNA (X63/Vpre-B1/A5/p130). Surface expression of the Vpre-B1, α5, and p130 molecules on these stable transflectants were examined by flowcytometry. The results of these experiments are shown in Fig. 3.

p130 is expressed on the surface of X63/Vpre-B1/A5/p130 transflectants, and α5 is strongly expressed on these cells as well. A small amount of α5 is detected on the surface of X63/Vpre-B1/A5 transflectants. However, the α5 surface expression is increased by concomitant p130 expression, so that the mean fluorescence intensities are 4-fold higher. These results indicate that p130 expression up-regulates α5 surface expression.

The interaction of p130 with SL chain could also be seen in co-immunoprecipitation experiments. To test this interaction 38B9 pro-B cells were surface iodinated, then lysed, and finally immunoprecipitated with specific antibodies. As shown in Fig. 4A, reciprocal co-precipitation was observed. α5-specific as well as Vpre-B1-specific mAbs co-precipitated 130-kDa protein with
21.5-kDa λ5 and 16-kDa Vpre-B1, whereas p130-specific mAb co-precipitated 21.5- and 16-kDa proteins together with 130-kDa p130. Control immunoprecipitations with rat normal Ig did not precipitate the 130-, 21.5-, and 16-kDa bands. The identities of Vpre-B1, λ5, and p130 proteins in the bands on the gels were confirmed by Western blots, showing that the 130- and 21.5-kDa bands specifically react with p130-specific and λ5-specific mAbs, respectively (Fig. 4B).

The ratios of p130 protein to SL chain, however, are different in the precipitations with the different antibodies. Estimation of the quantities of radioactivity in the bands indicates that the ratio of λ5 to p130 is roughly 1:2 for the case of precipitation with λ5-specific mAb, whereas it is 1:50 in the case of p130-specific mAb. Possible interpretations for these differences will be discussed under “Discussion.”

Interaction between Vpre-B1/λ5 and p130 is also seen when
the Vpre-B1, λ5, and p130 protein expressed in the transfectants are analyzed by immunoprecipitation after surface iodination. In the transfectants described in Fig. 3, reciprocal co-precipitation with λ5-specific and p130-specific mAbs could be seen in X63/Vpre-B1/λ5/p130 (Fig. 4C).

**p130 Mediates Homotypic Adhesion without Catenin-binding Motif**—p130 lacks the catenin-binding motif in its cytoplasmic domain, which, in classic type cadherins, is thought to be the main functional part of the molecules mediating homotypic adhesion. To examine any homotypic adhesion activity mediated by the p130 molecule, we transfected the *Drosophila* cell line SL3 with full-length p130 cDNA, in which the expression of p130 is controlled by the metallothionein promoter. Stable transfectants were isolated, and possible self-aggregation of the cells was examined. As shown in Fig. 5, Ca²⁺-dependent self-aggregation is induced in the transfected SL3 cell line, whereas the control cells, in which p130 expression is not induced by the addition of CuSO₄, do not show any self-aggregation. These results indicate that p130 shows cadherin functions. It can mediate Ca²⁺-dependent homotypic cell-cell adhesion even without a classical catenin-binding motif.

**Expression Pattern of p130 Message**—To examine the expression pattern of p130 on mRNA level, Northern blot analyses and semi-quantitative RT-PCR were carried out with RNA samples obtained from a variety of tissue and cells representing the B lineage and other cell lineages. Northern blot analyses of tissues showed that p130 message is expressed in spleen, bone marrow, lung, testis, and, most strongly, in the intestine (Fig. 6A). Liver, kidney, heart, brain, skeletal muscle, and thymus did not express detectable amount of the message. Because p130 message is not detectable, even by the PCR, in liver our results indicate a clear difference from the expression pattern of rat LI-cadherin (rat liver-intestine-specific cadherin) (41).

