Precursor B Cell Receptor Signaling Activity Can Be Uncoupled from Surface Expression

F. Betul Guloglu and Christopher A. J. Roman

*J Immunol* 2006; 176:6862-6872; doi: 10.4049/jimmunol.176.11.6862

http://www.jimmunol.org/content/176/11/6862

---

**References**

This article cites 68 articles, 43 of which you can access for free at:

http://www.jimmunol.org/content/176/11/6862.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Precursor B Cell Receptor Signaling Activity Can Be Uncoupled from Surface Expression

F. Betul Guloglu* and Christopher A. J. Roman2*†

Signals from the precursor BCR (preBCR) cause proliferation and differentiation of progenitor (pro-) B cells into pro-B cells. Given the very low amounts of surface preBCRs and the demonstrated cell autonomy of preBCR signaling, we examined the possible occurrence of preBCR signal propagation from intracellular membranes such as the endoplasmic reticulum (ER) and the trans-Golgi network (TGN) in transformed and primary pro-B cells. PreBCRs composed of normal Ig µ or truncated Dµ heavy chains (HCs) were redirected to intracellular sites via localization sequences appended to the HC cytoplasmic tail. PreBCR complexes retained in the TGN or shuttled from the TGN to lysosomes were as or 50% as active as the corresponding wild-type preBCRs in directing preBCR-dependent events, including CD2 and CD22 expression and proliferation in primary pro-B cells. This occurred despite their low to undetectable surface expression in transformed cells, which otherwise allowed significant surface accumulation of wild-type preBCRs. In contrast, ER-retained preBCRs were inactive. These results suggest that preBCR signaling is remarkably tolerant of dramatic changes in its subcellular distribution within post-ER compartments and support the possibility that the preBCR can activate signaling pathways in the TGN as well as the plasma membrane. The Journal of Immunology, 2006, 176: 6862–6872.

*School of Graduate Studies, Program in Molecular and Cellular Biology and †Department of Microbiology and Immunology and Morse Institute for Molecular Genetics, State University of New York–Downstate Medical Center at Brooklyn, NY

Received for publication January 26, 2006. Accepted for publication March 17, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked

1This work was supported in part by Research Project Grant 00-269-01-LBC from the American Cancer Society (to C.A.J.R.), by the New York City Council Speaker’s Fund of the New York Academy of Medicine (to C.A.J.R.), and by the State University of New York.

2Address correspondence and reprint requests to Dr. Christopher Roman, Department of Microbiology and Immunology, State University of New York–Downstate Medical Center at Brooklyn, 450 Clarkson Avenue, Box 44, Brooklyn, NY 11203. E-mail address: Christopher.Roman@Downstate.edu

Abbreviations used in this paper: HC, H chain; preBCR, precursor BCR; LC, L chain; SLC, surrogate L chain; PM, plasma membrane; S/LC, surrogate and/or conventional light chain; ER, endoplasmic reticulum; TGN, trans-Golgi network; Endo Hf, endoglycosidase; WT, wild type; WGA, wheat gluten; UR, unique region; Lamp-1, lysosome-associated membrane protein 1.

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$02.00
the ER because it could not associate with the SLC (11). Similarly, preTCR complexes retained in the ER via an ER retention sequence did not signal in vivo (13). However, whether the preBCR signal can be triggered by SLC-assembled preBCRs in the ER or from other intracellular compartments, such as the trans-Golgi network (TGN) or endosomes, is not known.

To address these issues, we have created a panel of preBCR complexes that were redirected within the secretary/endosomal system and tested their signaling activity in primary and transformed pro-B cells. Our results show that preBCR signaling activity can be relatively indifferent to dramatic changes in surface levels. They are consistent with the model that cell-autonomous signaling can be initiated from both intracellular, post-ER membranes and the cell surface.

Materials and Methods

Expression constructs

The creation of cDNAs encoding mouse μ HC 17.2.25, Dμ HC, human μHC TG.SA, and λ1 has been described previously (12). The cDNAs were subcloned into MiG (14), a murine retroviral construct that contains the marker gene GFP linked to the cDNA of interest via an internal ribosome entry site. cDNAs encoding mouse μ and Dμ IgHcs with ER, TGN, or lysosome localization signals fused to the cytoplasmic tail (Table I) were created using standard overlap PCR mutagenesis and were verified by sequencing (Geneviz; oligonucleotides and strategies available upon request).

Cells and in vitro cell culture

The v-abl-transformed Ragg−/−A5−/− and Rag1−/− pro-B cell lines were described previously (15). Short-term primary IL-7-dependent pro-B cell cultures were established by harvesting and plating total bone marrow of 4- to 6-wk-old Ragg−/− (16) and Rag1−/−A5−/− mice (17) in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Invitrogen Life Technologies), antibiotics (1% penicillin-streptomycin, 1-glutamine), 5 × 10−4 M 2-ME, and rIL-7 (100 U/ml) for 2 days at 37°C. Cells were seeded at a density of 0.5–2 × 105 cell/ml and maintained in culture for 2–3 days before retroviral infections (18, 19).

