New Alternatively Spliced Form of Galectin-3, a Member of the β-Galactoside-binding Animal Lectin Family, Contains a Predicted Transmembrane-spanning Domain and a Leucine Zipper Motif

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Osteoclasts or their precursors interact with the glycoprotein-enriched matrix of bone during extravasation from the vasculature, and upon attachment prior to resorption. Reverse transcriptase-PCR studies showed that two new alternatively spliced forms of chicken galectin-3, termed Gal-3TM1 and Gal-3TR1, were enriched and preferentially expressed in highly purified chicken osteoclast-like cells. Gal-3TM1 and Gal-3TR1 mRNA were also detected in chicken intestinal tissue, but not in kidney, liver, or lung. Gal-3TM1 and Gal-3TR1 messages both contain an open reading frame encoding a predicted 70-amino acid TM1 sequence inserted between the N-terminal Gly/Pro repeat domain and the carbohydrate recognition domain (exons 3 and 4). Gal-3TR1 mRNA contains an additional 241-bp sequence, which encodes a truncated open reading frame between the 4th and 5th exons, and, whose translation is expected to terminate within the carbohydrate recognition domain encompassing exons 4, 5, and 6. Immunoblotting and affinity chromatography showed that purified osteoclast preparations and intestinal homogenates contained a 36-kDa lactose-binding galectin. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analyses on chymotryptic peptides from the 36-kDa lectin confirmed its identity as Gal-3TM1. The TM1 insert contains a single transmembrane-spanning region and a leucine zipper-like stalk domain that is predicted to position the intact carbohydrate recognition domain of Gal-3TM1 on the exterior surface of the plasma membrane. Immunofluorescent staining of chicken osteoclasts confirmed the expression of Gal-3TM1 at the plasma membrane. Gal-3TM1 is the first example of a galectin superfamily member capable of being expressed as a soluble protein and as a transmembrane protein.

Carbohydrate on the outer membrane surface of cells has long been suggested as a determinant of specific cell-cell and cell-matrix recognition. In bone, carbohydrate receptors on osteoclasts and osteoclast precursors could play a functional role in mediating cell-matrix interactions. Osteoclasts arise from mononuclear hematopoietic precursors in bone marrow and share the same stem cell origin as granulocytes and monocyte/macrophages. A variety of carbohydrate receptors are known to be expressed by monocyte/macrophages, including selectins (1), galectin-3 (Mac-2, IgEBP) (2), mannose receptor (3), and sia-loadhesin (4). To resorb bone, osteoclast precursor cells must leave the vasculature, crawl to the resorption site, fuse with other precursors, and attach to the bone surface delimiting the area of bone to be resorbed and form a sealing zone. Most research on osteoclast attachment has focused on the vitronectin receptor and its -RGD- containing ligands (5–9). Although these studies clearly demonstrate that the vitronectin receptor and its ligands play an important role in osteoclast attachment, several pieces of conflicting data are most easily explained by the existence of one or more additional cell adhesion receptors (7, 10, 11).

The vascular basement membrane contains the glycoprotein laminin, whereas primary bone matrix contains two major glycoproteins, bone sialoprotein (12) and BAG-751 (13). Sato et al. (14) showed that BAG-75 was able to block bone resorption by an RGD-independent mechanism when either added directly to osteoclasts and bone slices, or adsorbed first to the bone target prior to addition of osteoclasts. Colucci et al. (15, 16) also showed that osteoclasts bind to laminin by means of an RGD-independent mechanism. Kukita et al. (17) found surface-adsorbed laminin was able to block osteoclast differentiation in rat bone marrow cultures. Finally, Niida et al. (18) and Taka-hashi et al. (19) were the first to demonstrate that galectin-3 was expressed by osteoclasts and TRAP-positive mononuclear precursors. Galectin-3 is the major non-integrin laminin binding protein of macrophages (20), which share a common lineage with osteoclasts. Galectin-3 is a galectin superfamily member and displays a single carbohydrate recognition site specific for

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) AF479564 and AF479565.

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1 The abbreviations used are: BAG-75, bone acidic glycoprotein-75; TRAP, tartrate resistant acid phosphatase; PVDF-P, polyvinylidene difluoride; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; RT, reverse transcriptase; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; TM, transmembrane; MOPS, 4-morpholinepropanesulfonic acid; CLI I, chicken lactose lectin-I.
phosphorimaging with a Storm system (Molecular Dynamics, Inc.) or autoradiography.

