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Examination of Whole Cell Galectin Binding by Solid Phase and Flow Cytometric Analysis

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Abstract

We have utilized simple flow cytometric and fluorescence-based solid phase assays to study the interaction of glycan-binding proteins (GBP) to cell surface glycoconjugates. These methods utilize commonly employed flow cytometry techniques and commercially available streptavidin-coated microplates to immobilize various biotinylated ligands, such as glycopeptides, oligosaccharides, and whole cells. Using this approach, fluorescently labeled GBPs, in particular, members of the galectin family, can be interrogated for potential interactions with cell surface carbohydrates, including elucidation of the potential impact of alterations in glycosylation on carbohydrate recognition. Using these approaches, we present examples of flow cytometric and fluorescence-based solid phase assays to study galectin–carbohydrate interactions.

Keywords

Galectin-1; Solid phase assay; Biotinylation; Fluorescence labeling; Immobilization; Binding affinity

1 Introduction

Glycan-binding proteins (GBPs) often regulate a wide variety of biological processes through the recognition of highly modifiable terminal residue recognition. However, in addition to the potential impact of terminal modifications, the core backbone of many glycan structures can influence terminal glycan binding [1–3]. As a result, binding data obtained following analysis on glycan microarrays that primarily contain relatively simple terminal glycan motifs may not fully illustrate the impact of core glycan structure on overall binding interactions [4, 5]. To overcome these limitations, various approaches have employed natural carbohydrates harvested from glycoproteins or glycolipids [4, 6–11]. However, in addition to the glycan backbone, cell surface presentation of glycans can also significantly impact galectin–carbohydrate interactions [12]. As a result, in addition to examining galectin–carbohydrate specificity using defined arrays populated with synthetic or naturally occurring glycans, interrogating galectin-binding specificity toward intact cells can provide a complementary tool to investigate the potential impact of cell surface glycan alterations on the binding interactions of various galectin family members.

Although previous studies have employed a variety of formats to examine galectin binding toward cell surface carbohydrates, flow cytometric analysis of galectin interactions with

mammalian cells or microbes likely reflects the most commonly employed method [13, 14]. This approach enables a relatively high-throughput method of evaluating significant changes in galectin engagement following genetic or enzymatic alterations in cell surface glycans (Fig. 1) [12, 15–20]. As different cell populations may express distinct galectin ligands, this approach also allows examination of potential galectin interactions with distinct populations of cells within a complex mixture, providing a useful tool to not only examine the potential impact of changes in glycosylation on galectin binding and function but also to characterize previously unrecognized cellular subsets that may express distinct carbohydrate ligand for various members of the galectin family.

While flow cytometric approaches provide a useful tool to examine general interactions between galectins and cell surface carbohydrates, the concentrations of galectins utilized in this format rarely saturate cell surface counter receptors prior to inducing significant agglutination. Galectin-induced agglutination can induce artifacts during flow cytometric analysis secondary to cell fragmentation, as previously shown for antibodies capable of agglutinating cells [21], which can significantly alter the cellular profile and apparent density of ligands. Although sub-agglutinating concentrations of galectins are often employed to avoid this limitation, these sub-saturating concentrations also limit complete analysis of binding as saturation can rarely be achieved in order to fully appreciate the impact of ligand density and other potential changes on binding affinity. In addition, the examination of galectin binding in the linear range of ligand engagement can result in significant inter-assay variability as a result of subtle differences in the concentration of galectins employed in different assay conditions. By contrast, most flow cytometric analyses purposely employ monoclonal antibodies at saturating levels to ensure complete engagement of target ligands in order to adequately identify potential alteration in ligand density between cellular subsets and following potential ligand modifications [22–24]. In addition, flow cytometric analysis does not necessarily provide a measurement of affinity to determine whether, in addition to alterations in ligand density, the actual affinity of galectins toward carbohydrate ligands may differ between unique cell populations decorated with different glycan ligands.

