Membrane Topology of Bves/Pop1A, a Cell Adhesion Molecule That Displays Dynamic Changes in Cellular Distribution during Development*

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We investigated the membrane topology of Bves/Pop1A as a foundation to dissect the molecular basis and function of Bves/Pop1A trafficking during development. Bves contains two asparagine-linked glycosylation sites within the amino terminus and three putative membrane domains. Therefore, glycosylation assays were performed to determine if the amino terminus of Bves is delivered into the endoplasmic reticulum lumen and glycosylated. We establish that Bves from chick heart and transfected cells is glycosylated, implying that the amino terminus of cell surface molecules is extracellular. Three biochemically distinct approaches were utilized to determine the orientation of the carboxyl terminus of Bves. First, glycosylation of Bves at exogenous sites within the carboxyl terminus was only observed in a construct that lacked the third membrane domain, which presumably reversed the orientation of the carboxyl terminus. Second, co-expression of full-length Bves with soluble, carboxyl-terminal Bves constructs that reside in different subcellular compartments revealed that Bves-Bves interactions occur in the cytoplasm. Third, the immunoreactivity of endogenous Bves at the cell surface of epicardial cells was dramatically enhanced with detergent. These results suggest that the membrane topology of cell surface Bves/Pop1A is composed of an extracellular amino terminus, three transmembrane domains, and a cytoplasmic carboxyl terminus. We therefore hypothesize that the carboxyl terminus regulates the cellular distribution of Bves/Pop1A during coronary vessel development.

Bves (blood vessel epicardial substance) was identified from a subtractive cDNA screen to identify clones enriched in heart tissue (1). Chick Bves is a 357-amino acid protein that contains two consensus asparagine-linked glycosylation sites in the amino terminus, three hydrophobic regions that are potential membrane domains, and a large 247-residue carboxyl terminus. Antibodies against chick Bves have labeled the primordial origin of coronary vessels, the proepicardial organ, the epicardium that surrounds the heart, migrating mesenchymal cells derived from the epicardium, and smooth muscle (1). Interestingly, the cellular distribution of Bves changes dynamically from the cell surface in epicardium to a more intracellular location in migrating mesenchyme and later reappears at the cell surface in vascular smooth muscle (2). Furthermore, it has been proposed that Bves functions as a cell adhesion molecule based on the results of transfected 1-cells (2). Thus, we sought to elucidate the membrane topology of Bves to distinguish intracellular regions that may be involved in this dynamic, intracellular trafficking from extracellular regions that may directly participate in cell-cell interactions.

The Popeye family of transcripts was independently identified and found to be highly expressed in cardiac and skeletal muscle (3). Two Popeye genes were identified in chick (POP1 and POP3). In addition, four alternatively spliced chick POP1 transcripts were isolated (POP1A through -1D), and POP1A was found to be identical to Bves (3). Although POP1 knockout mice did not exhibit an overt phenotype, treatment of mice that lack POP1 with cardiotoxin displayed delayed skeletal muscle regeneration relative to wild-type mice (4).

Establishing the membrane topology of Bves/Pop1A would provide a foundation for identifying and characterizing functional domains at the cellular level. The membrane topology of Bves/Pop1A is unknown, but two distinct models have been proposed (2, 4). Although both groups assumed that all three hydrophobic domains span the membrane, their models present different locations of the termini. Wada et al. (2) proposed that the amino terminus is cytoplasmic and the carboxyl terminus is extracellular. In contrast, Andrée et al. (4) proposed that the amino domain is extracellular and the carboxyl domain is intracellular. Here, we document that the amino terminus of Bves/Pop1A from chick heart and transfected cells is glycosylated at asparagine-linked sites. Furthermore, the orientation of the carboxyl terminus was examined using exogenous glycosylation sites, Bves-Bves interactions, and immunocytochemistry. Our results support a three transmembrane model of Bves/Pop1A in which the amino terminus of cell surface molecules is exposed to the extracellular environment, whereas the carboxyl terminus is exposed to the cytoplasm.

EXPERIMENTAL PROCEDURES

Chick Bves cDNA and Mutants—Bves/POP1A was subcloned into the Xhol-Sall sites of pCI-neo (Promega, Madison, WI). PCR was used to add epitope tags and generate mutants. The reagents consisted of the plasmid containing chick Bves/POP1A, recombinant Ftu polymerase (Stratagene, La Jolla, CA), and the appropriate primers (Invitrogen, Carlsbad, CA). The primers used to generate all Bves constructs described in this study are listed in Table I, and a schematic of representative constructs is illustrated in Fig. 1. The FLAG epitope (DYKD-DSDK) was added to the extreme carboxyl terminus, yielding Bves-FLAG. The HA1-epitope tag (YPYDVPDYA) was incorporated into the

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‡ The abbreviations used are: HA, hemagglutinin; TBS, Tris-buffered saline; WT, wild-type.

