Differences in the apical and basolateral pathways for glycosaminoglycan biosynthesis in Madin–Darby canine kidney cells

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Serglycin with a green fluorescent protein tag (SG-GFP) expressed in epithelial Madin–Darby canine kidney cells is secreted mainly (85%) into the apical medium, but the glycosaminoglycan (GAG) chains on the SG-GFP protein core secreted basolaterally (15%) carry most of the sulfate added during biosynthesis (Tveit et al., 2005). Here we report further differences in apical and basolateral GAG synthesis. The less intensely sulfated chondroitin sulfate (CS) chains on apically secreted SG-GFP are longer than CS chains attached to basolateral SG-GFP, whereas the heparan sulfate (HS) chains are of similar lengths. When the supply of \(3\)-phosphoadenosine-5'-phosphosulfate (PAPS) is limited by chlorate treatment, the synthesis machinery maintains sulfation of HS chains on basolateral SG-GFP until it is inhibited at 50 mM chlorate, whereas basolateral CS chains lose sulfate already at 12.5 mM chlorate and become longer. Apically, incorporation of \(35\)S-sulfate into CS is reduced to a lesser extent at higher chlorate concentrations than basolateral CS, although apical CS is less intensely sulfated than basolateral CS in control cells. Similar to what was found for basolateral HS, sulfation of apical HS was not reduced at chlorate concentrations below 50 mM. Also, protein-free, xyloside-based GAG chains secreted basolaterally are more intensely sulfated than their apical counterpart, supporting the view that separate apical and basolateral sorting platforms organize earlier in the secretory pathway than the trans-Golgi network (TGN), before the Brefeldin A block, whereas CS chain synthesis is completed in the TGN (Spiro et al., 1991; Sugumaran et al., 1992; Uhlin-Hansen and Yanagishita, 1993; Calabro and Hascall, 1994). The TGN is the exit site of apical and basolateral vesicular carriers from the Golgi apparatus and is also regarded as the Golgi compartment where most sorting processes take place (Griffiths and Simons, 1986). However, indications that more stable membrane microdomains might form earlier in the secretory pathway exist (Bagnat and Simons, 2002; Emery et al., 2003; Paladino et al., 2004; Alfalah et al., 2005), and we have recently promoted the idea that apical and basolateral sorting platforms organize earlier in the Golgi apparatus than the TGN in MDCK cells (Tveit et al., 2005).

An epithelial tissue depends on a proper, basolaterally localized extracellular matrix with HSPGs that carry GAG chains sufficiently sulfated to be able to bind growth factors and other signaling molecules. We have previously shown that MDCK cells secrete most of the newly synthesized HSPGs basolaterally, in terms of incorporated \(35\)S-sulfate label (Svennevig et al., 1995), whereas CSPGs are almost exclusively secreted apically (Kolset et al., 1999). Inhibition of sulfate activation to \(3\)-phosphoadenosine-5'-phosphosulfate (PAPS) by the competitive inhibitor chlorate has shown that HS sulfation resists higher chlorate concentrations than sulfation of CS and proteins (Humphries and Silbert, 1988; Fjeldstad et al., 2002). However, it has previously been difficult to study protein core-independent regulatory differences in the synthesis of apical and basolateral GAGs in epithelial cells, because a good model PG that could be quantitatively retrieved after metabolic labeling has been unavailable.