To overcome inherent limitations associated with flow cytometric analysis, we have also utilized a simple fluorescence-based solid phase assay on a microplate format to study interactions between galectins and cell surface glycoconjugates. This approach appears to avoid potential artifacts that may be introduced when using saturating concentrations of galectins [25–27]. To accomplish this, biotinylated human HL-60 cells or T cells were immobilized onto the streptavidin-coated microplates and probed with fluorescently labeled galectins (Fig. 2) [26, 15]. A portion of cells were also treated sequentially with different glycosidases before immobilization into the microtiter plates to gather structural information regarding the impact of carbohydrate modification on cell surface binding [26, 15]. Parallel binding experiments were performed with fluorescently labeled plant lectins with known binding specificity to control for the effect of glycosidases on cells [26, 15, 28, 29]. By using serially diluted galectins in solid phase assay with immobilized cells, apparent binding affinity of galectin to cell surface glycoconjugates was obtained [26, 27]. Thus, this approach not only facilitates examination of the impact of alterations in glycosylation on

galectin binding but also provides a useful approach to determine the relative affinity of galectins toward various cell surface-associated glycans.

Regardless of the approach used, galectin typically requires labeling using a variety of fluorescent probes that react with different functional groups of a protein to facilitate flow cytometric or fluorimeter detection following potential binding reactions. Fluorescent probes can be covalently attached in a variety of ways, including via primary amines, reduced cysteine residues, or oxidized carbohydrate residues. The fluorescent probe should be selected carefully, because covalent derivatization of amino acid residues involved in ligand binding may result in galectin inactivation. Therefore, it is important to test the activity of the labeled galectins before using it in an assay. If a monoclonal antibody is available to a galectin under study, an antibody can be fluorescently labeled and used to detect the bound galectin in the assay. Alternatively, if a recombinant galectin has been produced as an IgG fusion protein [30], commercial fluorescently labeled anti-IgG monoclonal antibody can be used for detection. Taken together, these methods provide a variety of approaches to elucidate galectin interactions with highly modifiable cell surface glycans.

2 Materials

2.1 Biotinylation and Fixation of HL-60 Cells

1. HL-60 cells (other types of cells can also be used).
2. Phosphate buffered saline (PBS) and PBS, pH 8.0
3. 8 % paraformaldehyde in PBS, pH 7.2 (prepare fresh or store at -20°C).
4. EZ-LinkTM Sulfo-NHS-LC-Biotin (Pierce).
5. Burker chamber (or suitable equipment) for counting cells.

2.2 Glycosidase Treatments of Biotinylated and Fixed HL-60 Cells

1. *Arthrobacter ureafaciens* neuraminidase (e.g., Roche Diagnostics, Mannheim, Germany).
2. *Escherichia freundii* endo- β -galactosidase (e.g., V-Labs, Inc., Covington, LA).
3. Jack bean β -galactosidase (e.g., Glyko).
4. PBS (standard) pH 7.4.
5. PBS, pH 5.0 containing 1 mg/ml BSA.

2.3 Fluorescence Labeling of Gal-1 Through Primary Amines

1. Recombinant human dimeric Galectin-1.
2. Alexa Fluor 488 carboxylic acid succinimidyl ester (Invitrogen).
3. 0.1 M lactose in PBS.
4. 14 mM β -mercaptoethanol in PBS.
5. PD-10 (or equivalent) small gel filtration column (GE Healthcare).

6. α -Lactosyl-agarose (Sigma) (1–2 ml volume).

2.4 Fluorescence Labeling of Gal-1 Through Cysteines

1. Recombinant human dimeric galectin-1.
2. Alexa Fluor 488 C₅-maleimide (Invitrogen).
3. 0.1 M lactose in PBS.
4. 14 mM β -mercaptoethanol in PBS.
5. PD-10 (or equivalent) small gel filtration column (GE Healthcare).
6. α -Lactosyl-agarose (Sigma) (1–2 ml volume).

2.5 Fluorescence Labeling of Tomato Lectin (LEA) Through Carbohydrates

1. *Lycopersicon esculentum* (tomato) agglutinin (LEA) (Vector Laboratories).
2. Sodium *m*-periodate.
3. Alexa Fluor 488 hydrazide (Invitrogen), prepare fresh solution 2 mg/ml in PBS.
4. PBS (standard) pH 7.4.
5. PD-10 (or equivalent) small gel filtration column (GE Healthcare).