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amino terminus between Leu220 and Lys229 of Bves/Pop1A, yielding HA-
Bves. HA-Bves-FLAG was used to compare the immunoreactivity of anti-
anti-FLAG (M2; 1:1000), polyclonal anti-HA (1:250), and monoclonal
Canada.
provided by Dr. Hoda Eid at the University of Ottawa, Ottawa, Ontario,
from the ATCC. The rat epicardial cell line EMC (7, 8) was kindly
blasts, and mouse skeletal muscle precursor C2C12 cells were obtained
determined at the DNA Core in the Stahlman Cardiovascular Research
site at Asn39.2 PCR fragments were digested and ligated into Bves
cleavable signal sequence and an endogenous Asn-linked glycosylation
Met128-FLAG was made with primers that correspond to the end of the
sense, containing a portion of the coding region that starts at Met128,
construct Met128-FLAG was generated with primers Xho-Met128
rCNT1 insert to Ala yielded rCNT1-N/A. The carboxyl-terminal Bves
the construct labeled rCNT1. Mutation of the Asn residue within the
cotransporter (5), was used to replace amino acids 117
amino terminus between Leu27 and Lys28 of Bves/Pop1A, yielding HA-
5′-nucleoside cotransporter (5), was used to replace amino acids 117
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**TABLE I**
Sequences of primers used to generate Bves constructs

| Primer | Direction | Sequence (5′ → 3′) |
|--------|-----------|-------------------|
| pClneo (1009) | Sense | CTTTCTCCTCC ACAGGTGTCG AACTCCAGATG CA |
| Xho-5′-cBves | Sense | AGGCTTCGAGA CAGCGGGGACA GAGATCCTCA AGATG |
| BioEI (640) | Antisense | ATCACTTGCG ATATGCTGC GGTCATCTCA AGT |
| EcoRI (280) | Antisense | CTGGACCTAT ATGCTGTCG ATTCCTGATT TG |
| cBves-FLAG-Sal-3′ | Antisense | GGTCGACTCT ATATTGCTG ATGTCGTCG TCGATG |
| cBves-Sal-3′ | Antisense | CTGTCATCG TCAGTGCAGT TTAAAGCGG CTGGTCGAGC |
| N20A | Sense | GTATTCCGAC ACTTAAAGGC TGGACCATCT GTGCTTTCTC |
| N20A | Antisense | AGAAAGGGAC AGGTTGGGCA CTTTTAGATC TGGAATAGT |
| N27A | Sense | GCACTCTGCT GTTCCCTCCT TAACCAAACT GCCCTTTGATC GACAGCTTCT |
| N27A | Antisense | AGCCCTGACG AGAAGAAGTG AGAAACACT CATGGTCCCT CA |
| HA-5′f short | Antisense | CCGTATGCGA GAGGGCTGAT AGGATAGATA TACCAAGTTC GACAGCTTCT |
| HA-3′f short | Sense | TATGCGAATG ATCGACCTCC TGGCCTTCTC AAC |
| HA-3′f long | Sense | TATCCTTACG ACGTTCCTGA CTATGCGAAA AATGCCACCT CTGTTG |
| KE1/SNETS (119) | Sense | TCTCTTTGAG ACCTGGCTTG TCCTATCTCT TAATCTGATC GTGCTG |
| KE1/SNETS (119) | Sense | AGACGGTAGG CAGATTGAGG TGGACAGCG GCGGATACAG CAT |
| MFEP/MINLT (129) | Antisense | TGGACGAGCA TGGATGCTTT CATTCTCTTC TGGATAGATC CT |
| MFEP/MINLT (129) | Antisense | AGCCCTGACG AGAAGAAGTG AGAAACACT CATGGTCCCT CA |
| QRLT7QNL7 (141) | Antisense | GCAGATGTTG CGAATTGGAG TGGAAATAGT CTTGCGGAGC AC |
| QRLT7QNL7 (141) | Sense | GTGCCTGGAG AGCATTATCC AAACCTTAC GACAGCTTCT |
| EDKT/KNET (162) | Antisense | CCGTACATG ACTGATGTTC ATATGCGAAA AATGCCACCT CTGTTG |
| EDKT/KNET (162) | Sense | GTTCAAGCTT ATGCTGCAAA GAATGAAACA TCAGTTGATG ACAGG |
| GEK/NET (209) | Antisense | ATAGTGGACC TGATAGCTTG CATCTGATGCT TATTGCGTAC |
| cBves (280) | Sense | TATCCTTACG ACGTTCCTGA CTATGCGAAA AATGCCACCT CTGTTG |
| cBves (280) | Sense | TATCCTTACG ACGTTCCTGA CTATGCGAAA AATGCCACCT CTGTTG |
| rCNT1 5′f short | Antisense | GCCAAGATGC CGAATTGGAG TGGAAATAGT CTTGCGGAGC AC |
| rCNT1 3′f short | Sense | AACAACACTG TCCATATCC GAGAATATCC GGGCATCAGC AT |
| rCNT1 3′f short | Sense | AACAACACTG TCCATATCC GAGAATATCC GGGCATCAGC AT |
| rCNT1 3′f short | Sense | AACAACACTG TCCATATCC GAGAATATCC GGGCATCAGC AT |
| D5T 5′f | Antisense | GAT CAGGCTTC TCTGGTACG AGCCTATGATG CCAAAATGAT GAACAATGC |
| D5T/CNT1 3′f | Sense | TGGGCTCAGC TCAGAGACAG GCTAAAGGG TGGGAGATG |
| rCNT1-N/A | Sense | GTCGAGATGG AAGCGGTCTG CTCCCTCCTC CAAACTTGGC TA |
| rCNT1-N/A | Antisense | TAGGACAGTT TGGGACAGGA GGGACGGCG TCTTCTCTCC AC |
| Xho-Met128 | Sense | CCTGCAGATG ATGCTGACCC CTCTGGGAGC |
| Xho-Met128 | Sense | CCGTGACGAA CTAGTGGTCA GCTGTCAA TC |
| 2C/Met128-5′f | Antisense | TGCTCACGAA ATATGCGGG ACGCTAAGCA GGGCCACCA TC |
| 2C/Met128-3′f | Sense | CGATCCCGAG ATATGTTGGA GCCATCCCAT GTGCTCAGG AG |

