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13. ABSTRACT *(Maximum 200 words)*

Cell-extracellular matrix (ECM) interaction is important for cell differentiation in mammary epithelial cells. This cell-ECM interaction is mediated by integrins, a family of cell adhesion receptors that mediate cell-cell and cell-ECM interactions, and play a critical role in cell migration, proliferation, and differentiation. The integrin $\alpha 2\beta 1$ functions as a collagen and/or laminin receptor. Previous reports suggest that $\alpha 2\beta 1$ plays a critical role in normal mammary cell differentiation as well as in the pathogenesis of breast cancer. Therefore, $\alpha 2\beta 1$-ligand interaction can be a key factor in controlling breast cancer differentiation. The purpose of this study is to clarify the role of $\alpha 2\beta 1$-ligand interaction in breast cancer differentiation, and to elucidate the molecular mechanism of $\alpha 2\beta 1$-ligand interaction. During the first year of this project, I found that (1) ligand binding-defective mutant $\alpha 2$ can be stably expressed in a poorly differentiated mammary carcinoma cell line, Mm5MT, and (2) the collagen-contact sites in $\alpha 2\beta 1$ are localized in the three separate loop structures surrounding the MIDAS (metal ion dependent adhesion site) motif in the I(A) domain of $\alpha 2$. These findings will form the basis for the remainder of this study.

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Tetsuji Kamata 8/27/98

PI - Signature Date

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INTRODUCTION

Cell-extracellular matrix (ECM) interaction has a profound impact on cell structure and function (1). There is overwhelming evidence that the ECM influences mammary epithelial cell differentiation (2). When mammary epithelial cells are cultured in reconstituted basement membrane gels, they undergo morphogenesis and exhibit many of the characteristics of alveoli *in vivo* (3). Cell-ECM interactions are altered in malignant neoplastic cells. The loss of normal cell-ECM interaction is associated with a loss of cellular differentiation and a loss of adhesion-dependent regulation of cell growth (4). Cell-ECM interaction is mediated through a variety of cell surface receptors including integrins, which play a major role in cell-ECM interactions. Integrins are a supergene family of adhesion receptors that consist of at least 15 α and 8 β subunits (5-9). Each integrin molecule is composed of structurally different α and β subunits and functions as a receptor for extracellular matrix proteins or cell surface ligands. Structurally, each α or β subunit consists of a large extracellular domain, a transmembrane domain, and a short cytoplasmic domain. The ligand binding specificity of each integrin is determined by its combination of α and β subunits. Many integrins have overlapping binding specificities. For example, the integrins $\alpha1\beta1$ and $\alpha2\beta1$ both bind to collagen and laminin. Besides binding to the ECM and cellular ligands, integrins contribute to the regulation of cell shape, morphology, and motility by assembling cytoskeletal proteins through cytoplasmic domains. Also, integrins activate a variety of signal transduction pathways(10).

What role do integrins play in mammary differentiation? Mammary cells acquire a glandular morphology and express mammary-specific genes when cultured in Matrigel(3, 11). The first clue to suggest the role of integrins in these processes comes from a paper by Streuli et al(2) in which they report that β-casein expression in mammary epithelial cells requires $\beta1$ integrins. Another clue comes from a paper on the analysis of $\alpha2\beta1$ expression in normal and neoplastic carcinoma tissues(12) in which it is reported that, while normal mammary epithelial cells express high-level $\alpha2\beta1$ integrin, neoplastic mammary tissue expresses less $\alpha2\beta1$ as it becomes less differentiated. Berdichevsky et al. showed that collagen-induced morphogenesis of mammary epithelial cells in a monolayer culture could be inhibited by anti-$\alpha2$ or anti-$\beta1$ monoclonal antibodies(13). This same group also showed that the

expression of c-*erb*B2 in mammary epithelial cells causes a decrease in α2β1 expression and impairs the cells' ability to undergo morphogenesis in a collagen gel(14). More direct evidence of α2β1 participation in mammary cell differentiation comes from a paper by Zutter et al(15) in which they report that the expression of α2β1 integrin in poorly differentiated mammary carcinoma cells resulted in a phenotypic alteration from fibroblastoid, non-contact-inhibited, invasive cells to epithelioid, contact-inhibited, less invasive cells. Expression of α2β1 also restored the cells' ability to differentiate into gland-like structures in three-dimensional matrices, and markedly reduced their tumorigenicity. The same group also reported that the reduction of α2β1 expression in well differentiated mammary carcinoma cells resulted in the loss of morphogenesis in collagen gels(16). These reports clearly indicate that α2β1 is critically involved in the differentiation of normal mammary cells as well as that of mammary carcinoma cells. However, the molecular mechanism that leads to differentiation is still not clear. Is α2β1-collagen interaction required, or is the expression of α2β1 itself enough for differentiation to take place? If α2β1-collagen interaction is required, what is the role of the α2β1 cytoplasmic domains? Are the α2β1 cytoplasmic domains specifically required? And if the α2β1 cytoplasmic domains are required, what kind of signaling molecules are specifically involved in this process? I intend to answer these questions by expressing ligand-binding defective α2 (α2 that has mutations in its cytoplasmic domains) in a poorly differentiated mammary carcinoma cell line. Finding the answers to these questions will bring us closer to developing therapeutic interventions against breast cancer.