Retroviral infections

Retroviruses were produced by cotransfection of HEK293 cells (by calcium phosphate) with the retroviral plasmids plus pMD1.Luc . Retroviruses were produced by cotransfection of HEK293 cells (by calcium phosphate) with the retroviral plasmids plus pMD1.Luc . Retroviruses were produced by cotransfection of HEK293 cells (by calcium phosphate) with the retroviral plasmids plus pMD1.Luc . Retroviruses were produced by cotransfection of HEK293 cells (by calcium phosphate) with the retroviral plasmids plus pMD1.Luc . Retroviruses were produced by cotransfection of HEK293 cells (by calcium phosphate) with the retroviral plasmids plus pMD1.Luc .

Detection of tyrosine-phosphorylated proteins

Stimulation of v-abl-transformed cells was performed according to Mielcz et al. (11). Briefly, cells (5 × 105/ml) were incubated in complete serum-free RPMI 1640 medium for 2 h at 37°C. Cells were washed and stimulated with 350 μM H2O2 and 100 μM sodium orthovanadate (in 1×PBS) for 2–3 min. Stimulation was terminated with cold 1×PBS. Cells were lysed in digitonin lysis buffer (1×digitonin, water soluble; Sigma-Aldrich), 150 mM NaCl, 10 mM tris(hydroxymethyl)amino-methane (Tris), 1 mM EDTA (pH 7.5), 1% protease inhibitor mixture (complete tablet; Roche) at 4°C for 15 min. Supernatant was incubated with 2 μl of anti-Igβ (or anti-Igμ) Ab overnight. Overnight, supernatant was incubated with 20 μl of protein G beads for 1 h, washed three times with digitonin lysis buffer, and eluted by boiling in reducing 1×SDS sample buffer. Eluates were resolved on 10%–15% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked with anti- Igβ or anti-Igμ Abs. Protein deglycosylation with endoglycosidase H (Endo H) or PNGase F (New England Biolabs) was performed according to the manufacturer’s protocols.

Ab uptake experiment

Following Natarajan and Lindstad (23), HC-expressing and control cells were incubated with RPE-conjugated anti-mouse IgM Ab at 37°C (experimental) or on ice (control starting amount). After 30 min, cells incubated at 37°C were treated with 5 volumes of ice-cold acid buffer (PBS and HCl, pH 2.0) for 45 s, then acidic buffer was neutralized by the addition of 2 volumes of 1 M HEPES (pH 7.6), followed by fixation with the addition of 0.5 volumes of 4% parafomaldehyde. The relative amount of Ab internalized at that time point relative to the starting amount (defined as 100%) was determined by flow cytometry.

Drug treatment

To block degradative pathways, v-abl-transformed and primary cells were treated with 40 mM NH4Cl, 0.1 mg/ml leupeptin (Roche), or vehicle only (DMSO) for 5–6 h at 37°C. To block endocytosis of surface receptors, primary and v-abl-transformed cells were incubated with 10–25 μM cytochalasin D (Sigma-Aldrich) for 1–6 h.

Confocal imaging

WT and “redirected” μHCs were visualized in HC-infected primary and v-abl-transformed cells via staining with anti-IgM-Cy5 and one of the following compartmental markers: Lysotracker, to probe late endosomes/lysosomes; wheat gluten (WGA), to detect Golgi, early endosomes, and endosomes; transferrin, to stain early endosomes; and anti-GFP, to detect the ER. For Lysotracker and transferrin staining, cells were incubated in fresh medium with 5 μM Lysotracker Red DND-99 (Molecular Probes and Invitrogen Life Technologies) for 15 min or with 10–20 μg/ml
rhodamine-conjugated mouse transferrin (Rockland) for 15–30 min at 37°C according to the manufacturer’s instructions. Cells were washed twice with 1× PBS and fixed in 2–4% paraformaldehyde for 15 min at room temperature. Following washing with 1× PBS, cells were incubated first in permeabilization buffer (0.1% saponin (Sigma-Aldrich), 0.1% sodium azide, and 5% FBS in 1× PBS) for 15 min and an additional 15–45 min in permeabilization buffer with 10–75 μg/ml Cy5-conjugated anti-mouse IgM (The Jackson Laboratory) at room temperature. For ER and Golgi stains, 1–10 μg/ml tetramethylrhodamine isothiocyanate-conjugated WGA (Molecular Probes) or 0.2 μg/ml rabbit anti-GRP78 (1/1000, H-129; Santa Cruz Biotechnology) was added along with anti-mouse IgM at this step. Cells labeled with anti-GRP78 were visualized using Rhodamine Red-X-conjugated Phalloidin (anti-rabbit IgG (The Jackson Laboratory)). Following washing several times with 1× PBS, cells were resuspended in mounting medium (Prolong Gold antifade reagent; Molecular Probes) and 20 μl was placed on slides (VWR Superfrost Plus). Images were analyzed with a confocal imaging system (Bio-Rad MRC-1024 krypton/argon laser and Olympus IX70 inverted microscope).