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2 J. R. Backstrom, unpublished data.

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**Extraction of Protein from Cells**—Detergent-soluble protein was extracted from cells as described previously (6). Protein concentrations were estimated with the BCA assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the protein standard. Blots of 30 μg samples were incubated overnight at 37 °C for 3 h. A 4× solution of SDS-sample buffer (250 mM Tris, pH 6.8, containing 8% SDS, 40% glycerol) was added to each tube, and protein concentrations were estimated with the BCA assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the protein standard. Blots of 30 μg samples containing 1% 2-mercaptoethanol were probed with anti-Bves (B846) and immunoreactive bands detected with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Pierce).

**Transfection of Cells**—Cells were co-transfected with DNA prepared in LipoFectAMINE (Invitrogen). The DNA solution was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and incubated overnight at 37 °C in a humidified chamber containing 5% CO2. NIH/3T3 cells were electroporated with 2 μg of DNA (6) and grown as per the COS and C2C12 cells. For the experiments that involved preventing Asn-linked glycosylation in COS cells, the DNA solution was replaced with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 0.2 μg/ml tunicamycin (Calbiochem, La Jolla, CA). Cells were washed once with Hank’s buffered saline solution containing CaCl2 and MgCl2 (Invitrogen) before extracting protein.

**Extraction of Protein from Cells**—Detergent-soluble protein was extracted from cells as described previously (6). Protein concentrations were estimated with the BCA assay (Pierce).

**Biotininylation of Cell Surface Protein**—COS cells transfected with either Bves-FLAG or rat 5-HTg receptor cDNA (negative control) were incubated with 0.5 mM NHS-LC-biotin (Pierce) in Hanks’ buffered saline solution as per the manufacturer’s protocol. Protein was immunoprecipitated with anti-FLAG and subjected to electrophoresis under reducing conditions. Separate lanes were probed with either anti-FLAG to detect total cellular protein or with 0.5 μg/ml streptavidin-phosphatase (Pierce) to detect cell surface protein.

**C-terminal Localization**—COS cells were co-transfected with 4 μg of HA-Bves and 4 μg of either Met128-FLAG or 2C/Met128-FLAG. Anti-FLAG (2 μg) was added to tubes containing 1% Triton-soluble protein.

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**Analysis of Bves from Chick Heart**—Chick hearts from day 6 embryos were used to examine glycosylation of Bves. Cell extracts prepared essentially as described (6) were treated with buffer or recombinant N-glycosidase F (Glyko, Novato, CA) and incubated at 37 °C for 3 h. A 4× solution of SDS-sample buffer (250 mM Tris, pH 6.8, containing 8% SDS, 40% glycerol) was added to each tube, and protein concentrations were estimated with the BCA assay (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as the protein standard. Blots of 30 μg samples containing 1% 2-mercaptoethanol were probed with anti-Bves (B846) and immunoreactive bands detected with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Pierce).
Fig. 1. Schematic of representative Bves constructs used in this study. All constructs were tagged with either the HA epitope in the amino terminus (triangle) and/or the FLAG epitope (FLAG) at the extreme carboxyl terminus. The "Y" in the amino terminus corresponds to the consensus Asn-linked glycosylation sites at Asn20 and Asn27. Both glycosylation sites were mutated (N20A/N27A; small oval) to analyze glycosylation at exogenous sites within the carboxyl terminus. The 14-amino acid rCNT1 insert (small box) was based on a glycosylated region of the rat concentrative Na+/nucleoside cotransporter (5). The three putative membrane domains are denoted with large boxes. Deletion of the third membrane domain yielded constructs denoted as "D3T." The carboxyl terminus is predicted to begin near Lys311, and the Met128-FLAG construct starts at Met128. The chimeric construct 2C/Met128 contains the amino terminus of the rat serotonin 5-HT2C receptor fused to Met128-FLAG. The 2C region contains an endogenous Asn-linked glycosylation site. Details are described under "Experimental Procedures.”

and incubated with rocking at 4 °C. After 4 h, 20 μl of anti-mouse Protein G beads (1:2 slurry) was added. The beads were prepared from cross-linking goat anti-mouse IgG to Protein G at a final concentration of 3.2 mg of antibody/ml of beads using Seize-X (Pierce). After an overnight incubation with rocking at 4 °C, supernatants were discarded, and the beads were washed three times with TBS containing 0.1% Triton X-100. Protein was eluted from the beads with 50 μl of SDS-PAGE sample buffer containing 1% 2-mercaptoethanol and subjected to incubation in a 95 °C water bath for 1 min. Two blots were used to analyze 20-μl aliquots of each sample elution (40% of total) and a sample of starting extract (4% of input).