RT-PCR—In initial work, total RNA was isolated from chicken giant cells derived from bone marrow cells cultured for 5 days (31). The reverse transcriptase step was carried for 50 min at 42 °C using an oligo(dT)12-18 primer and Superscript II reverse transcriptase (Invitrogen, Inc.). After inactivation of the transcriptase, the cDNA was used as a template in PCR with chicken galectin-3 primers 1 and 5 (see “Materials”).

In subsequent studies, total RNA was purified from osteoclasts and tissues isolated from chicks according to Collin-Osdoby et al. (26). This purification, which includes an immunofluorescence purification step with osteoclast-specific 121F antibody, produces >95% pure osteoclast preparations. RNA was then digested with DNase I (15–40 min at 30 °C) and cDNA produced using an oligo(dT) primer and SuperScript II reverse transcriptase according to a protocol provided by the supplier (Invitrogen). This reaction mixture and associated control mixture (without reverse transcriptase) were then treated with RNase H for 20 min at 37 °C and used as templates in PCR runs.

Synthesis and Cloning of TM1 Insert cDNA Sequence—Two partially overlapping oligonucleotides of 105 and 107 bp representing the majority of the TM1 insert sequence were chemically synthesized and purified by polyacrylamide gel electrophoresis (IDT, Inc.). The oligos were filled in by incubating with 4 units of Klenow fragment (Roche Molecular Biochemicals), the enzyme was then heat-inactivated at 75 °C, and this double-stranded template was diluted 1/50 and used in a polymerase chain reaction with specific primers (#9 and 4, see “Materials”). A resultant band of 197 bp was isolated and labeled as described above and used for Northern blotting.

Analysis of Hydrophobicity—Hydropathy analyses were carried out by applying the TMpred (35) and TopPred2 (36) programs to galectin-3TM1 and other protein sequences of interest.

Marrow Ablation Surgery and Immunohistochemical Staining of Primary Bone or Purified Osteoclasts—Rats were anesthetized, and bilateral tibial marrow ablation was performed as described by Gorski et al. (37). Tibias were removed intact from rats on days 8–10 following ablation surgery and fixed/calcified for 2 days in Bouin’s solution and then for 6–10 days in 4% formaldehyde containing 0.85% sodium chloride and 10% acetic acid (changing the latter solution every 2 days). Longitudinal sections of ablated tibias were immunostained using an affinity-purified rabbit anti-rat galectin-3 antibody (4 µg/ml IgG) (38) and a Vectastain ABC goat secondary antibody kit with a glucose oxidase detection system and colorimetric substrate kit (Vector Laboratories, Inc.). In some cases, sections were also either double- or single-stained for TRAP (Sigma Chemical Co.) following instructions from the supplier.

Purified chicken osteoclasts were fixed briefly in 3% (w/v) paraformaldehyde in Hanks’ balanced salt solution, permeabilized with 0.5% (w/v) Triton X-100 in 20 mm Hepes, 300 mm sucrose, 50 mm sodium chloride, 3 mm magnesium chloride, and then blocked 30 min in 1% bovine serum albumin in phosphate-buffered salt solution. Cells were incubated with primary anti-chicken recombinant retinal galectin antibodies (1/200), rinsed, incubated with fluorescein-conjugated secondary antibodies, rinsed again, and then stained with 0.3 µg/ml 4′,6-diamidino-2-phenylindole (Molecular Probes, Inc.) prior to viewing. Alternatively, purified chicken osteoclasts were cultured overnight after isolation and then surface-labeled without permeabilization as follows. After rinsing with wash buffer, cells were fixed in the cold for 15 min, rinsed with phosphate-buffered saline, and then blocked for 1 h as described above. Cells were sequentially incubated with anti-chicken retinal galectin antibodies (1/200), rinsed, incubated with fluorescein-conjugated secondary antibodies, rinsed again, and then stained with 0.3 µg/ml 4′,6-diamidino-2-phenylindole (Molecular Probes, Inc.) prior to viewing. Alternatively, purified chicken osteoclasts were cultured overnight after isolation and then surface-labeled without permeabilization as follows. After rinsing with wash buffer, cells were fixed in the cold for 15 min, rinsed with phosphate-buffered saline, and then blocked for 1 h as described above. Cells were sequentially incubated with anti-chicken retinal galectin antibodies (1/200), rinsed, incubated with fluorescein-conjugated secondary antibodies, rinsed again, and then stained with 0.3 µg/ml 4′,6-diamidino-2-phenylindole (Molecular Probes, Inc.) prior to viewing. 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RESULTS

Chicken Osteoclasts Preferentially Express Larger Alternatively Spliced Forms of Galectin-3 mRNA—As shown in Fig. 1A (left panel), RT-PCR studies with RNA from marrow-derived chicken osteoclasts yielded a larger than expected 753-bp product for galectin-3. A diagram indicating the position of primers used in relation to the published chicken galectin-3 sequence (27) is shown in Fig. 1B. A band at 543 bp, the predicted product size, was barely detectable (Fig. 1A, left panel).