Results

Creation and localization of redirected preBCR complexes

Fusion of known ER, TGN, and lysosomal targeting/retention sequences to the cytoplasmic tail of IgHCs was used as a strategy to redirect preBCR complexes to different subcellular membranes (Table I). Fusions were made to a representative normal SLC-dependent μ HC that forms functional preBCRs and supports B cell development in vivo and in vitro (21, 24), and Δμ (25), a truncated HC that makes signaling-impaired preBCR complexes that do not progress through the secretory pathway as efficiently as μ preBCRs (12, 26). The efficacy of intracellular retention of the redirected μ HCs and the corresponding preBCR complexes was first tested in Abelson lines, which, unlike primary pre-B cells, accumulate readily detectable amounts of surface preBCRs (Refs. 8 and 21; Figs. 1 and 2). As such, the efficacy of the retention/localization sequences was clearly evident in these cells.

μ HCs appended with different ER-retention sequences showed variable degrees of ER localization and retention. PreBCR complexes with μER1, μER2, and μER3 HC s still reached the cell surface, although at lower levels than preBCRs with wild-type μ (μWT, Fig. 1, A and B, and data not shown; Table II). In contrast, little, if any, μER4 was detected on the cell surface. Western blot analysis showed that μER4 was expressed as well as μWT, but no mature (TGN-modified, Endo-Hf-resistant) species was detected (Fig. 1C, lane 5). Confocal microscopy revealed that little to no μER4 colocalized with WGA, which stains the PM and Golgi, whereas discreet areas of overlap were evident with μWT (Fig. 1D, cf. WGA second and third panels from the left). Conversely, coincident staining of μER4 with calnexin and GRP78 (BiP) showed extensive overlap and confirmed ER localization (Fig. 1D, lower panels, and data not shown). Mutation of KK to AA in the μER4 retention sequence (μER4mut) allowed WT levels of the resultant preBCR surface expression and mature HC species (Fig. 1, A and B), suggesting that preBCR assembly was not perturbed by the additional cytoplasmic sequences. Thus, only ER4 efficiently retained μ-preBCR complexes in the ER.

Among the four TGN localization sequences tested, only TGN4 enforced low levels of μ and preBCR surface expression (Fig. 1, A and B, and data not shown; Table II). Western blot showed this was not due to reduced protein expression (Fig. 1C), μHCs/preBCRs with mutations of critical amino acid residues in the TGN4 localization sequence (μTGN4mut) exhibited WT levels of surface expression (Fig. 1, A–C). Although μTGN4 was expressed dramatically less on the surface than μWT and μTGN4mut, all three proteins showed similar amounts of mature Golgi-modified forms (Fig. 1C). Similarly, confocal imaging showed that μTGN4 was found nearly exclusively in WGA-positive, calnexin-negative intracellular compartments (Golgi), but not in the PM (Fig. 1D).

These retention sequences also retained Δμ complexes. However, Δμ preBCRs are ordinarily not transported out of the ER with the same efficiency as μ preBCRs (12, 27), and attachment of the ER1 localization signal was sufficient to abolish the already low surface expression of Δμ (Fig. 1, A and B). This corresponded to the lack of mature Δμ species by Western blot (Fig. 1C). The effects of the ER1 localization sequence were reversed by mutation (ΔμER1mut). Similarly, the TGN4 localization signal also abolished Δμ surface expression (Fig. 1, A and B, bottom row), but unlike ER1, the proportion of the mature, Golgi-modified form of ΔμTGN4 was the same as Δμ (Fig. 1C, lanes 10–14). Confocal imaging showed extensive overlap of intracellular ΔμTGN4 and WGA, supporting the idea that it is primarily localized in the Golgi (Fig. 1D).

μ and Δμ HC s were also linked to a lysosomal targeting motif of lysosome-associated membrane protein 1 (Lamp-1), an integral lysosomal membrane protein (28). Most of Lamp-1 was shown to be directly routed to lysosomes from the TGN rather than indirectly by endocytosis from the plasma membrane (29–31). IgM and preBCR staining of transformed cells infected with ΔμLamp or μLamp showed no detectable HC on the surface (Fig. 1, A and B). Unlike the TGN4 fusions, Western blot showed that there were very low levels of Golgi-modified mature forms of μLamp and ΔμLamp compared with the WT counterparts (Fig. 1C).

Table I. Amino acid sequence of cytoplasmic domains of chimeric μ HC clones with ER, TGN- and lysosomal localization signal sequences attached to the cytoplasmic region of WT μ