Immunocytochemistry—Cells grown in 8-well chamber slides were either fixed and permeabilized with Histochrome MB (Amresco, Solon, OH) or fixed with 1% paraformaldehyde in phosphate-buffered saline followed by permeabilization with 0.1% Triton X-100 in TBS. The wells were washed once with 2% bovine serum albumin in TBS and blocked with the same solution overnight at 4 °C. Cells were treated with primary antibodies followed by the appropriate secondary antibodies, each for 1 h at room temperature. After three washes with TBS and two washes with water, slides were dried and coveredslipped with Aqua Polymount (Polysciences, Inc., Warrington, PA). Fluorescent images were captured using an Olympus BX60 microscope in the Stahlman Cardiovascular Research Laboratories.

RESULTS

Glycosylation of Bves from Chick Heart—Chick Bves/Pop1A contains two consensus Asn-linked glycosylation sites within the amino terminus (Asn20 and Asn27; Fig. 1). Because these are the only Asn-linked sites (Asn-X-Ser/Thr), evidence of glycosylation would establish that the amino terminus of Bves reached the lumen of the endoplasmic reticulum, which corresponds to the extracellular environment of cell surface molecules. Thus, extracts prepared from chick heart were incubated in the absence or presence of N-glycosidase F. Protein was subjected to SDS-PAGE and blots were probed with anti-Bves antibodies (B846). In the absence of N-glycosidase, a diffuse pattern of bands were detected with masses of 56–58 kDa (Fig. 2, lane 1). In contrast, N-glycosidase decreased the size of immunoreactive bands to 43 kDa (lane 2), which is similar to the predicted mass of 41 kDa. An additional minor band was also observed at 50 kDa, which may represent a partially deglycosylated form of Bves.

Glycosylation of the Amino Terminus of Bves from Transfected Cells—Glycosylation of Bves was next examined from transfected COS kidney epithelial cells (Fig. 3, top panel), NIH/3T3 fibroblasts (middle panel), and skeletal muscle precursor cells (bottom panel). Wild-type (WT) Bves displayed a predominant immunoreactive band with a mass of 47 kDa (lane 1). Point mutations were made at either one or both consensus Asn-linked sites. Whereas the N20A (lane 2) and N27A (lane 3) mutations produced major bands with masses of 45 kDa, mutation of both sites (N20A/N27A, lane 4) yielded a major band at 43 kDa. These results from transfected cells and chick heart establish that the amino terminus of Bves is glycosylated.

Glycosylation at Exogenous Sites within the Carboxyl Terminus of Bves—The glycosylation studies were extended to examine the possibility that the carboxyl terminus of Bves also reaches the lumen of the endoplasmic reticulum. To ensure that this assay would indicate glycosylation at an exogenous site, the endogenous sites within the amino terminus were mutated to alanine (N20A/N27A; Fig. 1). Exogenous glycosylation sites (Asn-Glu-Thr) or (Asn-Ala-Thr) were introduced at five different positions within the carboxyl terminus (Fig. 4A). An additional construct (N20A/N27A/rCNT1) was created that contains a 14-amino acid insert based on a region of the carboxyl terminus of rCNT1 that contains a functional Asn-linked glycosylation site (5). An increase in size was not observed with any of the constructs containing an exogenous site (Fig. 4B). Thus, either the structure of Bves prevented glycosylation or the carboxyl terminus is exposed to the cytoplasm.

We explored the possibility that removing the third membrane domain of Bves would reverse the orientation of the
carboxyl terminus and direct it into the endoplasmic reticulum. Our efforts focused on constructs that contain the rCNT1 insert for these sets of experiments. A D3T/rCNT1 construct was created that lacks the predicted third membrane domain (Figs. 1 and 5A) and transfected into COS cells. Deletion of the third transmembrane domain yielded bands on immunoblots with masses of 41 and 43 kDa (Fig. 5B, lane 3). The 2-kDa difference in mass was consistent with the results obtained for each site in the amino terminus of wild-type Bves (lane 1). Although the D3T/rCNT1 construct lacks endogenous glycosylation sites within the amino terminus, two strategies were employed to confirm that formation of the minor, 43-kDa band of D3T/rCNT1 construct lacks endogenous glycosylation sites within the carboxyl terminus, two strategies were employed to confirm that formation of the minor, 43-kDa band of D3T/rCNT1 construct was due to glycosylation of the 41-kDa protein. First, cells were grown in the presence of the Asn-linked glycosylation inhibitor tunicamycin to prevent glycosylation. Tunicamycin prevented the appearance of the larger immunoreactive bands in D3T/rCNT1 (lane 4) as well as in wild-type Bves (lane 2). Second, the Asn residue within the rCNT1 insert was mutated to Ala (D3T/rCNT1-N/A). Whereas two bands were detected from D3T/rCNT1 (Fig. 5C, lane 2), only a single immunoreactive band was observed from D3T/rCNT1-N/A (lane 3), confirming that the Asn residue within the rCNT1 insert provided a functional glycosylation site. These results provide evidence that the carboxyl terminus of wild-type Bves is located in the cytoplasm, and removal of the third membrane domain reverses its orientation into the endoplasmic reticulum lumen.