2.6 Gal-1, LEA, and GS-II Binding to Immobilized HL-60 Cells

1. Streptavidin-coated high-binding-capacity 96-well microtiter plates (Pierce).
2. PBS (standard) pH 7.4.
3. 1 % BSA in PBS.
4. Fluorescently labeled lectins: Alexa Fluor 488-labeled Gal-1, Alexa Fluor 488-labeled LEA, and fluorescein-labeled *Griffonia simplicifolia* lectin II (GS-II) (EY Laboratories Inc., San Mateo, CA).
5. Fluorescence microtiter plate reader with suitable filters (for Alexa Fluor 488 and fluorescein excitation at 485 nm and emission at 535 nm).
6. 8-channel (or 12-channel) manual pipet (250–300 μ l).

2.7 Determination of Gal-1 Binding Using Flow Cytometric Analysis

1. PBS.
2. 1 % BSA in PBS.
3. Fluorescently labeled lectins: Alexa Fluor 488-labeled Gal-1, Alexa Fluor 488-labeled LEA, and fluorescein-labeled *Griffonia simplicifolia* lectin II (GS-II) (EY Laboratories Inc., San Mateo, CA).
4. BD FACSCalibur.

2.8 Determination of Apparent Binding Affinity of Gal-1 for Immobilized HL-60 Cells

1. Streptavidin-coated high-binding-capacity 96-well microtiter plates (Pierce).
2. PBS (standard) pH 7.4.
3. 1 % BSA in PBS.
4. 1 % BSA and 20 mM lactose in PBS.
5. Alexa Fluor 488-labeled Gal-1.
6. Fluorescence microtiter plate reader with suitable filters (for Alexa Fluor 488 and fluorescein excitation at 485 nm and emission at 535 nm).
7. 8-channel (or 12-channel) manual pipet (250–300 µl).
8. Computer software capable to calculate nonlinear curve fitting (e.g., SigmaPlot).

3 Methods

3.1 Biotinylation and Fixation of HL-60 Cells

1. Wash HL-60 cells three times with PBS (*see* Note 1).
2. Suspend cells at a concentration of 20×10^6 cells/ml in PBS, pH 8.0.
3. Add 1.3 mg EZ-Link Sulfo-NHS-LC-Biotin per ml of cells and incubate at RT for 30 min.
4. Wash cells three times with ice cold PBS.
5. Add 8 % paraformaldehyde in PBS to final concentration of 2 % and incubate at RT for 30 min.
6. Wash cells three times with ice cold PBS.
7. After final wash suspend cells to 1 ml of PBS and count cells.

3.2 Fixation Alone of HL-60 Cells (for Enzymatic Treatment Followed by Flow Cytometric Analysis)

1. Wash cells three times with ice cold PBS.
2. Add 8 % paraformaldehyde in PBS to final concentration of 2 % and incubate at RT for 30 min.
3. Wash cells three times with ice cold PBS.
4. After final wash suspend cells to 1 ml of PBS and count cells.

¹When planning the experiment, it should be considered that relatively a large number of cells are required for solid phase assay. Here we use 100,000 cells/well on 96-well plate. Initial amount of harvested cells should be considerably larger because a number of centrifugations during the course of the experiment result in loss of cells.

3.3 Glycosidase Treatments of Biotinylated and Fixed (or Fixed Alone) HL-60 Cells

1. Suspend biotinylated and fixed HL-60 cells at the concentration of 5×10^6 cells/ml in PBS. Take a portion of cells for neuraminidase treatment (proceed to **step 2**), and leave the rest of cells untreated (proceed directly to **step 4**).
2. Add *Arthrobacter ureafaciens* neuraminidase to a final concentration of 100 milliunits/ml of cells in PBS and incubate at 37 ° C for 3 h.
3. Wash neuraminidase-treated cells three times with PBS and count cells.
4. Suspend neuraminidase-treated and neuraminidase-untreated cells (from **step 1**) into PBS, pH 5.0 containing 1 mg/ml BSA at the concentration of $2\text{--}5 \times 10^6$ cells/ml, and divide each into three equal aliquots.
5. Add *Escherichia freundii* endo- β -galactosidase (250 milliunits/ml, final concentration) to the first sample, Jack bean β -galactosidase (100 milliunits/ml, final concentration) to the second sample, and buffer alone (PBS, pH 5.0 containing 1 mg/ml BSA) to the third sample.
6. Incubate overnight at 37 ° C.
7. Glycosidase-treated cells and control cells can be immobilized directly into streptavidin-coated microtiter plates (Subheading 3.7) (see Note 2).