Modulation of α2β1-ECM interaction could affect mammary cell differentiation (13). Therefore, elucidation of α2β1-ligand binding mechanisms may lead to the development of therapeutic interventions against breast cancer. α2β1 integrin is a receptor for collagen and laminin. The ligand binding site(s) in α2β1 has been localized in the I (A) domain of the α2 subunit (17, 18). Although several amino acid residues that constitute the metal ion-dependent adhesion site (MIDAS) are critical for ligand binding (19), the specific contact site for each ligand is not well characterized. Based upon the crystal structure of the α2 I domain (20), I will try to identify the specific ligand binding site for collagen or laminin using domain-

swapping mutagenesis and site-directed mutagenesis. Once the specific mutations that block either collagen or laminin are determined, I will express these mutations in a breast cancer cell line and examine their effect on differentiation. The informaion obtained in this study may form the basis for the development of therapeutic interventions against breast cancer.

BODY

Experimental Methods and Procedures

Mutagenesis

Site-directed mutagenesis was carried out by unique restriction enzyme site elimination with a double stranded vector (21). Loop-swapping mutagenesis was carried out using the overlap extension polymerase chain reaction (22). The amino acid residues in α2 that were replaced with the corresponding αL residues are 152-157 (L βA-α1), 212-219 (L α3-α4), and 257-262 (L βD-α5). In del αC, residues 283-290 including the C-helix are deleted from α2. The entire I domain (residues 140-367) is deleted from α2 in del I.

Cell culture and transfection

The Murine poorly differentiated mammary carcinoma cell line Mm5MT was purchased from the American Tissue Culture Collection (ATCC). Fifty μg of wild-type or mutant α2 cDNA was transfected into Mm5MT by electroporation. Forty-eight hours after transfection, cells were harvested and surface-expression of α2 was examined by FACS analysis. For stable transfection, 50 μg of wild-type or mutant α2 cDNA was transfected together with 2 μg of pFneo containing a neomycin resistant gene. After transfection, cells were grown in selection medium containing 300 μg/ml G418. After 3 weeks, the resulting colonies were harvested with trypsin-EDTA and surface-expression of α2 was examined by FACS analysis.

Transfection of wild-type and mutant α2 into CHO-K1 cells was done as described in (18).

Adhesion Assay

Adhesion of CHO cells transfected with α2 to immobilized collagen type I was quantitated as previously described (18).

Results and Discussion

Wild-type and mutant α2 are surface-expressed in Mm5MT

We previously reported that mutation of Asp-151, Thr-221, and Asp-254 to Ala (D151A, T221A, and D254A, respectively) in α2 completely abolished α2β1 binding to collagen (17, 18). Mutant α2 cDNA subcloned into the mammalian expression vector pBJ-1 was transfected into the mouse mammary carcinoma cell line Mm5MT by electroporation. This cell line lacks endogenous α2β1 integrin and is known to undergo morphogenesis after α2β1 transfection. Fig.1 shows the expression of wild-type and mutant α2 in Mm5MT after transient transfection. All mutants showed expression comparable to that of wild-type (approximately 8.9-14.7% of cells express α2 on the cell surface). I also obtained Mm5MT stably expressing α2 mutants (data not shown). Although the proposed project did not progress as expected due to the difficulty in transfection, these results indicate the feasibility of the current project.