When the 753-bp cDNA product was subjected to automated DNA sequencing, a nearly complete identity was observed with the published chicken galectin-3 sequence (27) and extended from position 350 (near the translation start site) to 800 bp (Fig. 2). Only a single silent base substitution (G for A) was noted at position 722. However, an additional 210-bp open reading frame was found to be inserted between positions 770 and 771 of the chicken galectin-3 sequence (Fig. 2). Although the general structure of the chicken galectin-3 gene has yet to be determined, this region is the site of the junction between the third and fourth exons in both the human and mouse Lgals3 genes (45, 46) (Fig. 2). We designated this 210-bp open reading frame as insert TM1 and carried out additional studies to confirm that Gal-3TM1 was derived from expressed sequence.

Subsequently, use of RNA from highly purified chicken osteoclast preparations (26) consistently yielded RT-PCR products with the partial Gal-3TM1 sequence (Fig. 1A, middle panel). No products were obtained in the absence of reverse transcriptase, indicating a dependence upon mRNA template, rather than contaminating DNA. Finally, when a 634-bp product produced with a Gal-3TM1-specific primer (Fig. 1A, middle panel) was sequenced, the expected TM1 sequence was obtained (Fig. 2).

As illustrated in Fig. 1A after ethidium bromide staining (EthBr, right panel), RT-PCR analysis of the 3' end of the coding region (using primer pairs 6 + 8 and 7 + 8, see “Materials”) of the chicken osteoclast galectin-3TM1 message identified two major products, a predicted 534-bp cDNA and an unexpected 765-bp band, as well as a larger minor form. Controls without added reverse transcriptase were blank. All three products contained the TM1 insert as evidenced by Southern blotting with this probe (Fig. 1A, Autorad, rightmost panel). The sequence of the 534-bp cDNA overlapped with the terminal 20 bp of the TM1 insert and then displayed nearly complete identity with positions 772–1259 of the chicken galectin-3 sequence (Fig. 2). Only four changes were identified. The first three of these purine substitutions proved silent, whereas the 3'-most terminal substitution resulted in a Gly to Glu change (predicted 6th exon). The 765-bp cDNA sequence was identical with that for Gal-3TM1, except for an extra 241-bp insert (designated TR1), which followed the TM1 by 89 bp, and an A to G silent substitution on the 3'-side of the insertion site (Fig. 2). Comparisons with genomic human and mouse Lgals3 sequences predict that Gal-3TR1 protein should terminate 11 residues downstream of the TM1-insert-containing messages, 534 and 765 bp, expressed by highly purified osteoclasts. EthBr, ethidium bromide stained; Autorad, autoradiograph with 32P-labeled Gal-3TM1 probe. Numbers below gels refer to primer pairs used (see “Materials”).
However, a low level of expression of the 1.3-kb Gal-3 message would be difficult to detect in the presence of an excess of the 1.5-kb species. Expression of the 1.5- and 2.0-kb forms was found to rise dramatically in cultures treated for 5 days with vitamin D3 and UMR-106 osteoblast cell-conditioned media, conditions that foster differentiation of osteoclast-like cells.