In this work, we have taken advantage of the fact that the PG serglycin coupled to green fluorescent protein (SG-GFP), Golgi apparatus and/or in recycling endosomes (Rodriguez-Boulan and Musch, 2005). Proteins that traverse the secretory pathway are translocated into the lumen of the endoplasmic reticulum (ER) before most of them acquire glycan modifications that are trimmed off and/or extended throughout the Golgi apparatus. One important class of glycans that attaches to protein cores is glycosaminoglycans (GAGs), which are long linear repeats of characteristic disaccharide units, usually linked to serines with adjacent glycines in the core protein via a xylose-galactose-galactose-glucuronic acid tetrasaccharide (Prydz and Dalen, 2000). The nature of the disaccharide is decisive to whether the proteoglycan (PG) is a heparin/heparan sulfate (HS) or a chondroitin sulfate/dermatan sulfate (CS/DS) PG. PGs with more than one class of GAG chains are called hybrid PGs. Several studies with different cell lines indicate that synthesis of HS chains is completely before the trans-Golgi network (TGN), before the Brefeldin A block, whereas CS chain synthesis is completed in the TGN (Spiro et al., 1991; Sugumaran et al., 1992; Uhlin-Hansen and Yanagishita, 1993; Calabro and Hascall, 1994). The TGN is the exit site of apical and basolateral vesicular carriers from the Golgi apparatus and is also regarded as the Golgi compartment where most sorting processes take place (Griffiths and Simons, 1986). However, indications that more stable membrane microdomains might form earlier in the secretory pathway exist (Bagnat and Simons, 2002; Emery et al., 2003; Paladino et al., 2004; Alfalah et al., 2005), and we have recently promoted the idea that apical and basolateral sorting platforms organize earlier in the Golgi apparatus than the TGN in MDCK cells (Tveit et al., 2005).

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In this work, we have taken advantage of the fact that the PG serglycin coupled to green fluorescent protein (SG-GFP),
when expressed in MDCK II cells, is produced as a hybrid PG and secreted 85% apically and 15% basolaterally (Tveit et al., 2005). This allows us to study both HS and CS chain synthesis along the apical and basolateral secretory pathways. CS-GAG chains attached to SG-GFP secreted apically are longer than CS chains on SG-GFP secreted basolaterally, even if the apical GAGs are less sulfated. Protein-free GAG chains linked to xylosides recovered from the basolateral medium are also more intensely sulfated than their apical counterparts. When the supply of active sulfate (PAPS) is limited by chlorate treatment, priority is given to sulfation of HS on both apically and basolaterally secreted SG-GFP molecules, whereas sulfation of apical CS resists higher chloride concentrations than the basolateral counterpart. This differential response indicates that HS-GAG synthesis and sulfation in the apical and basolateral secretory pathways are regulated differently and is a further support for the idea that these pathways are segregated during GAG synthesis in the Golgi apparatus.

Results

We have previously shown that PGs secreted from the basolateral membrane domain of epithelial MDCK II cells are more intensely sulfated than those secreted into the apical medium. Even when the same PG protein core (serglycin with C-terminal GFP [SG-GFP]) was immune isolated from the two opposite medium reservoirs of transfected MDCK II cells (MDCKII), the covalently attached GAG chains on basolaterally secreted SG-GFP were several times more intensely sulfated than those on apically secreted SG-GFP, and the patterns of CS sulfation were different (Tveit et al., 2005). We now present experiments where we have investigated differences in chain lengths and regulation of sulfation of apical and basolateral GAG chains. To address such differences, the GAG chains have been removed from their SG-GFP protein core after immune precipitation, allowing separate studies of CS and HS chains. Figure 1 shows a Sepharose CL-6B chromatogram with a comparison of the chain lengths of GAGs derived from apically and basolaterally secreted SG-GFP. Clearly, the major peak of basolateral GAGs has a lower average $K_{av}$ than the apical counterpart, although basolateral GAGs are more intensely sulfated (Tveit et al., 2005). The average CS and HS chain lengths were estimated after differential degradation and by use of a standard curve with $K_{av}$ values for dextrins of different sizes (data not shown), giving apical CS-GAG chains with an average size of 67.0 ± 2.1 kDa and basolateral chains of 43.1 ± 2.0 kDa. HS chains derived from the hybrid SG-GFP PG were more similar in size after apical (34.1 ± 3.8 kDa) and basolateral (29.6 ± 2.4 kDa) retrieval (Table I).