**Surface Biotinylation of Bves**—Biotinylation experiments were performed to determine whether Bves reaches the cell surface of COS cells. Cells expressing either Bves-FLAG or a negative control construct were incubated with NHS-LC-biotin to label cell surface protein. Cell extracts were incubated with anti-FLAG antibodies and anti-mouse beads. Precipitated proteins were electrophoresed, and the resulting blots were probed with either anti-FLAG to detect total cellular Bves (Fig. 6, lane 1) or streptavidin-phosphatase to detect cell surface Bves (lane 2). These results document that Bves is localized primarily within intracellular compartments of COS cells, whereas only low levels are present at the cell surface. Therefore, approaches to determine the membrane topology of Bves in COS cells examine properties of Bves within intracellular compartments.

**Oligomerization of Bves**—During the course of characterizing immunoreactive Bves on immunoblots, we found that the banding pattern of Bves derived from cell extracts electrophoresed under reducing versus non-reducing conditions was different. Under reducing conditions (1% 2-mercaptoethanol), a predominant, monomeric form of Bves-FLAG was observed (Fig. 7, lane 1). Under non-reducing conditions, several higher molecular mass forms of Bves-FLAG were observed in addition to the monomeric form (lane 1). The amount of monomer from both electrophoretic conditions was compared with indirectly estimate the relative level of oligomers. The monomer from 4 μg of protein run under non-reducing conditions displayed equivalent immunoreactivity to that from 1 μg of protein run under reducing conditions (data not illustrated). These results imply that ~80% of the Bves protein from COS cells exists in an oligomeric form. Similar results were obtained from C2C12 skeletal muscle precursor cells transfected with Bves-FLAG (data not illustrated).

**Characterization of Bves Carboxyl-terminal Constructs**—Next, we examined whether oligomerization of Bves occurs within the carboxyl terminus. If so, we could exploit this interaction to determine the orientation of Bves. Similar to the results with full-length Bves-FLAG (Fig. 7, lane 1), a carboxyl-terminal Bves construct that lacks the membrane domains and
and 41 kDa for the N20A/N27A/D3T/rCNT1 mutant (lane 3). For the rCNT1 mutant containing Ala (lane 2), a single band was detected at 41 kDa for the rCNT1 mutant containing Asn (lane 1), or the N20A/N27A/D3T/rCNT1-N/A mutant (lane 3). Tunica-

mycin inhibited generation of the 47- and 45-kDa bands of wild-type Bves (lane 2) and the 43-kDa band of the N20A/N27A/D3T/rCNT1 mutant (lane 2). C, immunoblot of COS cell extracts from cells transfected with wild-type Bves (lane 1), the N20A/N27A/D3T/rCNT1 mutant (lane 2), or the N20A/N27A/D3T/rCNT1-N/A mutant (lane 3) and grown in the absence of tunicamycin. Two bands were observed at 43 and 41 kDa for the rCNT1 mutant containing Asn (lane 2), whereas only a single band was detected at 41 kDa for the rCNT1 mutant containing Ala (lane 3).

Fig. 5. Deletion of the third membrane domain of Bves promotes glycosylation at an exogenous site within the carboxyl terminus. A, carboxyl-terminal region of Bves that illustrates the sequence of the third membrane domain that was deleted in D3T mutants (dashed lines). The Asn residue within the rCNT1 insert (underlined) was mutated to Ala in the D3Tr/CNT1-N/A mutant. B, immunoblot of COS extracts from cells transfected with wild-type Bves (lanes 1 and 2) or the N20A/N27A/D3T/rCNT1 mutant (lanes 3 and 4) and grown in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of tunicamycin. Cells grown in the absence of tunicamycin yielded three bands at 47, 45, and 43 kDa for WT Bves (lane 1) and two bands at 43 and 41 kDa for the N20A/N27A/D3Tr/CNT1 mutant (lane 3). Tunicamycin inhibited generation of the 47- and 45-kDa bands of wild-type Bves (lane 2) and the 43-kDa band of the N20A/N27A/D3Tr/CNT1 mutant (lane 2). C, immunoblot of COS cell extracts from cells transfected with wild-type Bves (lane 1), the N20A/N27A/D3T/rCNT1 mutant (lane 2), or the N20A/N27A/D3T/rCNT1-N/A mutant (lane 3) and grown in the absence of tunicamycin. Two bands were observed at 43 and 41 kDa for the rCNT1 mutant containing Asn (lane 2), whereas only a single band was detected at 41 kDa for the rCNT1 mutant containing Ala (lane 3).