**FIG. 2.** cDNA and translated protein sequences for chick Gal-3TM1 and Gal-3TR1 isoforms. As described under "Methods," cDNAs were produced by RT-PCR with mRNA isolated from highly purified chicken osteoclasts and sequenced. Sequences were aligned using a ClustalW analysis (MacVector program, version 7.0) and regions of identity denoted by the boxed areas. The Gal-3TM1, Gal-3TR1, and chicken galectin-3 nucleic acid sequences are enclosed within a box outlined by dashed lines. Translated protein sequences are located immediately below each corresponding section of nucleic acid sequence and are compared with that for human galectin-3; these four protein sequences are enclosed within a box with solid borders. Numbers on the right side refer to the Gal-3TM1 cDNA and protein sequence. Numbers on the left refer to the chicken galectin-3 sequence. Numbers of the cDNA sequences, which starts at 333 bp, is based upon that for the published chicken galectin-3 mRNA sequence (27). The limits of individual exons for the human gene (45) are noted by arrows immediately below the human galectin-3 protein sequence; individual exons are identified by number. For comparison, the limits of the chicken TM1 and TR1 exons are also depicted by arrows. Chic Gal-3, chicken galectin-3; Hum Gal-3, human galectin-3; TM1, additional 210-bp open reading frame inserted between positions 770 and 771 of galectin-3 sequence; and TR1, additional 241-bp sequence inserted between positions 1069 and 1070 of Gal-3TM1 and encoding the 11-residue truncated carbohydrate recognition domain.
FIG. 3. Expression of Gal-3TM1 and Gal-3TR1 increases during osteoclast differentiation in culture. Total RNA was isolated from chicken marrow cultures on days 1 and 5 and Northern-blotted onto nylon, and the resultant membranes were hybridized with 32P-labeled Gal-3 or TM1 cDNA probe as described under "Methods." The left and right panels represent separate replicate blots hybridized with radiolabeled probes for either chicken galectin-3 or the TM1 insert, respectively. The middle panel depicts a similarly loaded gel stained with ethidium bromide. Size estimates were made by reference to the migration positions of 28 and 18 S rRNA bands.

FIG. 4. Messages for Gal-3TM1 and Gal-3TR1 exhibit restricted tissue expression. A, RT-PCR with splice variant-specific primer pair reveals that Gal-3TM1 and Gal-3TR1 are expressed in chicken intestine, but not liver, lung, or kidney tissue. RT-PCR with total RNA used primers 6 and 8 (see "Materials"); see "Methods" for experimental details. B, parallel studies with galectin-3-specific primer pair detects expression in all four chicken tissues, although the level in intestine and kidney seems higher than that for liver and lung. RT-PCR with total RNA used primers 2 and 3 (see "Materials"); +, RT-PCR with reverse transcriptase; −, negative control without reverse transcriptase; Intest, intestine.

FIG. 5. Human, mouse, and rat osteoclast-like cells express galectin-3 but not Gal-3TM1 and Gal-3TR1. Total RNA was either isolated from cultures of human and mouse marrow-derived osteoclast-like cells or from day 9 rat marrow ablation tissue enriched in osteoclasts, and used for RT-PCR studies with galectin-3TM1/Gal-3TR1-specific or galectin-3-specific primer pairs (Fig. 4). Replicate analyses indicate that Gal-3TM1 and Gal-3TR1 messages were expressed in intestine but not liver, lung, or kidney tissue (Fig. 4A). By comparison, galectin-3 expression was detectable in intestine and kidney (Fig. 4B).

Rat, Human, and Mouse Osteoclast-like Cells Express Galectin-3 but Not Galectin-3TM1—To determine whether expression of galectin-3 splice variants was restricted to specific tissues, total RNA was isolated from 4-week-old chick liver, lung, kidney, and intestinal tissues, and, used in RT-PCR studies with galectin-3TM1/Gal-3TR1-specific or galectin-3-specific primer pairs (Fig. 4). Replicate analyses indicate that Gal-3TM1 and Gal-3TR1 messages were expressed in intestine but not liver, lung, or kidney tissue (Fig. 4A). By comparison, galectin-3 expression was detectable in intestine and kidney (Fig. 4B).
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Purified chicken osteoclasts and whole intestinal homogenate contain functionally active 36-kDa galectin-3TM1 protein, whereas kidney homogenate does not. All results depicted were obtained with 4–20% gradient SDS gels (see "Methods"). A, Western blots of whole homogenates of chick kidney, intestine, and purified osteoclast preparations reveal 36-kDa Gal-3TM1 band cross-reactive with two polyclonal anti-chicken CLL I lectin antibodies. Intestine also contains 14-kDa immunoreactive band, whereas kidney homogenate contains 29- and 16-kDa bands. B, purification of osteoclast- and intestine-derived 36-kDa Gal-3TM1 by lactosyl-Sepharose affinity chromatography. The Coomassie Blue-stained fractions depicted all represent the pooled lactose eluate fraction from their respective chromatographic runs (see "Methods" for details). The kidney preparation contains predominantly CLL I and a trace of 29-kDa lectin, whereas the intestinal preparation contains primarily 36-kDa Gal-3TM1 and a small amount of 14-kDa lectin. The osteoclast-derived eluate contains only the 36-kDa Gal-3TM1 band. Individual bands shown were subsequently cut out, digested in the gel with trypsin or chymotrypsin, and analyzed by MALDI-TOF mass spectrometry. Kid, kidney-derived; Int, intestine-derived; OC, osteoclast-derived; CLL I, 16-kDa galectin; 1, result with antibody prepared by immunization with native CLL I; 2, result with antibody prepared by immunization with denatured CLL I; and, 14 kDa, band identified as CLL II by size and MALDI-TOF.

purred by this step (Fig. 6B). When a kidney supernatant fraction was treated similarly, the lactose-eluate contained predominantly the 16-kDa CLL I lectin along with a trace amount of the 29-kDa band (Fig. 6B). The identities of gel-purified lectin bands were then analyzed by mass spectrometry on peptide digests.

