Detection of L- and E-selectin ligand by ligand blotting using L- and E-selectin-IgM chimeric proteins

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Method Article

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Abstract

Introduction

Selectin-IgM chimeric proteins are commonly used to detect selectin ligands on tissue sections or cultured cells. There is no report to detect the ligands on membrane blot. Here we describe about ligand blotting using mouse E-selectin-IgM and L-selectin-IgM. E-selectin-IgM blotting is easier than L-selectin IgM blotting probably because of the higher affinity of E-selectin and its ligand.

Reagents

E- and L-selectin-IgM cDNA in pcDNA1 or equivalent vector HRP-conjugated anti-human IgM, Pierce Immobilon-P PVDF filter, Millipore Washing buffer: 10 mM HEPES-NaOH pH 7.4, 130 mM NaCl, 1.5 mM CaCl2, and 0.05% Tween 20. To remove precipitates from the buffer, filtrate the solution through 0.22 um filter after stirring for at least 20 min.

Procedure

Expression of E- and L-selectin-IgM chimeric protein in COS cells 1. Transfect COS-1 cells with pcDNA1-E/L-selectin-IgM. 2. Two to three days later, collect culture supernatant . 3. Concentrate the medium using Centriprep YM-30 or YM-100 for 10 to 50 fold. The concentration rate is dependent on each experiment. The chimeric proteins can be used without further purification. **Ligand blotting** 4. Perform SDS polyacrylamide gel electrophoresis of desired materials \(for example, lymph node lysates) and transfer proteins onto Immobilon-P by standard procedure. 5. Block the blot with 10 mg/ml bovine serum albumin in washing buffer for 30 min. 6. Incubate the filter with the concentrated culture supernatant containing E/L-selectin-IgM without dilution for 1 hr at 4°C. 7. Wash three times with washing buffer. Each wash should be 20 min long. We usually put ice in an ice basket, place a washing container on the ice and covered the basket with a lid. 8. Incubate the blot with 10,000 x diluted HRP-anti-human IgM for 1 hr at 4°C. 9. Wash as in 6. 10. Detect with chemiluminescent HRP substrate.

Timing

3 days to obtain E/L-selectin-IgM chimeric proteins. 24 hrs for ligand blotting.

Critical Steps

Filtration of washing buffer is essential to avoid dirty staining of the filter caused by insoluble calcium salt. Dilution of secondary antibody should be more than 10,000 times to prevent non-specific binding.

Troubleshooting

Probing with L-selectin-IgM is difficult because of weak binding of L-selectin and its ligands. If no signal is detected, incubate the blot with L-slectin or E-selectin chimera overnight. Also further concentrate the IgM chimeric protein.

Anticipated Results

When lymph node lysate was used as a source of E/L-selectin ligands, a strong signal of CD34 around 90 - 100 kD should be detected. Other minor signals around >200, 70, and 50 kD can be detected.

References

Mitoma, J., Bao, X., Petryniak, B., Schaerli, P., Gauguet, J. M., Yu, S.Y., Kawashima, H., Saito, H., Ohtsubo, K., Marth, J. D., Khoo, K. H., von Andrian, U. H., Lowe, J. B. and Fukuda, M. Critical functions of N-glycans in L-selectin-mediated lymphocyte homing and recruitment. _Nat Immunol_. **8**, 409-418. \(2007).

Acknowledgements

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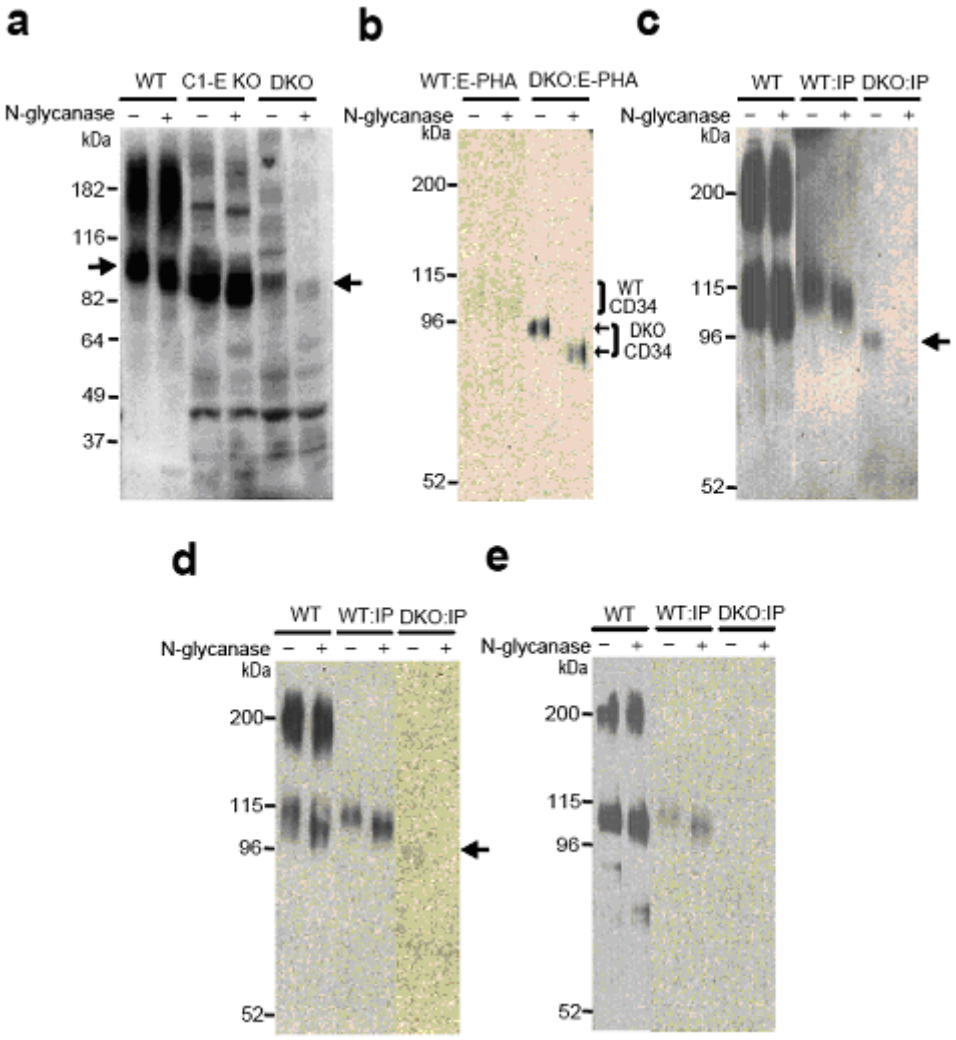
Figures

Figure 1

N-glycan-based L-selectin ligands detected by E- or L-selectin chimeric protein (a) E-selectin-IgM binding to lymph node stroma of indicated mice before and after N-glycanase treatment. Arrows denote migration of CD34. (b) E-PHA-bound proteins detected by the CD34-specific antibody. (c-e) CD34 from WT and DKO mice was immunoprecipitated, treated with or without N-glycanase, and blotted with E-selectin-IgM (c), L-selectin-IgM (d), and MECA-79 (e). Two left lanes in (a-e) are wild-type lymph nodes.


a
WT
C1-E KO
DKO
N-glycanase
kDa
182
116
82
64
49
37
b
WT:E-PHA
DKO:E-PHA
N-glycanase
kDa
200
115
96
52
WT CD34
DKO CD34
c
WT
WT:IP
DKO:IP
N-glycanase
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e
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WT:IP
DKO:IP
N-glycanase
kDa
200
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Figure 2