<table>
<thead>
<tr>
<th>μHCs</th>
<th>Cytoplasmic Sequence of μHCs</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>μWT</td>
<td>-KVK</td>
<td></td>
</tr>
<tr>
<td>μER1</td>
<td>-KVR- KYKRRSSFIDEDEKKMP</td>
<td>E19K (59)</td>
</tr>
<tr>
<td>μER1mut</td>
<td>-KVR- KYKRRSSFIDESSMP</td>
<td>E19K (59)</td>
</tr>
<tr>
<td>μER2</td>
<td>-KVR- SRRVKAHKSKTH</td>
<td>HPI (60)</td>
</tr>
<tr>
<td>μER3</td>
<td>-RTQGEAAARKAAA</td>
<td>ERGIC-53 (61, 62)</td>
</tr>
<tr>
<td>μER4</td>
<td>-KVR- SRRVKEFLKKTN</td>
<td>WBP1(63)</td>
</tr>
<tr>
<td>μERmut</td>
<td>-KVR- SRRVKEFLAXTN</td>
<td>WBP1(63)</td>
</tr>
<tr>
<td>μTGN1</td>
<td>-KVR- TKIIKKPPWLCLGGLCCTCSKRKRAERLKK</td>
<td>G12 (64)</td>
</tr>
<tr>
<td>μTGN2</td>
<td>-KVR- KKRKRPPASDYGQL</td>
<td>Furin (65)</td>
</tr>
<tr>
<td>μTGN3</td>
<td>-KVR- KKRKRPPASDINFQSFESRPPFL</td>
<td>DPAP (66)</td>
</tr>
<tr>
<td>μTGN4</td>
<td>-KVR- KKRKRPPASDQYQL</td>
<td>TGN38 (67)</td>
</tr>
<tr>
<td>μTGN4mut</td>
<td>-KVR- KKRKRPPASDADADQA</td>
<td>TGN38 (68)</td>
</tr>
<tr>
<td>μLamp</td>
<td>-KKRSHAGYUTT</td>
<td>Lamp-1 (28)</td>
</tr>
</tbody>
</table>

* Clones were constructed by PCR as described in Materials and Methods. Amino acids known to be important for localization in the ER, TGN, and lysosomes, are in bold.
To test whether mature TGN forms of \( \mu \)HCs were being made but rapidly shunted to lysosomes for degradation, transformed primary Rag1\(^{-/-} \)Abelson cells infected with WT or redirected \( \mu \)HCs were treated with a combination of ammonium chloride (NH\(_4\)Cl) and leupeptin. NH\(_4\)Cl affects trafficking of endosomal vesicles and increases the pH of acidic endosomes, and leupeptin inhibits lysosomal and proteosomal enzymes. Under these conditions, the mature forms of \( \mu \)WT, \( \mu \)TGN4, and \( \mu \)Lamp \( \mu \)HCs accumulated in both cell types (Fig. 2A, lanes 2, 4, 11, and 13). Cytochalasin D treatment, which inhibits actin filament formation and thus endosomal trafficking (32), also increased levels of mature \( \mu \)WT, \( \mu \)TGN4, and \( \mu \)Lamp proteins, most dramatically in primary cells (Fig. 2A). However, none of these treatments led to any change in corresponding preBCR surface expression by either cell type (Fig. 2B). These treatments did not affect \( \mu \)ER4, which remained immature in all cases. In contrast to \( \mu \), the mature forms of \( \mu \)ER1, \( \mu \)ER1 mutant, \( \mu \)Lamp, and \( \mu \)Lamp did not accumulate with these treatments (data not shown), suggesting \( \mu \) complex processes differently than \( \mu \) within the endosomal system.

Measurement of surface biotinylated preBCRs confirmed the flow cytometry results (data not shown). These data suggested that the \( \mu \)Lamp proteins were primarily localized to lysosomes and confirmed that the redirection sequences behaved similarly in primary and Abelson-transformed cells.

An anti-Ig\( \mu \) Ab uptake assay was used to further characterize any surface expression of redirected preBCRs (23). In this assay, the relative total amount of a conjugated anti-Ig\( \mu \) Ab internalized over a given time period is measured. In the Abelson-transformed Rag1\(^{-/-} \)Abelson cells, the amount of anti-Ig\( \mu \) Ab internalized by cells expressing \( \mu \)TGN4 preBCR complexes was only slightly higher than preBCR-negative cells and cells expressing \( \mu \)ER4, \( \mu \)ER1, or \( \mu \)TGN4 preBCR, whereas cells expressing \( \mu \)WT and \( \mu \)TGN4mut internalized severalfold higher amounts of Ab (Fig. 3A). No internalized Ab was detected in Abelson cells expressing \( \mu \)Lamp and \( \mu \)Lamp preBCRs. Also, no uptake was detected in primary cells with any HCs, consistent with the undetectable...
surface expression by flow cytometry (Fig. 3B). Both Ab uptake and internalization rate assays (8, 23) determined that \( /H9262 \) WT, \( /H9262 \) TGN4mut, and \( /H9262 \) ER4mut behaved similarly, demonstrating these activities were not affected by the extra cytoplasmic sequences (Fig. 3 and data not shown). This result also suggested that low to no detectable surface expression of \( /H9262 \) TGN4 and \( /H9262 \) ER4 was probably not due to faster internalization of these HCs from the cell surface.