MALDI-TOF analysis on chymotryptic peptides derived from the 36-kDa intestinal lectin is summarized in Table I. Nineteen predicted peptide masses for Gal-3TM1, including seven from the TM1 region, were found to match with experimental peaks and together comprise a 41% coverage of this sequence. When the Gal-3TM1 sequence was added to the NCBI data base, an MS-Fit search of all chicken proteins of 25- to 40-kDa found Gal-3TM1 to yield the highest number of peptide masses and the second highest MOWSE (molecular weight spectrometry) score. Galactin-3 was ranked 9th based on the number of peptide masses matched with the 36-kDa lectin band. In a similar way (results not shown), the 16-kDa kidney lectin (Fig. 6, right panel) was identified as CLL I (16-kDa lectin, CG-16; accession number P23668) (48). Also, the peptide mass spectrum and size of the 14-kDa intestinal lectin are consistent with that predicted for CLL II (C-14 lectin, accession number AAA48779) (not shown). CLL I and CLL II proteins share a 46% sequence identity, which would explain immunostaining of the intestinal 14-kDa band (Fig. 6A). MALDI-TOF results for the 36-kDa lectin, along with its analogous molecular weight, immunoreactivity with anti-chicken CLL I antibodies, and lactose-binding functional activity, establish its identity as Gal-3TM1.

To determine the intracellular distribution of Gal-3TM1, chicken osteoclasts were immunofluorescently stained with anti-chicken CLL I antibody #1 (Fig. 7). Confocal microscopy of unpermeabilized osteoclasts revealed that Gal-3TM1 antigenicity was localized at the plasma membrane (Fig. 7, A and B, arrow). Double staining of permeabilized osteoclasts with antibody and with 4',6-diamidino-2-phenylindole also reveals that the Gal-3TM1 content of chicken nuclei (Fig. 7D, arrow) is low relative to that in the cytoplasm (Fig. 7C, arrow). Clear spherical areas devoid of galectin-3TM1 antigenicity are presumed to represent intracellular vacuoles (Fig. 7, B–D, arrowheads). This situation is in contrast to that for galectin-3 in rat osteoclasts and may be due to the TM1 insert. Galectin-3 is clearly enriched in the nuclei of rat osteoclasts, which appear to actively resorb bone in the marrow ablation model (Fig. 7F). The latter distribution pattern was also observed in proliferating fibroblasts (38). Rat osteoclasts were defined on the basis of TRAP staining, size, multinuclearity (n > 2), and apposition to surfaces of bone trabeculae.

Encoded TM1 Protein Sequence Is Hydrophobic in Character—Fig. 2 compares the predicted protein sequences of chicken galectin-3TM1 and galectin-3TR1 with that for chicken and human galectin-3 (27, 28). To gauge the potential significance of the larger alternatively spliced insert sequence, the TM1 domain was scanned for characteristic protein motifs. The existence of a single transmembrane-spanning region encompassing residues 142 to 166 of Gal-3TM1 (represented by the horizontal line in Fig. 8) was predicted with the N terminus distributed intracellularly. Peak hydrophobicity values for this transmembrane segment approached those obtained for the transmembrane helices in bacteriorhodopsin (not shown) (49). Importantly, analyses of the chicken galectin-3 sequence (regions outside the two vertical bars in Fig. 8) did not yield a positive hydrophobicity score. Comparisons of the Gal-3TM1 sequence against the PROSITE data base also identified two overlapping sequences (residues 173–194 and 180–201), which fulfill the criteria for a leucine zipper motif containing a total of four consecutive leucine residues spaced seven residues apart. No recognizable homeodomain or basic DNA binding region is associated with this domain. The presumptive leucine zipper motif lies within the TM1 insert shared by Gal-3TM1 and Gal-3TR1 (Fig. 8). Gal-3TM1, but not Gal-3TR1, also contains a variant of the conserved NWGK motif (residues 261–264), which is required for apoptotic activity by bcl-2 family members (25, 50).