We investigated whether the apical and basolateral pathways for GAG synthesis would respond similarly or differently to a regulatory challenge, such as reduction of the cellular PAPS level by the competitive inhibitor of PAPS synthesis, chlorate (Baueuerle and Huttner, 1986; Humphries and Silbert, 1988). Treatment with 50 mM chlorate did not alter the secretion polarity (Figure 2A) or the CS:HS ratio of SG-GFP significantly (Figures 2B and 8B)

Table I. Molecular weights of GAG chains on secreted SG-GFP upon treatment with chlorate (50 mM) or BX (1 mM)

<table>
<thead>
<tr>
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<th>Apical (kDa ± SE)</th>
<th>Basolateral (kDa ± SE)</th>
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<tr>
<td>CS-GAG</td>
<td>67.0 ± 2.1</td>
<td>43.1 ± 2.0</td>
</tr>
<tr>
<td>HS-GAG</td>
<td>29.6 ± 2.4</td>
<td>34.1 ± 3.8</td>
</tr>
<tr>
<td>Chlorate</td>
<td>67.1 ± 5.7</td>
<td>66.0 ± 4.0</td>
</tr>
<tr>
<td>BX</td>
<td>37.0 ± 2.0</td>
<td>29.7 ± 1.7</td>
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but confirmed the finding that HSPG sulfation in general is more resistant to chlorate treatment than CSPG in MDCK cells (Fjelldstad et al., 2002). Treatment of MDCK II cells with 50 mM chlorate reduced the incorporation of $^{35}$S-sulfate into SG-GFP by 98% (Figure 3B), and CS-GAGs on SG-GFP secreted into the apical and basolateral media were now similar in size because the basolateral chains became longer (Table I). HS chains did not change significantly in length upon chlorate treatment and resisted dramatic reduction in the sulfation level until 50 mM chlorate was employed. To address the distribution of the remaining sulfate groups on SG-GFP after chlorate treatment of the cells, immune precipitation (IP) from media from one or two pooled filters was performed, followed by HS or CS degradation of individual samples. The SDS–PAGE gel (Figure 3A) shows that treatment of MDCK II cells with 50 mM chlorate essentially blocks sulfation of CS on both apical and basolateral SG-GFP. However, sulfation of CS on basolateral SG-GFP is close to maximally reduced already at 12.5 mM chlorate, whereas this chlorate concentration only reduces the sulfation of apical CS chains by...
35% (Figure 3B and C). In relative terms, more and more of the remaining sulfate is associated with HS chains with increasing chlorate concentrations, indicating that the cellular machinery prioritizes HS sulfation when the PAPS availability is low.

Another way to challenge the synthesis and transport machinery of PGs is by introduction of xylosides (Schwartz, 1979). The xylose portion allows polymerization of protein-free GAG chains, usually of the CS type (Schwartz et al., 1974; Galligani et al., 1975), whereas the hydrophobic portion facilitates access to the polymerizing enzymes. Exactly at what stage of GAG synthesis the xyloside becomes a competitor for the enzymatic machinery is not clear, but competition is evident at the level of GAG chain polymerization (Moses et al., 1999).

The benzyl-xyloside (BX) itself acquired GAG chains sensitive to c-ABC treatment (data not shown), as is generally the case for xylosides. These CS chains followed the trends observed previously (Figure 4A and B) by being predominantly secreted apically (Kolset et al., 1999), whereas the basolaterally secreted xyloside-based GAGs were more intensely sulfated (Tveit et al., 2005). The xyloside treatment had a dramatic effect on the apical secretion of SG-GFP protein cores. Whereas these are mainly secreted into the apical medium in control cells, the secretion is nonpolarized in the presence of BX (Figure 5). On the basis of confocal

Fig. 2. Chlorate treatment of MDCK II cells expressing SG-GFP. MDCK II cells were grown on filters and metabolically labeled with $^{35}$S-Cys/Met (A) or $^3$H-GlcN (B) in the presence or absence of 50 mM sodium chlorate. Apically and basolaterally secreted SG-GFP was immune precipitated and loaded onto a 4–12% SDS–PAGE gel after HNO$_2$ (H) or cABC (C) treatment or no treatment (Ctr) of $^3$H-GlcN-labeled samples. The gels were developed in a Typhoon 9400 PhosphorImager.