Tested with a panel of cell lines, expression of p130 message appears restricted to precursor B cells and myeloid cells (Fig. 6B). Pro-B and pre-B-I cell lines such as 38B9 and 63-12 express relatively high amounts of the message, as they do the λ5 message. Pre-B-II type cell lines, such as NFS5.3 expressed low levels of p130 message, whereas pre-B-II type cell line Ig6.3 did not express detectable amount of the message. The immature B cell line, WEHI231, expressed small amounts of the message, whereas no message was detectable in the mature B cell line A20, the myeloma X63, and the hybridoma SP6. In addition to B lineage cell lines, one of the macrophage lines, P388.D1,
expressed significant amount of the message, whereas more mature macrophage line, WEHI3, did not. It should be recalled that P388.D1 has been seen to be a line capable of myeloid as well as pre-B cell differentiation (42). This prompted an examination of p130 RNA expression in the progenitor cell lines, HAFTL-1. HAFTL-1 is a v-Ha-ras transformed fetal liver-derived progenitor B cell clone from which pre-B cell-like (clone 14) and macrophage-like (3G4, 2B7, 1G4) subclones were isolated by induced differentiation with LPS or interleukin-4 (43). Among these clones, the progenitor clone HAFTL-1 expressed strongly, the pre-B subclone expressed weakly, whereas the myeloid subclones did not express detectable amount of the message (data not shown). The T cell lines (SCFET27F, EL4, and B6.2.16.BU), stromal cell lines (PA6 and ST2), and fibroblast (NIH3T3) did not express detectable amounts of the message. These results suggest that p130 message is expressed strongly in pre-B and pre-B-I cell and less strongly in pre-B-II-type cell lines but probably not in mature macrophage, T cell, and stromal cell lines.

To examine expression of p130 message in normal cells of bone marrow and spleen, B-lymphocyte lineage subpopulations were purified by differential, preparative fluorescence-activated cell sorter, and RNA expression was examined by semi-quantitative RT-PCR (Fig. 6C). In bone marrow, B220⁺ / c-Kit⁺ pro/pre-B-I cells expressed high levels of the message. Interestingly, the message was not detectable in CD25⁻ large and CD25⁺ small pre-B-II cells but became detectable again in IgM⁺/IgD⁻ immature and IgM⁺/IgD⁺ mature B cell populations. In spleen, B220⁺ and Mac-1⁺ cells expressed the message, whereas CD4⁺ and CD8⁺ fractions did not. These results suggest that the expression of p130 message in vivo is confined to B lineage cells. The expression of p130 appears to be biphasic during the B cell development: on in pro- and pre-B cells, off in pre-B-II cells, and on again in immature and mature B cells.

Expression of p130 Protein—Expression of the p130 protein was examined with the p130-specific BD1B mAb. This mAb 1) immunoprecipitates 130 kDa protein, 2) reacts specifically with p130 transfectants, and 3) does not react with pre/pre-B-I cells from mice in which the p130-gene has been mutated by targeted disruption. In the bone marrow of 5-week-old C57BL/6 mice, about 15% of B220⁻ cells and 40% of B220⁺ cells are p130⁺ (Fig. 7A, panel a). In the B220⁻ population of cells, most of the p130⁺ cells are Mac-1⁻ cells. Further analysis of CD34⁺ early progenitors showed that the onset of p130 expression occurs already at these earlier stages of B lineage differentiation, i.e. in lin⁺/CD34⁺ cells, which might represent cells close to hematopoietic stem cells (Fig. 7A, panel b).

A small numbers of cells in the B220⁺ population were found to be p130⁻. Further dissection of this population with c-Kit- and CD25-specific mAbs revealed that B220⁺/c-Kit⁺ pre/pre-B-I cells are p130⁺, whereas B220⁺/CD25⁺ pre-B-II cells are p130⁻ (Fig. 7A, panels c and d). This is in line with the observed down-regulation of p130 mRNA in pre-B-II-like cell lines.

However, a large part of the B220hi more mature B lineage cells is p130⁺ again. The dissection of this population by IgM- and IgD-specific mAbs into immature and mature sIg⁺ B cells revealed that p130 expression is low in IgM⁺/IgD⁻ immature B cells and is up-regulated upon IgD expression (Fig. 7A, panel e). Interestingly, the expression is again down-regulated in the IgM⁺/IgD⁻ population. Collectively these results indicate that mature B cells in the bone marrow express the highest level of p130 protein.

In the spleen, most p130⁺ cells are B220hi (Fig. 7B). Among the B220hi cells, the CD21⁺/CD23⁻ cells express the highest amount of p130, with a mean fluorescent intensity that is 10 times higher than CD21hi/CD23⁻ or CD21⁻/CD23⁻ subpopulations (Fig. 7B, panel b). These CD21⁺/CD23⁻ cells most likely are marginal zone B cells. Consistent with this analysis is the histochemical analysis with p130-specific mAb on spleen sections, which showed that p130⁺ cells were mainly located in the marginal zone, and only scattered positive cells are in the primary follicles (Fig. 8). p130⁺ cells are almost absent from T cell rich zones in the peri-arteriolar lymphoid sheath.

**DISCUSSION**

We have identified a nonclassical cadherin of 130 kDa (called p130) that can be found noncovalently associated with SL chain in mouse pro- and pre-B-I cells. We call it BILL-cadherin, because it is expressed in B lineage cells, intestine, leukocytes, and liver. As far as we know, this is the first instance that such a cadherin has been found in B lineage cells. BILL-cadherin has characteristic extracellular cadherin domains and lacks
catenin-binding motifs in its cytoplasmic domain, as nonclassical cadherins do. The amino acid sequence is very similar to that of rat LI-cadherin (liver-intestine-specific cadherin; Ref. 41) and human HPT-1 (44), and, to a lesser extent, of rabbit kidney-specific cadherin (45).