**Signaling competency of redirected preBCR complexes**

The signaling competency of redirected preBCR complexes was tested in the Rag1 \(^{-/-} \) Abelson line and in primary IL-7-dependent pro-B cells from Rag1 \(^{-/-} \) mice. In these systems, preBCRs are composed of retrovirally expressed HCs that assemble with endogenous SLC and Ig \( /H9225/H9226 \) components (21). \( /H9224 \) WT-preBCR expression in primary cells results in a \( /H9224 \) 5-dependent induction of CD2 and CD22 (Fig. 4, A (Rag1 \(^{-/-} \) \( \lambda5^+ \) cells) and B (Rag1 \(^{-/-} \) \( \lambda5^-/- \) cells), middle and right bar graphs), increased relative growth (Fig. 4, A and B, left graphs), and increased survival in low concentrations of IL-7 compared with preBCR-negative (i.e., uninfected and control-infected) pro-B cells (Fig. 4C and Ref. 21). In Abelson lines incubated with phosphatase inhibitors, relative levels of total phosphotyrosine increase in cells expressing functional \( \mu \) WT preBCRs compared with preBCR-negative cells (Fig. 5A and Ref. 8).

In these assays, \( \mu \) ER4 preBCRs were either inactive or profoundly impaired, promoting little, if any, proliferation, induction of CD2 or CD22 surface expression (Fig. 4A), or survival (Fig. 4C) in primary Rag1 \(^{-/-} \) \( \lambda5^+ \) cells. Total phosphotyrosine levels in \( \mu \) ER4-expressing Abelson cells were much lower than those in \( \mu \) WT, but were still above control preBCR-negative cells (Fig. 5A). The lower levels were not due to impaired preBCR assembly, because \( \mu \) ER4 could be coimmunoprecipitated with Ig \( \beta \) as well as,
respectively, and SEs are shown. Comparison of metabolism and signaling competency of WT and redirected preBCRs with ER, TGN, and lysosomal targeting signals

Table II. Comparison of metabolism and signaling competency of WT and redirected preBCRs with ER, TGN, and lysosomal targeting signals

<table>
<thead>
<tr>
<th>Surface Expression</th>
<th>Internalization Rate</th>
<th>Ab Uptake</th>
<th>Proliferation</th>
<th>CD2</th>
<th>CD22</th>
</tr>
</thead>
<tbody>
<tr>
<td>μWT</td>
<td>+ + + +</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μER1</td>
<td>+ +</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>μER1mut</td>
<td>+ + + +</td>
<td>ND</td>
<td>ND</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μER2</td>
<td>+ +</td>
<td>ND</td>
<td>ND</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μER3</td>
<td>+ +</td>
<td>ND</td>
<td>ND</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μER4</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>μER4mut</td>
<td>+ + + +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μLamp</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>μTGN1</td>
<td>+ + + +</td>
<td>ND</td>
<td>ND</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μTGN2</td>
<td>+ + + +</td>
<td>ND</td>
<td>ND</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μTGN3</td>
<td>+ +</td>
<td>ND</td>
<td>ND</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μTGN4</td>
<td>+ +</td>
<td>+</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>μTGN4mut</td>
<td>+ + + +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
<td>+ +</td>
</tr>
<tr>
<td>Dμ</td>
<td>— / + b</td>
<td>—</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DμER1</td>
<td>—</td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DμER1mut</td>
<td>— / + b</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DμLamp</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DμTGN4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Experiments related to HC metabolism were performed in Abelson-transformed cells, whereas the functional analysis of HCs was done in primary Rag1−/− λ5+ cells. b = / + and superscript (‘) each represent −/2 +.

*The value of the preBCR minus control.

if not better than, the other HC alleles (Fig. 5B) and the SL156 preBCR epitope, which requires λ5 and μHC, was detected intracellularly in μER4-expressing cells (data not shown). Interestingly, despite lower overall tyrosine phosphorylation of higher m.w. proteins compared with μWT, phosphorylation of 32- to 40-kDa polypeptides that corresponded to Igβ/Igα dramatically increased in μER4-expressing cells. This suggests that preBCR-dependent ITAM phosphorylation can occur in the ER with functional HCs that can associate with λ5. However, this did not correlate with productive signaling events in primary cells. Consistent with their inability to completely retain preBCR complexes in the ER, the signaling activity of μER3 was comparable to WT μ, and μER1 and μER2 were ~50% less active (Table II).

In contrast to ER4, preBCR retention mediated by the TGN4 sequence had little effect on preBCR signaling activity. μTGN4 and DμTGN4/preBCRs were nearly as active as μWT and Dμ-preBCRs, respectively, for proliferation and CD2/CD22 induction in primary cells (Fig. 4A; the relative signaling efficiency of other HCs is shown in Table II). μTGN4 was also nearly as active at conferring a growth advantage to pro-B cells at lower IL-7 concentrations as μWT (Fig. 4C). The nearly WT levels of preBCR activity was also evident in Rag1−/− Abelson lines expressing the μTGN4 preBCR, in which preBCR-dependent increases in total phosphotyrosine levels of high m.w. proteins was similar to that of μWT-expressing cells (Fig. 5A). The activity of μTGN4 was indistinguishable from μTGN4mut in primary cells (Fig. 4A), even though the latter was expressed on the surface of the Abelson line at WT levels (Fig. 1A). Similarly, μLamp-preBCRs were active, exhibiting an intermediate activity that was comparable to μER1 and Dμ (Fig. 4A), despite there being no detectable surface expression (Fig. 1A). Interestingly, the profile of μLamp-dependent phosphotyrosine exhibited both the prominent 32-kDa phosphorylation characteristic of μER4 but also the higher m.w. proteins common with μWT and μTGN4 (Fig. 5A).