Discontinuous multiple loops in the I domain are required for collagen binding

Amino acid residues that constitute the MIDAS motif, e.g. Asp-151, Thr-221, and Asp-254 in α2, are critical for both divalent cation binding and ligand binding (17, 18). These residues are well conserved among different integrin I domains irrespective of their ligand binding specificities (19). These results suggest that there are amino acid residues that allow specific ligands to bind to particular integrins. By replacing short segments of the α2 I domain with the homologous residues of the αL I domain, which has different ligand binding specificities, and examining the effect on ligand binding, we will be able to identify the specific segments/amino acid residues in α2 that are required for collagen/laminin binding. Based on the crystal structure of the α2 I domain, I replaced loops surrounding the MIDAS motif with the homologous loops of αL (Fig.2). The βE-α6 loop contains an extra C-helix that can be seen only in collagen-binding integrin I domains. This C-helix is believed to create a groove on top of the MIDAS face in which a collagen triple helix docks (20). I created a deletion mutant which deletes most of the

βE-α6 loop including the C-helix. Fig. 3 shows the result of adhesion of clonal CHO cells expressing mutant α2 to immobilized collagen type I. The L βA-α1 and L α3-α4 mutants almost completely abolished collagen adhesion even though the MIDAS motif is maintained. The L βD-α5 mutant partially affected collagen adhesion. Conversely, the del αC mutant did not have any impact on collagen adhesion, even though αC is predicted to have a collagen contact site. These results suggest that the collagen contact sites are localized in the βA-α1, α3-α4, and βD-α5 loops of the α2 I domain, and that the C-helix does not contain any critical collagen contact sites. These results are consistent with the fact that the von Willebrand factor A3 domain that does not contain a C-helix also binds to collagen (23). I introduced multiple alanine point mutations within the residues in the βA-α1, α3-α4, βD-α5, and βE-α6 loops to identify specific residues that are required for collagen binding. Preliminary results suggest that Ileu-156 and Tyr-157 in βA-α1; Gln-215, Gly-217, and Gly-218 in α3-α4; and Gly-254 in βD-α5 are critical for collagen binding, even though mutations in the amino acid residues in the βE-α6 loop did not affect collagen binding (data not shown).

Recomendations in Relation to the Statement of Work

I encountered an unexpected difficulty in transfecting α2 cDNAs into the mammary carcinoma cell line Mm5MT that caused a delay in this project. While a low transfection efficiency could be overcome by increasing the amount of cDNA used and the electroporation voltage, reliably obtaining a stably transfected cell line is still a problem. Although the current method is working, I intend to try other selection markers and vectors to improve transfection stability and efficiency.

Although the identification of collagen/laminin binding sites in the α2 I domain was not included in the original proposal, this information may be needed to manipulate mammary cell differentiation. The specific residues required for laminin binding can be identified by using laminin as an adhesion substrate. Once the binding site(s) for collagen and laminin are identified, the specific roles of collagen and laminin in breast cancer cell differentiation can be determined using the proposed assay.

CONCLUSIONS

1) Mutant α2 can be stably expressed in a poorly differentiated mammary carcinoma cell line that lacks endogenous α2β1. I will be able to use this system to determine the role of α2β1 in breast cancer differentiation as stated in the original proposal.

2) The α2β1-collagen contact face has been localized in the 3 separate loop structures surrounding the MIDAS motif. The α2b1-laminin binding face will be determined using the same methodology. The separate roles of collagen and laminin in α2β1-induced breast cancer differentiation can be clearly determine d by using mutants that specifically blocks either collagen or laminin binding.

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Legends to the figures

Fig.1. **Surface-xpression of wild-type and mutant α2 in Mm5MT.** Forty-eight hours after transfection, cells were harvested and incubated with mouse IgG, HAS-3 (anti-α2 N-terminal), or 12F1 (anti-α2 I domain), followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin. Stained cells were analyzed by flowcytometry. Percent α2 expression as calculated by HAS-3 positivity are 4.36, 14.71, 9.99, 9.83, 8.90, 13.99 for Mm5MT, wild-type, D151A, T221A, D254A, del I, respectively.

Fig. 2. **Alignments of human α2 and αL I domains.** The β strands
and α helices of the α2 I domain are underlined. Swapped regions of α2 and αL (L βA-α1: 152-157; L α3-α4: 213-219; L βD-α5: 257-262) are outlined in the box. Deleted region in a2 (del aC) is also outlined.

Fig. 3. **Adhesion of the mutant α2-expressing CHO cells to collagen.** Clonal CHO cells stably expressing wild-type or mutant α2 were added to the wells coated with BSA (□), collagen type I (■), or fibronectin (▨). After 1 hour at 37 C, non-adherent cells were removed and the adherent cells were quantitated by endogenous phosphsatase assay.