3.4 Fluorescence Labeling of Gal-1 Through Primary Amines

1. Human galectin-1 (Gal-1) (1–2 mg) can be labeled through primary amines using Alexa Fluor 488 carboxylic acid succinimidyl ester according to manufacturer's instructions with minor modifications as described in the following steps.
2. Incubate Gal-1 with reactive dye in PBS containing 0.1 M lactose for 1 h at room temperature or by continuing incubation overnight at 4 ° C with stirring.
3. Remove free dye and lactose from the labeled Gal-1 using a PD-10 column equilibrated in PBS containing 14 mM β -mercaptoethanol.
4. To separate functionally active Gal-1 from inactive protein, pass labeled Gal-1 over a small lactosyl-agarose column (1–2 ml volume) in PBS. Wash unbound (inactive) Gal-1 from the column with 3–5 column volumes of PBS. Bound (active) Gal-1 is eluted with 0.1 M lactose in PBS. Before each experiment, lactose must be removed using a PD-10 column equilibrated in PBS containing 14 mM β -mercaptoethanol.
5. Gal-1 samples labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester can be stored in 0.1 M lactose in PBS at +4 ° C for few months. Lactose must be removed using a PD-10 column in PBS containing 14 mM β -mercaptoethanol before each experiment (see Note 3).

²In the present experiments, we treated biotinylated and fixed cells with glycosidases before capturing cells on streptavidin plates. We did not observe significant differences in the binding assays, if glycosidase treatments were performed on the plate after capturing biotinylated and fixed cells.

³Alternatively, labeled Gal-1 can be stored without lactose in PBS containing 14 mM β -mercaptoethanol at 4 ° C for a few days.

3.5 Fluorescence Labeling of Gal-1 Through Cysteines

1. Accessible cysteine residues on human galectin-1 (Gal-1) (1–1.5 mg) can be labeled using thiol reactive Alexa Fluor 488 C₅-maleimide according to manufacturer's instructions with minor modifications as described in the following steps.
2. Incubate Gal-1 with tenfold molar excess of thiol reactive Alexa Fluor 488 C₅-maleimide in PBS containing 0.1 M lactose overnight at 4 °C under stirring.
3. Remove free dye and lactose from the labeled Gal-1 using a PD-10 column in PBS containing 14 mM β -mercaptoethanol.
4. To separate functionally active Gal-1 from inactive protein, pass labeled Gal-1 over a small lactosyl-agarose column (1–2 ml volume) in PBS. Wash unbound (inactive) Gal-1 from the column with 3–5 column volumes of PBS. Bound (active) Gal-1 is eluted with 0.1 M lactose in PBS. Before each experiment, lactose must be removed using a PD-10 column equilibrated in PBS containing 14 mM β -mercaptoethanol.
5. Gal-1 samples labeled with Alexa Fluor 488 C₅-maleimide can be stored in PBS containing 14 mM β -mercaptoethanol at 4 °C for at least 3 months without losing activity (*see* Note 4).

3.6 Fluorescence Labeling of Tomato Lectin (LEA) Through Carbohydrates

1. Dissolve *Lycopersicon esculentum* (tomato) agglutinin (LEA) in PBS to the final concentration of 4 mg/ml.
2. Weight out solid sodium *m*-periodate (to obtain final concentration of 100 mM in the sample).
3. Add LEA in PBS to the tube containing solid sodium *m*-periodate, and incubate for 30 min at room temperature in the dark to oxidize *cis*-diols of carbohydrates to aldehydes.
4. Remove sodium *m*-periodate using a PD-10 gel filtration column in PBS. Collect 0.5 ml fractions and measure absorbance at 280 nm for each fraction. Pool fractions containing protein (=oxidized LEA).
5. Add Alexa Fluor 488 hydrazide solution (2 mg/ml in PBS) to oxidized LEA sample to the final concentration of 100 μ g/mg lectin.
6. Incubate for 1.5–2 h at room temperature under stirring.
7. Remove free dye using a PD-10 column in PBS. Collect 0.5 ml fractions and measure absorbance at 280 nm and 494 nm for each fraction. Pool fractions containing protein (*see* Note 5).