It is very possible that BILL-cadherin is the mouse homologue of rat LI- or human HPT-1 cadherin. However, in contrast to rat LI-cadherin, mouse BILL-cadherin message is not detected in adult mouse liver, although B cells in mouse fetal liver do express the molecule.

The cadherin family is divided into two subgroups, classic and nonclassic types, based on the presence or absence of a catenin-binding site in their cytoplasmic domain. About 20 classic cadherins have been identified in a single vertebrate species and have been shown to be involved in many phases of morphogenesis. In the nervous system, for example, most of 20 classic cadherins show “regional” expression and are thought to confer region-specific adhesiveness during the neural morphogenesis and synaptic plasticity (46–48). Adhesiveness is a Ca$^{2+}$-dependent process. In homotypic adhesion two cells of the same or of different differentiation lineages at the same or different stage of development adhere to each other through contacts by the same molecule, e.g. cadherin, expressed on the two cells. Heterotypic adhesion involves one molecule, e.g. cadherin, to a different molecule, e.g. integrins. For example, E-cadherin on mucosal epithelial cells was seen to interact with $\alpha E\beta 7/\alpha M 290/\beta 7$ integrin on T cells (49, 50). The sites for homotypic and for heterotypic interactions of E-cadherin have been found to be different (51).

Adhesion via classic cadherins induces signal transduction via catenin family molecules that find a binding domain in the cytoplasmic domain of the cadherins (reviewed in Ref. 52). This signal transduction is thought to be involved in the morphogenetic changes that the cells undergo during their differentiation. We have not yet investigated whether BILL-cadherin is associated with some of the proteins that are found in the complex in addition to SL chain coprecipitated from pro-B and pre-B-I cells. Because BILL-cadherin lacks the catenin-binding
domain, it would be interesting to examine whether another component of the complex (105- and 35–65-kDa) exerts a signaling function.

The nonclassic cadherins, which lack a catenin-binding motif, are further divided into three subgroups, protocadherins (53), CNR (54), and cadherins that are different from either of
the two. Interestingly, genes for protocadherins and CNRs represent an organized genomic structure like those for immunoglobulins and T cell receptors, suggesting the possibility of diversifying their specificities by gene rearrangement-like mechanism (55, 56). BILL-cadherin appears to differ from CNR and protocadherins because it has a particularly short cytoplasmic domain. It is unclear whether nonclassic cadherins can induce signals, and if they do by which molecular modes, although CNR molecules are associated with Fyn tyrosine kinase in their cytoplasmic domains (57). These molecules are also expressed regionally in various tissues including brain and thought to be involved in “sorting out” of the cells (47). However, it is clear that rat LI-cadherin (58) and BILL-cadherin (this study) are able to exert Ca^{2+}-dependent homotypic adhesion. Hence, BILL-cadherin could be involved in the “sorting” or “homing” of cells during the B cell development in primary lymphoid organs such as fetal liver and bone marrow as well as during immune responses of mature B cells in secondary lymphoid organs.

Our RT-PCR and flow cytometric analyses show that BILL-cadherin is expressed in two phases during B cell development in the bone marrow. Lin<sup>-</sup>/CD34<sup>-</sup> and Lin<sup>-</sup>/CD34<sup>+</sup> populations do not express p130 whereas Lin<sup>-</sup>/CD34<sup>-</sup> and Lin<sup>-</sup>/CD34<sup>+</sup> populations express p130. Panels c and d, the expression of p130 in pro/pre-B cells. Total bone marrow cells were stained with anti-c-Kit (PE-labeled) (panel c) and anti-CD25 (PE-labeled) (panel d) as well as anti-B220 (Cy5 labeled) and anti-p130 (biotinylated BD1 detected with RED613-Streptavidin). The gates indicated are analyzed for the p130 expression. Note that c-Kit<sup>+/220<sup>lo</sup></sup> pre/B-I cells express p130 whereas CD25<sup>+/B220<sup>lo</sup></sup> pre/B-II cells do not. Panel e, the expression of p130 in immature/mature B cells. Total bone marrow cells were stained with anti-IgM (Cy5), anti-IgD (FITC), and anti-p130 (biotinylated BD1 detected with PE-Streptavidin). Note that p130 is expressed most strongly in IgM<sup>lo/IgD</sup> population. The expression of p130 in spleen cells. Panel a, total spleen cells were stained with PE-Streptavidin. The majority of p130 cells are B220<sup>+</sup>. Panel b, total spleen cells were stained with anti-CD21 (FITC), anti-CD23 (PE), and anti-p130 (biotinylated BD1 detected with RED613-Streptavidin). Note that CD21<sup>+/CD23<sup>+</sup></sup> B cells express the highest amount of p130.
small pre-B-II cells, and up-regulated again in immature and mature B cells.