DISCUSSION

The data presented here support the following conclusions. First, highly purified chick osteoclasts produced in vivo and osteoclasts produced by differentiation of marrow precursors in culture exclusively express two new alternatively spliced mRNA forms of chicken galectin-3. These forms each contain a 210-bp TM1 open reading frame but are distinguished by the presence or absence of an additional 241-bp TR1 insert sequence. TR1 encodes a stop codon and is predicted to yield a truncated Gal-3TR1 protein lacking a complete carbohydrate recognition domain. Second, Gal-3TM1 and Gal-3TR1 messages were also identified in chick intestinal tissue, but not in kidney, liver, or lung. Third, the results of homology searches with these sequences indicate that the 70-residue TM1 insert contains a single transmembrane-spanning region followed by a leucine zipper stalk domain, which is predicted to position the carbohydrate recognition domain of Gal-3TM1 on the exterior face of the plasma membrane. Fourth, the presence of a 36-kDa Gal-3TM1 protein in osteoclast and intestine homogenates was
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The table depicts results of MS-Fit analysis (Protein Prospector program) with a maximum number of three missed cleavages. The sum of peptides listed in the table represents 41% of the total Gal-3TM1 sequence.

| Predicted mass/charge | Actual mass/charge | Gal-3TM1 peptide sequences matched | Modifications<sup>a</sup> |
|-----------------------|--------------------|-----------------------------------|--------------------------|
| 1079.4706             | 1079.6213          | KVAVDALHLL (residues 292–301)     | 1 Cys-a                  |
| 1152.5195             | 1152.5182          | AMGTTGVCGL (145–155)<sup>b</sup>  | 2 Met-ox, 1 Cys-a        |
| 1227.5556             | 1227.5437          | MQAMKARCW (1–9)                   |                          |
| 1299.5830             | 1299.5713          | SSSSLCLMAGTW (138–150)<sup>b</sup> | 1 Met-ox                |
| 1315.7818             | 1315.5682          | SSSSLCLMAGTW (138–150)<sup>b</sup> | 1 Met-ox                |
| 1456.2517             | 1455.6435          | CLAMGTTGCGL (143–155)<sup>b</sup> | 1 Met-ox, 2 Cys-a       |
| 1616.2825             | 1615.8597          | KVAVDALHLLFNF (292–305)           |                          |
| 1638.2489             | 1637.8494          | CIAIDITLSVTMLTM (317–332)         |                          |
| 1688.3033             | 1688.7612          | MQAMKARCQPHW (1–13)               | 1 Met-ox                |
| 1881.5241             | 1880.8015          | GCGMSHMSHMSHPSPCL (156–173)<sup>b</sup> | 2 Met-ox, 1 Cys-a      |
| 1951.5182             | 1951.8386          | GCGMSHMSHMSHPSPCL (156–173)<sup>b</sup> | 2 Met-ox, 2 Cys-a      |
| 2219.7397             | 2220.1170          | NPRFKEHDHRVIVCNMF (241–258)       |                          |
| 2237.7230             | 2236.9897          | GCVSLGCMSSHMSHPSPCL (151–173)<sup>b</sup> | 1 Met-ox                |
| 2274.7615             | 2248.0756          | KEDHRVIVCNMFQGNNW (245–262)       |                          |
| 2291.7999             | 2291.5141          | NPRFKEHDHRVIVCNMF (241–258)       |                          |
| 2343.8224             | 2344.2811          | MPRLLITITTVSNPSNFSL (208–228)     | 1 Cys-a                  |
| 2359.8738             | 2360.2760          | MPRLLITITTVSNPSNFSL (208–228)     | 1 Met-ox                |
| 2449.9489             | 2450.2486          | MQAMKARCCQPHWMLPLPL (1–20)        |                          |
| 2449.9489             | 2450.1010          | GCVSLGCMSSHMSHPSPCL (151–173)<sup>b</sup> | 3 Cys-a                  |
| 2629.0151             | 2629.4962          | REKKLEITKLICADITLSVLL (306–328)   | 1 Cys-a                  |

<sup>a</sup> Met-ox and Cys-a represent oxidized methionine and acrylamide-modified cysteine residues, respectively.

<sup>b</sup> Peptides contained entirely within the TM1 insert region.