Fig. 3. Effect of chlorate on SG-GFP GAG sulfation in MDCK II cells. MDCK II cells were grown to confluency on filters and metabolically labeled with $^{35}$S-SO$_4^{2-}$ in the absence or presence of 12.5–50 mM chlorate. Apically and basolaterally secreted SG-GFP was isolated by IP and loaded onto SDS–PAGE gels (A) or Sepharose CL-6B columns (B and C) after treatment with HNO$_2$ or cABC for HS or CS degradation, respectively. Panel A shows the effect of 50 mM chlorate, and panels B and C show the effect of 12.5–50 mM chlorate. For IP of SG-GFP secreted from control cells and cells treated with 12.5 mM chlorate, single filters were used, whereas for cells treated with 25 or 50 mM chlorate, two filters were pooled for each experiment to enhance the signal of $^{35}$S-sulfate.

Fig. 4. Sulfation of xyloside-based GAGs. MDCK II cells were grown on filters, labeled with $^3$H-GlcN or $^{35}$S-SO$_4^{2-}$ in the presence of 1 mM BX. Labeled macromolecules in the apical and basolateral media were purified by Sephadex G-50 fine gel filtration, and one-tenth of each sample was loaded onto 4–20% SDS–PAGE gels (A). The $^{35}$S-sulfate per $^3$H-GlcN ratio in xyloside-based GAGs (low-molecular-weight region) was calculated using ImageQuant (B).

Fig. 5. Apical secretion of SG-GFP protein cores. Where these are mainly secreted into the apical medium in control cells, the secretion is nonpolarized in the presence of BX (Figure 5). On the basis of confocal
Apical and basolateral glycosaminoglycan pathways

In this work, we have studied differences that occur in post-translational processing of a model PG in the apical and basolateral pathways. Laser fluorescence microscopy of the SG-GFP, it seems that the protein core to some extent accumulates in the ER in the presence of BX (data not shown), indicating a reduced capacity for entry of SG-GFP into the apical pathway. The incorporation of $^3$H-glucosamine ($^3$H-GlcN) and $^{35}$S-sulfate is reduced for both apical and basolateral SG-GFP (Figure 6A and B), manifested as shorter CS-GAGs (Table I). These shorter GAGs contain lesser $^{35}$S-sulfate per incorporated $^3$H-GlcN, whereas apical SG-GFP still carries less intensely sulfated GAGs than the basolateral counterpart (Figure 6C). The amount of CS attached to xylosides was (20 times that of CS on SG-GFP apically and 16 times that of CS on SG-GFP basolaterally. An additional observation is that treatment of MDCK-II cells with BX reduces the extent of HS modification more than that of the CS modification on basolateral SG-GFP; in other words, the relative fraction of CS chains on SG-GFP is increased, seen as more HNO$_2$-resistant and less chondroitina se-A,B,C (cABC)-resistant material in the basolateral medium of BX-treated cells (Figure 7). This is contrary to the observation that xylosides generally knock down CS synthesis (Klein et al., 1989; Margolis et al., 1991) and is surprising because MDCK cells tend to maintain HS synthesis/sulfation as long as possible when challenged by other inhibitors (Fjeldstad et al., 2002). At the apical side, the CS : HS ratio on SG-GFP was not changed by BX treatment (Figure 8B and C), although the total incorporation of $^{35}$S-sulfate and $^3$H-GlcN is reduced to 20–30% of controls (Figure 8A).

In sum, we have challenged the biosynthesis of one PG (SG-GFP) in different ways, and the apical and basolateral pathways have responded differently. This adds to the accumulating evidence that these two pathways are separated before the TGN and thus might be regulated differently.