Figure 1

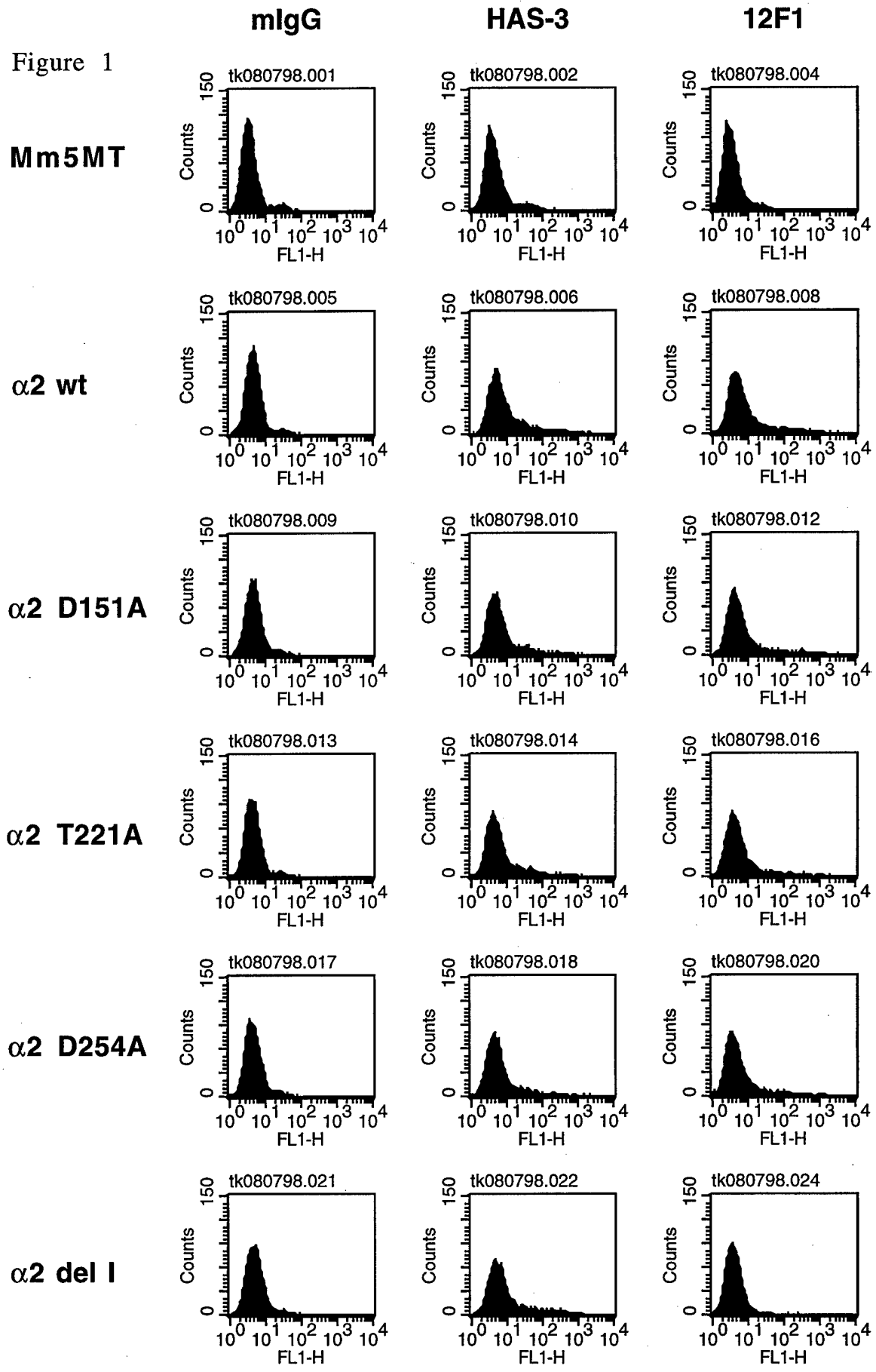
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Figure 2