**FIG. 7.** Comparison of representative immunostaining patterns of Gal-3TM1 and galectin-3 with chicken and rat multinucleated osteoclasts, respectively. A and B, confocal image of non-permeabilized, chicken osteoclast displays predominantly plasma membrane staining for Gal-3TM1 (green). A, vertical optical slice from mid-region of cell. B, vertical optical slice from the same cell representing surface-attached, basal membrane region. Arrow, exterior plasma membrane face; arrowhead, clear areas are presumed to be vacuoles. Original magnification, ×1000. C and D, permeabilized chicken osteoclast exhibits intracellular and peripheral membrane staining, but not intranuclear or vacuolar staining for Gal-3TM1. C, permeabilized, immunofluorescently stained osteoclast (green). D, the same osteoclast with intranuclear 4′,6-diamidino-2-phenylindole-stained DNA signal (light blue) overlaid upon immunofluorescent image (green). Arrow. individual nucleus; arrowhead, vacuole. Original magnification, ×600. E, negative, double-stained control. Non-immune serum was substituted for anti-galectin antibody and used in an identical immunofluorescence protocol with chicken osteoclasts, which were also stained with phalloidin-Texas Red for identification. The image shows a representation of a control immunofluorescent signal (green) and Texas Red (red) signals. Original magnification, ×1000. F, multineurated rat osteoclasts in marrow-ablated bone colorimetrically stained for galectin-3 antigen (black) and TRAP. The large arrows denote an osteoclast whose five nuclei are preferentially immunostained for galectin-3. Scale bar, 50 μm. Note: A–E were stained with rabbit anti-chicken CIL I antibody #1; F was stained with goat anti-rat galectin-3 (see “Methods” for more details).

**FIG. 8.** Hydrophobicity plot of galectin-3TM1 sequence identifies presumptive transmembrane spanning domain. The hydrophobicity of the Gal-3TM1 sequence was analyzed by TMpred program (35), and the results are plotted with the N terminus on the left side. The heavier tracing reflects the preferred model with the N terminus inside. The vertical bars on the graph denote the limits of the 70-residue TM1 insert domain. The horizontal bar on the figure indicates the position of the predicted single transmembrane-spanning region involving residues 142–166; an alternative overlapping region (residues 134–157) exhibited a slightly lower hydrophobicity score.

confirmed on the basis of its size, its purification via lactosyl-Sepharose chromatography, reactivity with antibodies cross-reactive with chicken galectin-3, and its peptide masses which matched those predicted for the TM1 insert and other regions. Confocal immunofluorescent staining of chicken osteoclasts confirmed the expression of Gal-3TM1 at the plasma membrane. Gal-3TM1 is the first example of a galectin family lectin capable of being expressed as a soluble protein and as a transmembrane protein with its carbohydrate recognition domain expressed on the exterior face of the plasma membrane (or the luminal side of intracellular membrane systems). The presence of a leucine zipper-like motif provides a potential mechanism to form homo- and/or heterodimeric complexes at these sites.

Although the work of Niida et al. (18), Colnot et al. (51), as well as results presented here, clearly demonstrate the presence of galectin-3 protein in human, mouse, and rat osteoclasts in vivo, this report is the first to identify and detect the expression of alternative splice forms Gal-3TM1 and Gal-3TR1 by osteoclasts isolated from calcium-deficient chickens. This distinctive expression pattern and the role of osteoclasts and...
intestinal cells in regulation of serum calcium raises the possibility that Gal-3TM1 and Gal-3TR1 may have been induced by exposure to calcium deficiency in vivo. For example, calcitonin receptor expression on chicken osteoclasts seems to depend upon the serum calcium level of the birds used to prepare the osteoclasts. Calcitonin receptors can be demonstrated biochemically and/or functionally on osteoclasts from deficient hosts (32, 52). However, osteoclasts isolated from chickens fed a normal calcium diet do not express calcitonin receptors or respond to salmon calcitonin (53). Additional studies will be necessary to test this rationale for Gal-3TM1 regulation.

Definition of Gal-3TM1 and Gal-3TR1 as alternatively spliced messages is based upon positioning of insert sequences at conserved exon-intron junctions for the mouse and human Lgals3 genes (45, 46) and the open reading frame of the TM1 insert sequence. Also, the size of intron sequences in the mouse Lgals3 gene range from 409 to 1404 bp (46), which are all longer than either of the 210- to 241-bp TM1 and TR1 insert sequences. Average chicken intron size is comparable to that for mammalian species (54). Finally, support also comes from the fact that the low affinity IgE Fc receptor (CD23), itself a C (calcium-dependent)-type lectin, is expressed as a soluble and transmembrane domain containing alternatively spliced forms (55).