**Discussion**

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basolateral secretory pathways of epithelial MDCK cells. MDCK II cells transfected to express the human SG variant in frame with the GFP (SG-GFP) were metabolically labeled with 35S-sulfate, 3H-GlcN, or 35S-cysteine/methionine (35S-Cys/Met) before the secreted PG was immune isolated from the apical and basolateral media. SG-GFP is secreted both as a hybrid PG, with CS and HS chains, and as a pure CSPG. We have previously found that the fraction of SG-GFP (85%) secreted apically acquires GAG chains with less sulfate than the basolaterally secreted SG-GFP (15%). We have now demonstrated another difference between apical and basolateral CS chains on SG-GFP—the apical chains are significantly longer (Table I). Also, xyloside-based CS chains followed the general pattern—those secreted basolaterally contained more sulfate than those secreted to the apical medium. A reduction in the supply of active sulfate, by chlorate treatment, had different effects on the apical and basolateral pathways. Sulfation of HS chains on the basolaterally secreted SG-GFP was maintained, whereas CS chains rapidly lost sulfate. SG-GFP secreted at the apical membrane carried CS chains that resisted higher chlorate treatments, but GAG chains on both apical and basolateral SG-GFP are shorter in the presence of BX; again the apical pathway is affected the most (Table I). One effect of BX that had a predominant basolateral manifestation was an inhibitory effect on HS-GAG synthesis on basolateral SG-GFP. In sum, our data support the view that the apical and basolateral secretory pathways are segregated during GAG synthesis in the Golgi apparatus. The regulatory challenges introduced in the experimental system did all affect the two pathways differently. It has recently been shown that units of the Golgi complex of imaginal disk cells in Drosophila containing the UDP-sugar transporter Fringe connection are distinct from those containing the sulfotransferase Sulfateless (Yano et al., 2005). A separation of the Golgi apparatus into several, separate functional units could explain much of the diversity observed for glycan modification and would allow a more precise regulatory response. It has been proposed that sorting into the apical and basolateral pathways might occur at exit from the ER (Rodriguez-Boulan and Musch, 2005). A novel detergent extraction technique, employing Tween 20, makes separation of apical and basolateral proteins with mannose-rich N-glycans possible (Alfalah et al., 2005), indicating that these pathways are segregated at an early stage of the secretory pathway. A concern regarding the differences in modification of apical and basolateral SG-GFP observed in this work and in previous work (Tveit et al., 2005) could be changes occurring to the PGs after their secretion into the medium, during the remainder of the metabolic labeling period (24 h in total). There is, however, a very limited endocytic uptake and transport to a degrading compartment at the apical pole of MDCK cells (Bomsel et al., 1989; Parton et al., 1989), making a chain shortening or sulfatase activity acting on re-endoctyosed SG-GFP unlikely to contribute to the observed differences. Still, further experiments are needed to address questions...
concerning the physical segregation of the apical and basolateral pathways in epithelial cells.

Materials and Methods

Construction of plasmid

The plasmid sergly-pEGFP was made as described earlier (Tveit et al., 2005). Expand Long Template PCR System (Roche, Mannheim, Germany) was used together with 5′-ATCGGAATTCTAGTGCAGAAGATCTCAA-3′ and 5′-TTGCAACGTACGATGGATCTAACATAAAATC CTCTT-3′ to amplify the coding sequence of SG. The PCR product was ligated into the EcoRI and BamHI restriction sites in the expression vector pEGFP-N3 (Clontech, Palo Alto, CA), making sergy-pEGFP.

Cell culture and transfections

MDCK II cells were grown in Dulbecco’s Modified Eagle’s medium (DME) with 5% FBS (PAA, Pasching, Austria), 1% antibiotics and L-glutamine (Biowhittaker, Verviers, Belgium) at 37°C, and 5% CO2. The cells were stably transfected (MDCK II), as previously described (Tveit et al., 2005), with 4 µg sergy-pEGFP and 12 µL Fugene 6 (Roche) for 72 h. For selection of transfected colonies, 1 mg/mL of G-418 (Duchefa, Harlem, The Netherlands) was supplemented to the medium. Several colonies were selected and screened by IP with polyclonal anti-GFP (Abcam, Cambridge, UK). One resulting clone was used for the experiments presented here.

Metabolic labeling and treatment of MDCK II cells

MDCK II cells (10^6) were seeded on 4.7 cm^2 filters (Costar 3412) in 1.6 mL growth medium added apically, with 2 mL in the basolateral well. The cells were grown to confluency (4 days), before metabolic labeling was carried out with 1 mL medium apically and 2 mL basolaterally, with 0.3 µCi/mL 35S-Cys/Met (PerkinElmer, Boston, MA) using DME without glucose (Gibco). All metabolic labeling experiments were of 24-h duration. Apical and basolateral media were harvested, and cell fractions were treated for CS degradation at 37°C overnight, before addition of 60 µL (50–50 slurry) of protein-A-Sepharose beads before rotating 3 h at 4°C. The beads were then washed six times with IP wash solution (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Triton X-100) with 1% BSA and four times without BSA.