However, signaling by Dμ preBCRs was intolerant to the other redirection sequences. In contrast to μ, DμER1 and DμLamp did not mediate any preBCR-dependent signaling (Fig. 4A and data not shown). This corresponded to the absence of mature forms of these Dμ HCs by Western blot (Fig. 1C, lanes 11 and 14) even in the presence of endosomal/lysosomal inhibitors (data not shown).

FIGURE 3. Measurement of surface preBCR expression by uptake of anti-IgM Abs. Control (−) or HC-infected Rag1−/− Abelson-transformed (A) or primary cells (B) were incubated with R-PE labeled anti-IgM Ab and the relative amount of this Ab internalized by cells during a 0.5- to 1-h period was measured as described in Materials and Methods. Note that in A, the μα1 sample was from Rag1−/−λ5−/− cells infected with both the μ HC and a λ1 LC virus; all other samples were infected with the HC only. The scale of the bars in B is ~2-fold greater than those depicted in A. n = 6 and n = 3 for transformed and primary cells, respectively, and SEs are shown.
In general, any signaling mediated by the redirected HCs was, like the parental HCs, dependent on the SLC complex. Lamp and ER HCs did not show any activity in primary Rag1/−/A5/+ cells (Fig. 4B). A low level of activity, substantially lower than in A5/+ cells, was detected in primary Rag1/−/A5/+ cells infected with TGN4, TGN4mut, and ER1mut HCs (Fig. 4B). This corresponded to a minor fraction of mature HC species detected by Western blot analysis of Rag1/−/A5/+ transformed cells that expressed these HCs (data not shown). However, no HCs could be detected on the surface of these cells by flow cytometry (data not shown). This suggested that the appended cytoplasmic sequences of these particular HCs may have actually promoted some low level of A5/SLC-independent HC export from the ER to the TGN, in turn causing a low level of signaling in A5’s absence. Thus, the low level of A5-independent signaling is consistent with SLC-independent enhanced ER export driven by the redirection sequences. For ER1, this was evident when the retention functions were inactivated (ER1mut).

Activity of redirected BCR complexes

Targeting sequences also affected the intracellular trafficking of BCR complexes. Previously, we showed that Rag1/−/A5/+ and D/−/A5+ BCRs and their redirected counterpart BCRs in Rag1/−/A5/+ (A) and Rag1/−/A5/+ cells (B) with respect to relative growth (left), CD2 induction (middle), and CD22 induction (right). In all graphs, the bar showing the activity of the normal μ (μWT) preBCRs is black; and the dotted line represents the activity of the normal Dμ-preBCR. Cells were infected with the indicated control (−) or HC-containing retroviruses; the relative growth of infected (GFP−) and uninfected (GFP+) CD19+ cells in each culture was measured as in Materials and Methods (Rag1/−/A5+/− n = 14 for empty vector, Dμ, μWT, μER4, μTGN4; n > 3 for the rest, Rag1/−/A5+/− n ≥3 for all HCs; SE bars are shown). The values shown in the middle and right graphs represent the percentage of CD19+ GFP+ cells that were CD2+ or CD22+. A, Rag1/−/A5+/− cells, n = 17 (CD2) and n = 21 (CD22) for empty vector, Dμ, μWT, μER4, μTGN4; n > 3; B, Rag1/−/A5+/− cells, n ≥3 (CD2 and CD22) for all HCs; SE bars are shown. C, Relative abilities of normal and redirected preBCRs to sustain growth under limiting IL-7 in 5 days. The numbers plotted are the relative growth of the CD19+ GFP+ population at the indicated concentration of IL-7 divided by the relative growth of the CD19+ GFP+ population in 100 U/ml IL-7 cultures.
transformed and primary pro-B cells coexpressing HCs and the \( \text{A1LC} \) expressed high levels of surface receptors primarily because HCs can leave the ER more efficiently in association with comparable amounts of each HC was expressed, but the highest A, undetectable on the surface (Fig. 6A). For example, transgenically expressed IgHCs were more effective at enforcing allelic exclusion when expressed in homozygosity, and in the absence of VpreB-1, efficiency of pre-B development was proportional to VpreB-2 gene and protein dose (35–37). Similarly, in our system we have observed that substantially lowering \( \mu \)WT expression resulted in proportionally lower signaling activity and surface levels (F. B. Guloglu, unpublished observations). In contrast, relative levels of surface expression of the redirected preBCRs did not always predict their preBCR signaling activity. Rather, a better correlation did exist between redirected preBCR signaling activity and the relative amounts of constituent mature, TGN-modified HCs. For \( \lambda \)Lamp, these mature forms were revealed after inhibition of the lysosomal system. Taken together, these data support the model that surface expression per se may not be critical for signaling, and that signaling may be propagated from intracellular, post-ER membranes such as the TGN.