Fig. 6. Cell surface biotinylation of Bves. COS cells were surface-biotinylated, and Bves was immunoprecipitated from cell extracts with anti-FLAG antibodies. Equal volumes of the eluate were used to analyze total cellular Bves (lane 1) or cell surface Bves (lane 2). Only minor levels of Bves reach the cell surface of COS cells. The background was determined from parallel experiments utilizing 5-HT2C receptor cDNA (data not illustrated).

starts at Met128 (Met128-FLAG; Fig. 1) also formed oligomers under non-reducing conditions (lane 2) and monomers under reducing conditions (lane 5).

We postulated that wild-type Bves would interact with a carboxyl-terminal Bves construct if the construct was delivered to the subcellular compartment where the carboxyl-terminal tail of wild-type Bves resides. For example, if the carboxyl terminus of Bves is located in the cytoplasm, it could potentially interact with Met128-FLAG, but not if Met128-FLAG was delivered to the lumen of the endoplasmic reticulum. Thus, a chimeric protein was created that contains the amino terminus of the serotonin 5-HT2C receptor fused to Met128-FLAG (2C/Met128-FLAG; Fig. 1). The 2C region contains an endogenous Asn-linked glycosylation site; therefore, glycosylation of 2C/Met128-FLAG would confirm delivery into the endoplasmic reticulum lumen. Importantly, 2C/Met128-FLAG formed oligomers under non-reducing conditions (Fig. 7, lane 3) and monomers under reducing conditions (lane 6).

Immunocytochemistry was utilized to determine whether the gross cellular distribution of the carboxyl-terminal constructs Met128-FLAG and 2C/Met128-FLAG are clearly different. COS cells were transiently transfected with the Bves constructs and labeled with mouse anti-FLAG followed by Cy3-labeled anti-mouse (Fig. 8). Full-length Bves-FLAG (A) displayed a similar fibrous labeling pattern as the chimeric carboxyl-terminal construct 2C/Met128-FLAG (B). Furthermore, the results from double-labeling experiments revealed that the distribution of Bves-FLAG and 2C/Met128-FLAG were strikingly similar to the endoplasmic reticulum protein calnexin (data not illustrated). In contrast, the carboxyl-terminal construct Met128-FLAG (C) displayed a diffuse labeling pattern consistent with a cytoplasmic location.

Fig. 7. Wild-type and carboxyl-terminal Bves constructs generate oligomers. COS cell extracts containing wild-type Bves or carboxyl-terminal forms of Bves were prepared in sample buffer without (–) or with (+) 2-mercaptoethanol at a final concentration of 1%. The amount of protein loaded from the non-reduced samples was three times more than that from the corresponding reduced samples. In the absence of reducing agent, Bves-FLAG (lane 1) and the carboxyl-termi-

nals of the serotonin 5-HT2C receptor fused to Met128-FLAG would confirm delivery into the endoplasmic reticulum lumen. Importantly, 2C/Met128-FLAG formed oligomers under non-reducing conditions (Fig. 7, lane 3) and monomers under reducing conditions (lane 6).

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A glycosylation assay was used to confirm that 2C/Met128-
FLAG reaches the lumen of the endoplasmic reticulum. COS cells transfected with either Bves-FLAG or 2C/Met128-FLAG were incubated in the absence or presence of tunicamycin. In the absence of tunicamycin, the fully glycosylated form of Bves-FLAG had the expected mass of 47 kDa (Fig. 9, lane 2), whereas that of 2C/Met128-FLAG was 41 kDa (lane 4). In the presence of tunicamycin, Bves-FLAG and 2C/Met128-FLAG had smaller masses of 43 kDa (lane 1) and 39 kDa (lane 3), respectively. In summary, these results in COS cells establish that Met128-FLAG localizes to the cytoplasm, whereas 2C/Met128-FLAG reaches the lumen of the endoplasmic reticulum and both carboxyl-terminal constructs form oligomers.

Co-immunoprecipitation of Bves—Because Met128-FLAG is localized in the cytoplasm, whereas 2C/Met128-FLAG reaches the endoplasmic reticulum lumen (illustrated in Fig. 10A), selective co-immunoprecipitation of HA-Bves would delineate the cellular location of its carboxyl terminus. Initial characterization studies of the antibodies directed against the HA and FLAG epitopes were performed to determine which antibody should be used for immunoprecipitation and which antibody should be used for detection on blots. HA-Bves-FLAG, containing both epitope tags (Fig. 1), was expressed in COS cells and the cell extracts used to test the antibodies. Mouse anti-FLAG antibody immunoprecipitated ~6-fold more immunoreactive HA-Bves-FLAG protein relative to the rabbit anti-HA antibody (data not illustrated). In contrast, the anti-HA antibody was only ~2-fold less reactive on blots against HA-Bves-FLAG relative to anti-FLAG (data not illustrated). Additionally, anti-HA detected HA-Bves but not Bves-FLAG on blots (data not illustrated). Therefore, anti-FLAG was used to immunoprecipitate the carboxyl-terminal FLAG-tagged constructs from cell extracts and anti-HA was used to detect potential associated HA-Bves.