Immune precipitations

Before IP, the apical and basolateral media and the cell fractions were precleared with 60 µL (50–50 slurry) of protein-A-Sepharose (Amersham Biosciences, Buckinghamshire, England, UK) for 1 h at 4°C. IP was performed as described previously (Tveit et al., 2005). All samples were added 1 µL anti-GFP/mL at 4°C overnight, before addition of 60 µL (50–50 slurry) protein-A-Sepharose beads before rotating 3 h at 4°C. The beads were then washed six times with IP wash solution (50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Triton X-100) with 1% BSA and four times without BSA.

SDS–PAGE and quantification of SG-GFP

After labeling and harvesting, the apical and basolateral media and cell fractions were run on Sephadex G-50 Fine (Amersham) chromatography columns to remove unincorporated radioactive molecules (Spiro et al., 1991). Aliquots (usually 50 µL) of each fraction were then added Insta-Gel II plus (Packard, Groningen, CA) counted in a scintillation counter, and one-tenth of each fraction (1.5 mL total) was analyzed by SDS–PAGE (BioRad, Hercules, CA). IP beads were either added SDS sample buffer (XT, BioRad) directly and run on a 4–12% XT SDS–PAGE with MOPS buffer (BioRad) or first treated for selective GAG degradation before running the gels. The gels were fixed, treated with Amplex (Amersham), and dried. Gels with 3H-GlcN-labeled samples were subjected autoradiography with Hyperfilm ECL (Amersham). 35S-Cys/Met and 35S-4SO42– labeled samples in gels were exposed to PhosphorImager screens, scanned (Typhoon 9410 PhosphorImager, Amersham), and quantified by ImageQuant (Amersham).

Selective GAG degradation

After IP of apical and basolateral medium aliquots, each sample was divided into three equal volumes; one for control, one for cABC (Seikagaku, Tokyo, Japan) (75 µu/mL) treatment for CS degradation at 37°C overnight, and one for HNO2 treatment for HS degradation (10 min at room temperature), as described (Shively and Conrad, 1976).

Alkaline 3-elimination treatment

Beads with IP SG-GFP were resolved in 0.15 M NaCl in 0.05 M Tris–HCl buffer, pH 8.0, and alkaline treated by addition of one-tenth of the sample volume of 5 M NaOH before incubation at room temperature for 20 h release GAGs from the core proteins. The reaction was stopped by neutralizing with HCl to pH 7–8 and buffered with 1 M Tris–HCl.

Gel filtration chromatography

The released GAGs were treated for degradation with cABC or HNO2 as described. Treated and untreated samples were applied to columns (1 cm diameter x 40 cm) of Sepharose CL-6B (Amersham Biosciences), along with blue dextran and K2CrO4 as internal standards. The column was eluted with 0.15 M NaCl in 0.05 M Tris–HCl buffer pH 8.0 and 0.1% Triton X-100, at a rate of 6 mL/h. Fractions of 1 mL were collected, and aliquots of each fraction were analyzed for radioactivity in a scintillation counter. The length
of CS and HS-GAG chains was determined from the remaining fraction volumes after HNO$_2$ or cABC treatment. Standard dextran series of 670, 410, 270, 50, and 12 kDa (Fluka, Buchs, Switzerland) were used for determination of the molecular masses of the GAGs. The fractions with dextran were identified by the phenol sulfuric acid method (Dubois et al., 1951).

Acknowledgments

The skillful technical assistance of Supunnee Sokboonya is highly appreciated. This work was supported by The Research Council of Norway, the Norwegian Cancer Society, and The Blix Foundation.

Abbreviations

BX, benzyl-xyloside; cABC, chondroitinase-A,B,C; CS, chondroitin sulfate; Cys/Met, cysteine/methionine; DME, Dulbecco’s Modified Eagle’s medium; ER, endoplasmic reticulum; GAG, glycosaminoglycan; GFP, green fluorescent protein; GlnC, glucosamine; HS, heparan sulfate; IP, immune precipitation; MDCK, Madin–Darby canine kidney; MDCK$, transfected Madin–Darby canine kidney; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PG, proteoglycan; SG, serglycin; TGN, trans-Golgi network.

References