⁴Gal-1 labeled with Alexa Fluor 488 C₅-maleimide is more stable during long-term storage than Gal-1 labeled with Alexa Fluor 488 carboxylic acid succinimidyl ester.

⁵The protein concentration and degree of labeling can be calculated using absorbance readings at 280 and 494 nm according to Alexa Fluor 488 hydrazide manufacturer's instructions. The degree of labeling of LEA was significantly higher by labeling through carbohydrates than in commercial fluorescently labeled LEA.

3.7 Gal-1, LEA, and GS-II Binding to Immobilized HL-60 Cells

1. To immobilize equal amount of biotinylated HL-60 cells in microtiter well, adjust cell density in each sample to 2×10^6 cells/ml using PBS (*see Note 6*).
2. Wash streptavidin-coated microtiter plates three times with 200 μ l of PBS.
3. Immobilize glycosidase digested and nondigested HL-60 cells to streptavidin-coated microtiter wells at equivalent densities (100,000 cells/well) in 50 μ l of PBS for 1.5 h at room temperature.
4. Wash the wells three times with 200 μ l of PBS containing 1 % BSA. After last wash remove buffer carefully by tapping the plate upside down gently against paper towel. Do not let the plate dry (*see Note 7*).
5. Add 50 μ l of fluorescently labeled Gal-1 (40 μ g/ml), LEA (100 μ g/ml), or GS-II (100 μ g/ml) in PBS containing 1 % BSA, and incubate for 1 h at room temperature.
6. Wash the wells four times with 250–300 μ l of PBS containing 1 % BSA (*see Note 7*).
7. Add 100 μ l of PBS to each well and read the fluorescence by fluorescence microplate reader. Figure 3 shows results on the binding of Gal-1, LEA, and GS-II to immobilized HL-60 cells.

3.8 Determination of Gal-1 Binding to HL-60 Cell Using Flow Cytometric Analysis

1. Prepare dilutions 0.04, 0.08, 0.15, 0.31, 0.75, 1.25, 2.5, and 5.0 μ M of fluorescently labeled Gal-1 in PBS with 1 % BSA and in PBS with 1 % BSA and 20 mM lactose (*see Note 8*).
2. Add 50 μ l of fluorescently labeled Gal-1 dilutions in PBS with 1 % BSA or PBS with 1 % BSA and 20 mM lactose to each sample containing 1×10^6 cells/ml (final concentration), and incubate for 1 h at room temperature. When analyzing nonfixed live cells, incubate the indicated concentrations of fluorescently labeled Gal-1 with 1×10^6 cells/ml for 30 min. at 4 ° C to avoid Gal-1 internalization or other alterations that may occur at room or higher temperatures during the binding assay.
3. Wash the cells three times with 250–300 μ l of PBS containing 1 % BSA.
4. Following the wash steps, evaluate cells using a microscope to ensure that the cells are not agglutinated.

⁶Samples should be prepared at least in triplicate, and the results should be calculated as a mean $\pm$ S.D.

⁷Microtiter plate washing machines should be avoided, and microtiter wells should be washed manually using 8-channel (or 12-channel) pipet. Fill wells gently with wash buffer using pipet, and empty wells into the waste by inverting the plate. After the last wash, remove buffer carefully by gently tapping the plate against paper towel. Do not let the wells to dry at any point during the experiment.

⁸Examination of potential Gal-1 interactions over a range of concentrations will allow a linear and sub-agglutinating range of concentrations to be established. Comparison between different potential cellular subsets, enzymatic treatments, or genetic changes in glycosylation should be done by examining potential differences in binding at the same concentration of galectin. As subtle changes in concentration of galectin may result in some degree of inter-assay variability, differences in binding between cell subsets within an assay may be best enumerated by examining percent changes in comparison to control cells.

5. Add 300 µl of PBS to each well and examine bound galectin by evaluating the mean fluorescence intensity of appropriately gated cells by using commonly employed flow cytometric analysis techniques. Figure 1 shows results on the determination of the binding of Gal-1 to HL-60 cells by flow cytometry.