HA-Bves was co-expressed with either Met128-FLAG or 2C/Met128-FLAG in COS cells. Mouse anti-FLAG was added to each cell extract and immunoprecipitated with goat anti-mouse cross-linked to Protein G beads. After washing the beads, protein was eluted with sample buffer containing 1% 2-mercaptoethanol. Separate blots were probed with anti-FLAG and anti-HA (Fig. 10B). The cell extracts contained considerably less Met128-FLAG relative to 2C/Met128-FLAG (top panel, lanes 1 and 2), whereas HA-Bves was expressed at similar levels from both extracts (bottom panel, lanes 1 and 2). In agreement with their expression levels, the anti-FLAG immunoprecipitates yielded lower levels of Met128-FLAG (top panel, lane 3) relative to 2C/Met128-FLAG (lane 4). However, co-immunoprecipitated HA-Bves was detected in association with Met128-FLAG (bottom panel, lane 3) but was not detected in association with 2C/Met128-FLAG (lane 4). Similar results were obtained when anti-HA was used with goat anti-rabbit beads to immunoprecipitate HA-Bves and anti-FLAG detected an association with only Met128-FLAG (data not illustrated). However, the level of co-immunoprecipitated Met128-FLAG was barely detected above background, which was consistent with the initial antibody characterization studies.

Immunocytochemistry of Endogenous Bves in Rat Epicardial Cells—The results thus far, based on properties of Bves within intracellular compartments, predict that the carboxyl terminus of cell surface Bves would be exposed to the cytoplasm rather than to the extracellular environment. To directly address this issue, we utilized an epithelial cell line derived from rat heart epicardium (epicardial cells) that expresses endogenous Bves. It was previously demonstrated that confluent cultures of epicardial cells express the majority of Bves immunoreactivity at the cell surface (2). Thus, we could determine the effect of non-ionic detergent on the availability of a carboxyl-terminal epitope. The optimal concentration of fixative was found to be 1% paraformaldehyde using antibodies against β-catenin as a control to label an intracellular protein localized at the cell surface (data not illustrated). Thus, epicardial cells were fixed with 1% paraformaldehyde and then treated in the absence or presence of 0.1% Triton X-100. Bves was labeled with rabbit anti-Bves (B846), directed against amino acids 263-289 of Bves (top panel, lanes 1 and 2), whereas robust labeling of both antibodies was detected in detergent-treated cells (panels C and D, respectively). In the second set of experiments, cells were double-labeled with both antibodies (data not illustrated). Enhanced immunoreactivity of anti-Bves and anti-β-catenin was again observed in detergent-treated cells relative to untreated cells. These results support the prediction that the carboxyl terminus of cell surface Bves is exposed to the cytoplasm.

DISCUSSION

Bves/POP1A is a member of the recently discovered Popeye (POP) family of transcripts that are expressed in several tissue types (1, 3). Whereas POP1, POP2, and POP3 transcripts have been discovered from mouse, only POP1 and POP3 have been isolated thus far from chicken (3). In addition, splice variants of chick POP1 have been identified, including POP1A (identical to Bves), 1B, 1C, and 1D (3). Interestingly, POP1C is predicted to
Fig. 10. The carboxyl-terminal Bves construct Met128-FLAG co-immunoprecipitates full-length HA-Bves. A, schematic of the distribution of wild-type and carboxyl-terminal Bves constructs interpreted from the preceding results. B, COS cells were co-transfected with HA-Bves and either Met128-FLAG or 2C/Met128-FLAG. Similar levels of HA-Bves were observed from both cell extracts (bottom panel, lanes 1 and 2). Lower levels of Met128-FLAG were detected in the cell extracts (top panel, lane 1) relative to that of 2C/Met128-FLAG (lane 2). The carboxyl-terminal constructs were immunoprecipitated from the cell extracts with anti-FLAG. The immunoprecipitates were blotted and probed with either anti-FLAG (top panel) to detect Met128-FLAG (lane 3) and 2C/Met128-FLAG (lane 4) or anti-HA (bottom panel) to detect co-immunoprecipitated HA-Bves. Although lower levels of Met128-FLAG were immunoprecipitated relative to 2C/Met128-FLAG, HA-Bves was detected in association with Met128-FLAG (bottom panel, lane 3).

encode a soluble protein homologous to a region of the carboxyl terminus, whereas the other isoforms contain hydrophobic membrane domains.

An overt phenotypic difference was not observed in POP1 knockout mice relative to wild-type littermates, but regeneration of cardiotoxin-treated skeletal muscle was delayed in mice that lack POP1 (4). Thus, it is possible that other Pop proteins compensate for a loss of Pop1 function in tissues that co-express more than one Pop member. It is important to resolve whether other Pop members compensate for the loss of Pop1 function during development as well as determine how Bves/Pop1A functions at the cellular level.