Before the SL chain forms the pre-B cell receptor with \(\mu\) heavy chain on large pre-B-II cells during B cell development (17), it is already expressed on the surface of \(\mu\)-chain nonexpressing pre-B and pre-B-I cells (23). Within the complex of 130-, 105-, and 35–65-kDa glycoprotein components, SL chain is associated in the pro- and pre-B-I cells. p130, a new nonclassic cadherin, may be the component to which SL chain binds. Recent crystallographic studies of E-cadherin have revealed a remarkable structural similarity with immunoglobulin domains, visible in the organization of the \(\beta\)-strand structure (61–63). The N-terminal domain of E-cadherin, which shows structural homology with Ig domain, is the one that is involved in the Ca\(^{2+}\)-induced rigidity and dimerization and that is thought to be the basis for homotypic adhesion mediated by cadherins (64). SL chain consists of a light chain constant region-like domain of the \(\lambda5\) protein, a \(\lambda\)-like \(\beta7\) strands of a V-like domain also contributed by the \(\lambda5\) protein, to which the Vpre-B1 protein associates in an Ig domain-like fashion (65, 66). Therefore, SL chain on BILL-cadherin could interact with each other in an Ig domain/Ig domain-like way.

Several experimental findings presented in this paper favor the interpretation that such an interaction between BILL-cadherin and SL chain exists. SL chain (\(\lambda5\))-specific mAb co-precipitate BILL-cadherin from pro-B and pre-B-I cell lines, as well as from myeloma cells co-transfected with the genes encoding SL chain and BILL-cadherin. The BILL-cadherin/SL-chain complex is expressed on pro-B cells of RAG2\(^{-/-}\) mice but not on pro-B and pre-B-I cells of \(\lambda5\)-null mice. Co-precipitation of the BILL-cadherin and SL chain-containing complex from myeloma cells transfected with the corresponding genes is almost equimolar with \(\lambda5\)-specific mAbs but not with BILL-cadherin specific mAb. The latter mAb might recognize a determinant that could be partially covered or otherwise nonavailable in the complexes. However, none of our experiments exclude the possibility that the interaction between BILL-cadherin and SL chain is indirect, within a complex of other proteins, especially the ones co-precipitated as 105 and 35–65-kDa bands.

Expression of BILL-cadherin is up-regulated in pro-B and pre-B-I cells, which is in parallel with the expression of SL chain. Interestingly, expression is down-regulated again at the stage when c-kit and CD43 are down-regulated, and CD25 is up-regulated, i.e. at the transition from pre-B-I to large pre-B-II cells. At this transition the pre-B cell receptor composed of \(\mu\) heavy chain and SL chains appear on the surface, and this leads to a subsequent down-regulation of SL chain expression. In principle, the parallel patterns of expression of BILL-cadherin and SL chain in precursor B cells would permit a mutual association throughout these cellular stages. In pre-B-II cells \(\mu\) heavy chains would replace BILL-cadherin in a complex with SL chain and terminate the expression of SL chain. It will be interesting to see whether the concerted expression of BILL-cadherin and SL chain is a consequence of shared gene expression controls.

BILL-cadherin expression is up-regulated again in later stages of B cell differentiation. Newly emerging mature B cells begin to express BILL-cadherin. In the spleen, the B220\(^+/\)CD21\(^+/\)CD23\(^{low}\) population, which corresponds to the marginal zone B cells (67), expresses the highest level of BILL-cadherin. This observation is consistent with the histological staining of spleen sections with BILL-cadherin specific mAb, which show the marginal zone as the most strongly stained structure. Some of the B cells in the marginal zone have been found to belong to the B1 lineage of B cell development and to be activated, possibly by cross-reactive autoantigens (16). Further analyses are aimed at identifying the antigen-recognizing repertoire of the p130\(^{hi}\) B cells in spleen. If they appear cross-reactive and autoreactive, then high level p130 expression might be used as an analytical tool for B1 cell identification.

In conclusion the biphasic expression profile of BILL-cadherin suggests at least two different functions of the molecule during B cell development and responses. These functions are now being explored.

Acknowledgments—We thank Dr. William Lane and David Avila for the intensive analysis of peptide sequencing, as well as Joachim Scherf for excellent technical assistance.

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