3.9 Determination of Apparent Binding Affinity of Gal-1 for Immobilized HL-60 Cells

1. Prepare dilutions 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, and 20.0 µM of fluorescently labeled Gal-1 in PBS with 1 % BSA and in PBS with 1 % BSA and 20 mM lactose (*see Note 9*).
2. Wash streptavidin-coated microtiter plates three times with 200–300 µl of PBS.
3. Immobilize neuraminidase digested and nondigested HL-60 cells to streptavidin-coated microtiter wells at equivalent density (100,000 cells/well) in 50 µl of PBS for 1.5 h at room temperature.
4. Wash the wells three times with 200 µl of PBS containing 1 % BSA. After last wash remove buffer carefully by tapping the plate upside down gently against paper towel. Do not let the plate dry (*see Note 7*).
5. Add 50 µl of fluorescently labeled Gal-1 dilutions in PBS with 1 % BSA or PBS with 1 % BSA and 20 mM lactose to each well, and incubate for 1 h at room temperature.
6. Wash the wells four times with 250–300 µl of PBS containing 1 % BSA. After last wash remove buffer carefully by tapping the plate upside down gently against paper towel (*see Note 7*).
7. Add 100 µl of PBS to each well and read the fluorescence by fluorescence microplate reader.
8. Calculate the apparent dissociation constants (K_d) using nonlinear curve fitting program. Figure 4 shows results on the determination of the binding affinity of Gal-1 for immobilized HL-60 cells.

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⁹Samples should be prepared at least in triplicate, and the results should be calculated as an average of three determinations.

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Fig. 1.

Gal-8N and Gal-8C display differential recognition of cell surface glycans. **(a)** Schematic representation of full-length Gal-8 and individual domains. **(b)** Binding of Gal-8N toward HL-60 cells with or without incubation 20 mM TDG or 20 mM sucrose. **(c)** Binding of Gal-8N toward HL-60 cells treated with *A. ureafaciens* neuraminidase. **(d)** Binding of Gal-8C toward HL-60 cells treated with *A. ureafaciens* neuraminidase. **(e)** Geometric mean fluorescent intensities (GeoMFI), a measure of mean fluorescent intensity on logarithmic scales, of Gal-8N binding before and after treatment of cells with *A. ureafaciens* neuraminidase. **(f)** GeoMFI of Gal-8C binding before and after treatment of cells with *A. ureafaciens* neuraminidase. **(g)** Comparison of Gal-8, Gal-8N, or Gal-8C binding toward HL-60 cells following treatment with *A. ureafaciens* neuraminidase. Bars represent the percent change in cell surface binding when compared with the mean fluorescent intensity of nontreated cells $\pm$ S.D. This research was originally published in the Journal of Biological Chemistry [16] with permission from the American Society for Biochemistry and Molecular Biology

Fig. 2. Galectin-1 binding to immobilized HL-60 cells as an example of fluorescence-based solid phase assay to study GBP–glycoconjugate interactions on streptavidin-coated microplates

Fig. 3. Binding of Gal-1, LEA, and GS-II to immobilized desialylated and nontreated HL-60 cells. A portion of biotinylated and fixed HL-60 cells first were desialylated. Nontreated and desialylated HL-60 cells were treated with endo- β -galactosidase or β -galactosidase and immobilized on streptavidin-coated microtiter wells (100,000 cells/well). Fluorescently labeled A, Gal-1 (40 μ g/ml); B, LEA (100 μ g/ml); and C, GS-II (100 μ g/ml) were incubated with the immobilized cells. All assays were performed in triplicate, and the results are the means $\pm$ S.D. of three determinations. This research was originally published in the Journal of Biological Chemistry [26] with permission from the American Society for Biochemistry and Molecular Biology

Fig. 4.

Binding affinity of Gal-1 for immobilized desialylated and nontreated HL-60 cells.

Biotinylated, fixed, desialylated HL-60 cells (a) and biotinylated, fixed, nontreated HL-60 cells (b) were immobilized on streptavidin-coated microtiter wells (100,000 cells/well).

Various concentrations of Gal-1 were incubated with the immobilized cells in buffer with or without 20 mM lactose. All assays were performed in duplicate, and the results are the average of two determinations. This research was originally published in the Journal of Biological Chemistry [26] with permission from the American Society for Biochemistry and Molecular Biology