In addition to muscle tissue, Bves/Pop1A is also found in cells that develop coronary vessels. Coronary blood vessels develop from transitory epithelium of the proepicardial organ. This epithelium provides the epicardial covering of the heart and precursors that form various differentiated cell types of coronary vessels. A subpopulation of epicardial cells delaminate from the epithelial cell layer, migrate into the subepicardial connective tissue and myocardium (9–12), and differentiate into vascular endothelium, vascular smooth muscle, or fibroblasts of coronary vessels (13, 14). Bves is found in the epicardium, migrating mesenchyme, and vascular smooth muscle (1). Furthermore, Bves at the cell surface of epicardial cells later accumulates in an intracellular compartment of migrating mesenchymal cells and then reappears at the cell surface of smooth muscle cells (2). Thus, Bves may be functional at only one cellular location or have a unique function at each location. Interestingly, Bves enhances the adhesive property of non-adherent l-cells, which suggests that Bves has a function at the cell surface (2). Clearly, it is critical to elucidate the membrane topology of Bves to identify regions that likely contain functional domains.

The findings that Bves promotes cell adhesion and its distribution is dynamically regulated during development suggest that several functional domains are involved. Thus, the goal of this study was to elucidate the membrane topology of Bves to discriminate extracellular regions that may directly participate in cell-cell interactions from intracellular regions that may be involved in trafficking. This functional distinction does not exclude the possibility that intracellular domains participate in regulating adhesive properties, which has been documented for the prototypic cell adhesion molecule E-cadherin (reviewed in Ref. 15). Nonetheless, defining the membrane topology of Bves/Pop1A would provide a basis for subsequent functional studies.

Two different models have been proposed for the membrane topology of Bves/Pop1A. André et al. (4) proposed that the amino terminus is extracellular. The data from in vitro translation of Bves/Pop1A in the presence of microsomes (2, 3) clearly demonstrated an increase in mass that could be due to Asn-linked glycosylation. Because the amino terminus of Bves is the only region that contains consensus Asn-linked sites, glycosylation would establish that the amino terminus of cell surface Bves is exposed to the extracellular environment. Here, we thoroughly examined glycosylation of Bves from chick heart and transfected cells. Whereas André et al. (4) proposed that the carboxyl terminus of Bves/Pop1A is intracellular, Wada et al. (2) proposed that it is extracellular, based on the results from antibody blocking experiments. Antibodies against the carboxyl terminus of Bves (B846) blocked cell migration in...
primary cultures of chick proepicardia, whereas antibodies against another carboxyl-terminal epitope (D033) were without effect (2). Here, we utilized three different approaches to determine the carboxyl terminus of Bves.

Glycosylation of Bves was examined to determine whether the amino terminus reaches the lumen of the endoplasmic reticulum and is glycosylated. Bves from chick heart was glycosylated, and mutating the two Asn-linked glycosylation sites prevented glycosylation of Bves. These results establish that the amino terminus of Bves/Pop1A, the only region that contains Asn-linked sites, reaches the lumen of the endoplasmic reticulum, implying that the amino terminus of cell surface molecules would be exposed to the extracellular environment.

Three biochemically distinct approaches were utilized to determine the orientation of the carboxyl terminus of Bves/Pop1A. The first two approaches examined Bves within intracellular compartments of transfected cells, whereas the third approach examined cell surface Bves in a native cell background. First, glycosylation assays were utilized to examine Asn-linked glycosylation at exogenous sites within the carboxyl terminus of Bves. Second, oligomerization of Bves within the carboxyl terminus was exploited to investigate interactions between wild-type Bves and a soluble, carboxyl-terminal construct that either resides in the cytoplasm or is delivered to the endoplasmic reticulum lumen. Third, immunocytochemistry was performed with antibodies against the carboxyl terminus of Bves to examine endogenous protein at the cell surface of epicardial cells. Although each approach has inherent caveats, together, the results provide compelling evidence that the carboxyl terminus of Bves is exposed to the cytoplasm.

The results from this study support a topological model in which the amino terminus of cell surface Bves/Pop1A is exposed to the extracellular environment, whereas the carboxyl terminus is exposed to the cytoplasm, a membrane topology consistent with three membrane domains. The data presented here agree with the model proposed by Andrée et al. (4) that was based on data that did not establish the orientation of the carboxyl terminus. In contrast, Wada et al. (2) proposed that the carboxyl terminus of Bves was extracellular based on their results from antibody blocking experiments. The same polyclonal anti-Bves carboxyl-terminal antibodies (B846) that blocked cell migration of chick proepicardial explants (2) were utilized here in the immunocytochemistry experiments with rat epicardial cells. A potential explanation for these apparently conflicting results is that the Bves antibodies neutralized a secreted Pop member, possibly Pop1C. We are currently investigating several avenues to reconcile this important issue. Significantly, the current results from three biochemically distinct approaches indicate that the carboxyl terminus of Bves/Pop1A is exposed to the cytoplasm.

The membrane topology of Bves/Pop1A provides regional discrimination of functional domains. First, we predict that direct cell-cell interactions occur between the amino termini of opposing Bves molecules. Second, we predict that the dynamic redistribution of Bves during coronary vessel development is regulated within the carboxyl terminus. Our goal is to identify the functional domain(s) within each of these regions. Establishing the membrane topology of Bves/Pop1A has provided a foundation to dissect the cellular functions of Bves/Pop1A.

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