Studies in different systems have demonstrated that receptors or signaling intermediates can signal from intracellular membranes (38–45). Many of these processes are known to require PM localization for ligand interaction and possibly to engage key signal transducers (46, 47). For example, the intensity of TCR stimulation regulates not only Ras isoform utilization but also the subcellular compartment from which Ras signals are propagated (48, 49). This may impact positive and negative selection during development or an immune response. However, mutated and oncogenic forms of receptor tyrosine kinases are no longer ligand dependent and can be active intracellularly (50, 51). In this way, the preBCR may be analogous to these constitutively active receptors, but unlike them

FIGURE 5. PreBCR-dependent induction of tyrosine phosphorylation and assembly of redirected preBCR complexes. A, Western blot to detect tyrosine-phosphorylated proteins in \( \text{Rag1}^{-/-} \) v-abl-transformed cells that were infected either with the indicated control (−) or \( \mu \) HCs retroviruses. Cells were serum starved, incubated with peroxidase/\( \text{H}_{2}\text{O}_{2} \), and then probed with either the 4G10 mAb, which detects tyrosine-phosphorylated proteins (top panel) or with an anti-IgB Ab (middle panel). Total extracts from the same cells were also probed directly by Western blot with an anti-Ig \( \beta \) or control (C) Ab (bottom panel) or with an anti-IgB Ab (middle panel) or with an anti-IgB Ab (bottom panel).

Transduction of the importance of surface expression and putative receptor-ligand interactions in signaling, we have created a panel of preBCRs with functional full-length HCs that were either not detectable on the surface (\( \mu \)ER4, \( \mu \)Lamp or were present over 10-fold less (\( \mu \)ER1, \( \mu \)TGN4) than WT on the surface of Abelson lines, which are permissive for WT preBCR surface accumulation. A combination of biochemical and immunofluorescent approaches showed that \( \mu \)ER4 was retained in the ER, whereas \( \mu \)Lamp and \( \mu \)TGN4 resided primarily in the Golgi and endosomes. That \( \mu \)ER4 was inactive supports the model that preBCR signaling cannot be propagated in the ER, at least not to induce proliferation and differentiation, and with normal HCs that can assemble into preBCR complexes. Surprisingly, \( \mu \)TGN4 preBCRs were as active as \( \mu \)WT complexes. Similarly, \( \mu \)Lamp preBCR retained ~50% WT activity, and \( \delta \mu \)TGN4 was as active as \( \delta \mu \). Thus, in contrast to the existing paradigm, relative levels of preBCR signaling by these HCs were disproportionate to their relative levels of surface expression.

One interpretation of these data is that preBCR signaling is tolerant of dramatic decreases in steady-state surface expression. However, in vivo studies have suggested that a linear relationship exists between preBCR signal strength and levels of IgHC and SLC expression, which are indirectly reflected in surface expression levels. For example, transgenically expressed IgHCs were more effective at enforcing allelic exclusion when expressed in homozygosity, and in the absence of VpreB-1, efficiency of pre-B development was proportional to VpreB-2 gene and protein dose (35–37). Similarly, in our system we have observed that substantially lowering \( \mu \)WT expression resulted in proportionally lower signaling activity and surface levels (F. B. Guloglu, unpublished observations). In contrast, relative levels of surface expression of the redirected preBCRs did not always predict their preBCR signaling activity. Rather, a better correlation did exist between redirected preBCR signaling activity and the relative amounts of constituent mature, TGN-modified HCs. For \( \lambda \)Lamp, these mature forms were revealed after inhibition of the lysosomal system. Taken together, these data support the model that surface expression per se may not be critical for signaling, and that signaling may be propagated from intracellular, post-ER membranes such as the TGN.

**Discussion**

Surface expression of preBCR complexes has been tightly linked to their signaling capability. IgHCs that cannot associate with the SLC, typically obligatory for receptor transport to the surface, do not contribute to B cell repertoire (33, 34). It is thought that preBCR expression in the plasma membrane may be critical to engage signal transduction pathways and extracellular ligands. To address the importance of surface expression and putative receptor-ligand interactions in signaling, we have created a panel of preBCRs with functional full-length HCs that were either not detectable on the surface (\( \mu \)ER4, \( \mu \)Lamp) or were present over 10-fold less (\( \mu \)ER1, \( \mu \)TGN4) than WT on the surface of Abelson lines, which are permissive for WT preBCR surface accumulation. A combination of biochemical and immunofluorescent approaches showed that \( \mu \)ER4 was retained in the ER, whereas \( \mu \)Lamp and \( \mu \)TGN4 resided primarily in the Golgi and endosomes. That \( \mu \)ER4 was inactive supports the model that preBCR signaling cannot be propagated in the ER, at least not to induce proliferation and differentiation, and with normal HCs that can assemble into preBCR complexes. Surprisingly, \( \mu \)TGN4 preBCRs were as active as \( \mu \)WT complexes. Similarly, \( \mu \)Lamp preBCR retained ~50% WT activity, and \( \delta \mu \)TGN4 was as active as \( \delta \mu \). Thus, in contrast to the existing paradigm, relative levels of preBCR signaling by these HCs were disproportionate to their relative levels of surface expression.