Searches of GenBank™ indicate that Gal-3TM1 is related to other transmembrane proteins. First, the TM1 sequence shares a 27–40% homology with several membrane transporter and channel proteins, e.g., the kidney urea transporter (56), skeletal muscle chloride channel (foot;f3;10), chicken rhodopsin (58), and the organic anion transporter polypeptide-related protein 3 (foot;f3;10). These homologies do not extend to the adjacent chicken galectin-3 sequence indicating the region of similarity is restricted primarily to the 70-residue TM1 insert. Second, similar sequence comparisons with other galectin super-family members and C-type lectins, including the liver asialoglycoprotein receptor, cd69, cd23 low affinity IgE Fc receptor, ly49c, murine C-type macrophage lectin, trout lectin (60), and ngk2-A failed to reveal significant homologies. Regardless, the latter type II transmembrane C-type lectins provide a useful structural paradigm (61, 62), because, like galectin-3, they do not contain N-terminal signal sequences, yet they possess a signal-anchor domain that acts as an internal signal sequence (63). In this way, we predict that Gal-3TM1, like the trout C-type lectin (60), exhibits a type II transmembrane topology with an N-terminal cytosolic domain, transmembrane domain, a leucine zipper stalk domain, and an extracellular C-terminal carbohydrate recognition domain.

Although secreted via a non-classical ER/Golgi-independent mechanism (21), galectin-3 is known to be associated with cell surfaces. The mechanism is thought to depend upon the propensity of galectin-3 to dimerize. The N-terminal half of galectin-3 has been shown to self-associate with itself; this domain is primarily composed of unusual Gly- and Pro-rich repeats (64). Because galectin-3 can be released from cells like macrophages with lactose, it has been assumed that galectin-3 dimers associate with cell surface glycoproteins with one of their two carbohydrate recognition binding sites. Recognition of extracellular lactosyl ligands is believed to be mediated by the remaining free binding site. For example, binding of IgE to macrophages and mast cells via galectin-3 is inhibited by lactose, whereas galectin-3 itself can be released by lactose (23). We speculate that the structure of Gal-3TM1 may have several functional implications. By forming homodimers via their shared leucine zipper domains, the moderate affinity of individual Gal-3TM1 carbohydrate recognition domains would be effectively increased through a multiplicity effect. The presence of a cytoplasmic N-terminal tail containing the Gly/Pro repeats also offers a mechanism to regulate the density, distribution, or lifetime of cell surface Gal-3TM1 receptors via interactions with the cytoskeleton.

Previous studies of lactose-binding lectins showed that galectin-4 (36 kDa), galectin-6 (33 kDa), galectin-9 (36 kDa), and CLL II (C-14) are expressed in intestine (57, 65–66). Studies by Iglesias et al. (42) and Beyer et al. (57) have demonstrated that chicken CLL I and CLL II are structurally and functionally distinct. Previous studies in chickens have not identified higher molecular weight forms of galectin-3 in intestine. We suggest that this may be due to prior use of adult intestinal tissue, which, in contrast to the 4-week-old calcium-deficient chick tissue used here, may not express Gal-3TM1 or Gal-3TR1.

Evidence for a functional role for osteoclast galectin-3 or Gal-3TM1 remains indirect. Analyses of osteoclastogenesis in culture have shown that galectin-3 is expressed by mononuclear osteoclast precursor cells prior to that for TRAP and calcitonin receptor (59), however, expression drops after formation of mature osteoclasts relative to precursor cells (34). Colucci et al. (15, 16) showed that osteoclast binding to laminin, which specifically recognizes galectin-3, occurs by means of an RGD-independent mechanism, but they did not test the effect of lactose. Also, Kukita et al. (17) found that surface-adsorbed laminin, like BAG-75 from bone matrix (14), was able to block osteoclast differentiation in rat bone marrow cultures. We suggest that Gal-3TM1 on chick osteoclast precursor cell surfaces participates in attachment to the substratum during migration across the vascular wall to bone and/or in recognition of partners during fusion into osteoclasts upon reaching bone.

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4 Recent quantitative studies show that purified rat galectin-3 binds as well to isolated bone acidic glycoprotein-75 as to monoclonal anti-DNP IgE, the commonly used standard ligand for this receptor. Binding is completely blocked in both cases by lactose but not sucrose (J. P. Gorski, F.-T. Liu, and P. Osdoby, unpublished results).
New Alternatively Spliced Form of Galectin-3, a Member of the β-Galactoside-binding Animal Lectin Family, Contains a Predicted Transmembrane-spanning Domain and a Leucine Zipper Motif
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