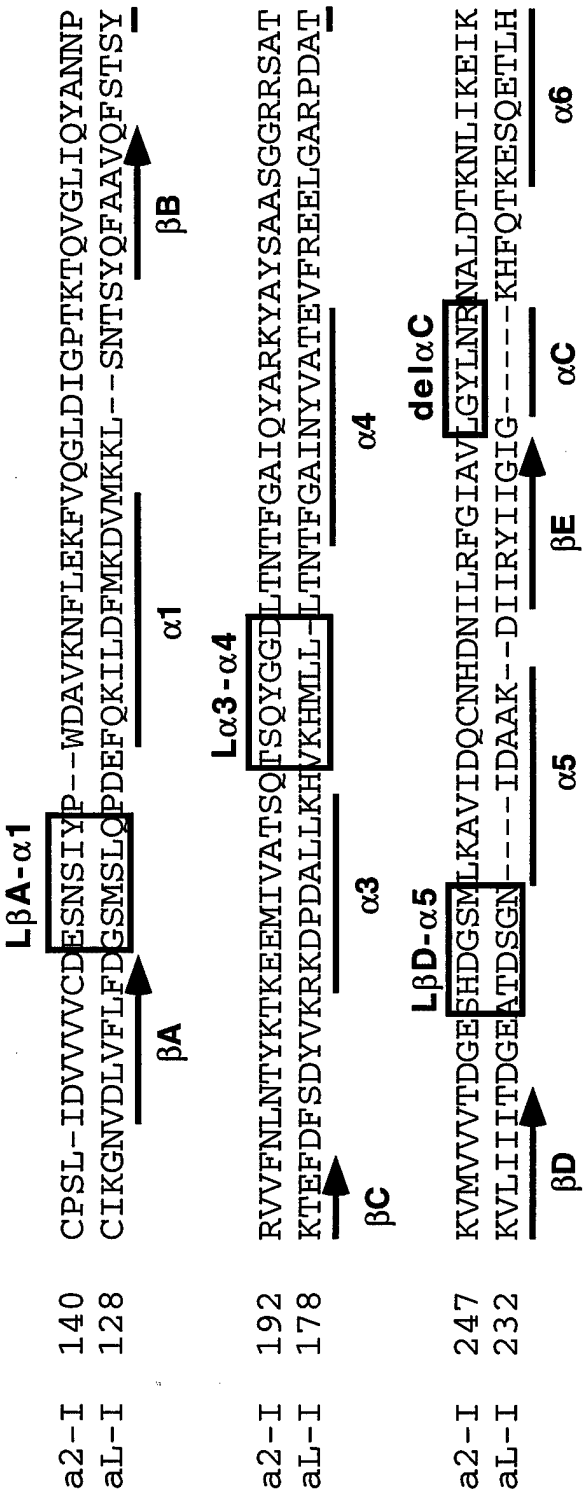
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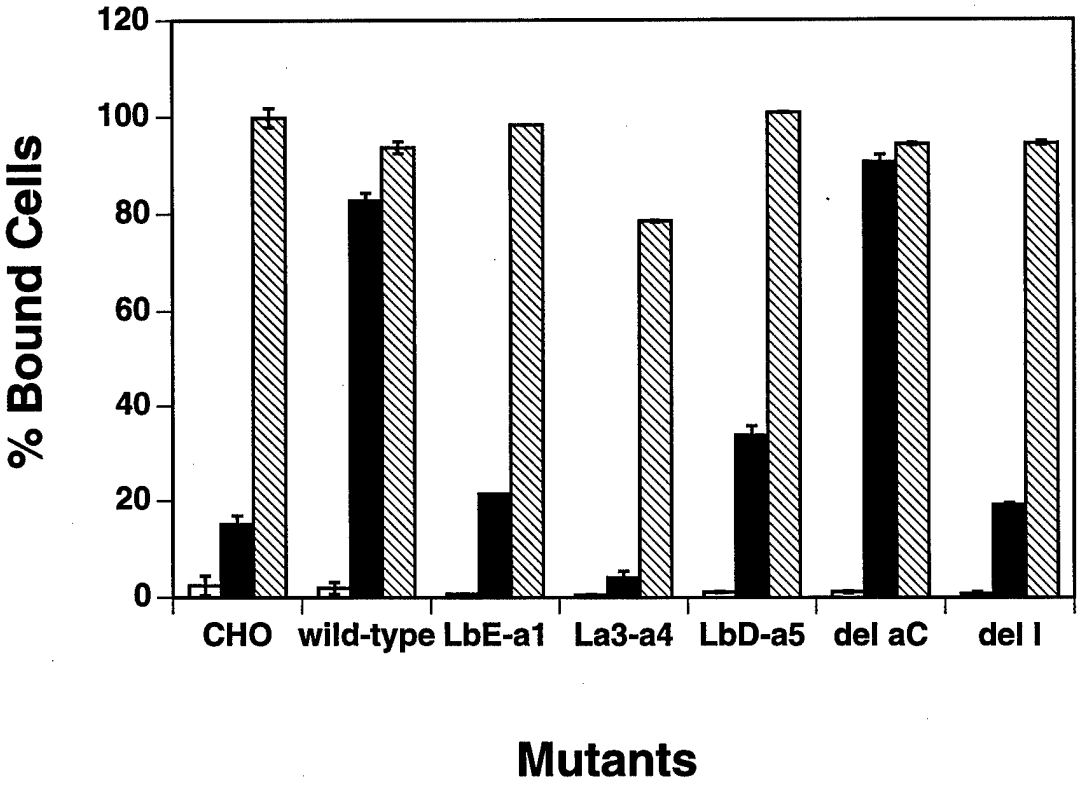
Figure 3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del I
Mutants