One interpretation of these data is that preBCR signaling is tolerant of dramatic decreases in steady-state surface expression. However, in vivo studies have suggested that a linear relationship exists between preBCR signal strength and levels of IgHC and SLC expression, which are indirectly reflected in surface expression levels. For example, transgenically expressed IgHCs were more effective at enforcing allelic exclusion when expressed in homozygosity, and in the absence of VpreB-1, efficiency of pre-B development was proportional to VpreB-2 gene and protein dose (35–37). Similarly, in our system we have observed that substantially lowering \( \mu \)WT expression resulted in proportionally lower signaling activity and surface levels (F. B. Guloglu, unpublished observations). In contrast, relative levels of surface expression of the redirected preBCRs did not always predict their preBCR signaling activity. Rather, a better correlation did exist between redirected preBCR signaling activity and the relative amounts of constituent mature, TGN-modified HCs. For \( \lambda \)Lamp, these mature forms were revealed after inhibition of the lysosomal system. Taken together, these data support the model that surface expression per se may not be critical for signaling, and that signaling may be propagated from intracellular, post-ER membranes such as the TGN.
can negatively regulate its own activity by driving its internalization and turnover and down-regulating SLC gene expression.

However, the data do not exclude the possibility that the PM has an important role in preBCR signaling. In one respect, this may be at the level of signaling output. It may be that preBCR expression in the PM is not important for CD2, CD22, and proliferation/survival, but is important for other preBCR-dependent phenomena not evaluated in this system. This includes allelic exclusion, a property which is lost both in v-abl-transformed cells and in our primary IL-7-dependent pro-B cultures with HCs expressed de novo via retroviruses (Ref. 52 and data not shown). Also not evaluated were hetero- and homotypic interactions that may promote later preBCR-dependent developmental changes (6, 7, 53, 54).

In another respect, it could be postulated that the Igαβ heterodimer, rather than the intact preBCR, may reach the PM and signal. Several recent studies on the BCR have indicated that Ag cross-linking induces Ig dissociation from the Igαβ heterodimer (55, 56). Whereas nearly all of the surface Ig is internalized, up to 30% of the Igαβ heterodimers remain on the PM and continue to signal. Based on these observations, it is postulated that key steps in common for WT and TGN/Lamp-redirected preBCRs are 1) transport Igαβ out of the ER and transit through the TGN and 2) “activation” of the Igαβ molecules in such a way to render them competent to productively engage signal transduction pathways. After activation, Igαβ may dissociate from the Ig components. HC-SLC components would be forwarded to lysosomes and activated Igαβ would continue to the PM and signal. Whereas the first model postulated that the TGN is where signaling pathways are first engaged, this alternative model incorporates the idea that PM expression of Igαβ may still be necessary for persistent and/or productive propagation of other subsequent preBCR-dependent developmental signals. A prediction from this and the first model is that preBCRs, by virtue of the λ5UR, would be most efficient at inducing this hypothetical Igαβ activation step, whereas λ5UR-deleted preBCRs and BCRs would be less efficient. Activation may be a preBCR-intrinsic property; however, if ligand interaction facilitates this, both models imply this ligand is intravesicular. This alternative model is consistent with the HC-SLC-independent signaling activity of PM targeted Igαβ fusion molecules (57, 58).

Nevertheless, these models do not exclude a role for an extracellular ligand in modulating preBCR signal transduction. Rather, several modes of preBCR signaling may coexist, which may depend on the individual properties of the preBCR clonotype. For example, ligand-dependent interactions may have a role in enhancing the intensity of preBCR-dependent signaling functions. This may be most important for certain HC clonotypes that may assemble with the SLC and transit through the secretory system but may not signal efficiently intracellularly via the cell-autonomous, possibly preBCR-intrinsic mechanism. A result may be that intact preBCRs that reach the surface are activated by SLC/preBCR ligand-dependent cross-linking (6).

The V(D)J recombination process often produces Ig genes that are nonproductive or that encode proteins that are structurally unsound. The “preBCR checkpoint” provides a functional screen for HC structure in the absence of any other information about future BCR properties. The primary and transformed pro-B cell culture systems here provide a rapid and effective means to systematically determine how the functionality of individual HC clonotypes with known biological properties is evaluated at this critical developmental stage. Expression of the redirected HCs in mice via transgenesis will help address the extent to which the resulting redirected preBCRs can support development in vivo or whether other interactions dependent on putative preBCR surface expression are necessary. In addition, the activity of redirected preBCRs in
progenitor cells defective in particular signaling molecules will provide further insight into how the membrane dynamics of the preBCR control its interfacing with different signaling pathways. These types of approaches will ultimately determine how the IgHC structure influences these processes so as to control repertoire establishment and influence disease states.

Acknowledgments
We gratefully thank Drs. S. Gottesman and W. Chirico (State University of New York–Downstate Medical Center) for critical reading of this manuscript and discussions. We are grateful to W. Oxberry (Department of Pathology Confocal Imaging Facility, State University of New York–Downstate Medical Center) for instruction and generosity and members of the Roman Laboratory for discussions.

Disclosures
The authors have no financial conflict of interest.

References


