HLA-E: strong association with β_2m and surface expression in the absence of HLA class I signal sequence-derived peptides

SUPPLEMENTAL MATERIAL

Elisa Lo Monaco, Leonardo Sibilio, Elisa Melucci, Elisa Tremante, Miloslav Suchànek, Vaclav Horejsi, Aline Martayan, and Patrizio Giacomini

Figure S1

Neuraminidase digestion of HLA-E heavy chains. W6/32 immunoprecipitates from the NP40 extracts of the indicated, HLA-E genotyped (see Materials and Methods) cell lines were either digested with neuraminidase (+), or mock incubated (-), and IEF blotted with MEM-E/02. A portion of the gel is shown. Charge variants due to HLA-E dimorphism and sialylation were assigned as described in Results.

Figure S2

Immunodepletion of HLA-B7-β_2m complexes in MEM-E/06 immunoprecipitates. NP40 extracts from non-radiolabeled 221.B7 transfectants were either mock-immunodepleted in the presence of an irrelevant antibody (-) or immunodepleted with BB7.1, an allele-specific antibody to conformed HLA-B7 heavy chains (Brodsky et al., Immunol. Rev. 47:3, 1979; fully referenced in the paper), and then immunoprecipitated with W6/32, BB7.1, Namb-1 and MEM-E/06, as indicated. Immunoprecipitates were resolved by SDS-PAGE, and blotted. HLA-E, HLA-B7 and β_2m were identified by staining filters with MEM-E/02, HC10 and Namb-1,

respectively. BB7.1 immunoprecipitated HLA-B7 but not HLA-E from 221.B7 soluble extracts (lanes 2 and 10), and did not appreciably deplete HLA-E heavy chains in Namb-1 and MEM-E/06 immunoprecipitates (compare lanes 4 to 7 and 5 to 8), as expected. However, BB7.1 partially depleted HLA-B7 heavy chains and β_2m immunoprecipitated by Namb-1 (compare lanes 12 to 15, and 20 to 23). Partial depletions are not surprising, since antibodies to polymorphic epitopes are known to bind more restricted populations of class I molecules as compared with antibodies to non-polymorphic epitopes (Brodsky et al., Immunol. Rev. 47:3, 1979). In addition, depletion of the entire β_2m pool by BB7.1 is impossible, since antibodies to β_2m, including Namb-1, bind β_2m even when free of class I heavy chains (Brodsky et al., Immunol. Rev. 47:3, 1979). Despite partial depletions, BB7.1 extensively depleted the HLA-B7 and β_2m components present in MEM-E/06 immunoprecipitates (compare lanes 13 and 16, and lanes 21 and 24), conclusively showing that the β_2m molecules present in MEM immunoprecipitates are mainly associated with HLA-B7 heavy chains.

Figure S3

Antibodies to HLA-E detect linear epitopes carried by denatured HLA-E heavy chains. NP40 extracts (100 μg/lane) of 221 cells, 221.B7, 221.AEH, and the murine cell line NIH-3T3 (selected across the species barrier as a suitable HLA-E-negative control) were boiled in reducing SDS-PAGE loading buffer containing 2% SDS and 2-mercaptoethanol, electrophoresed, transferred to replicate nitrocellulose filters, and blotted with the indicated antibodies to HLA-E, to classical class I heavy chains (HC10), and to human ERp57 (cross-reacting with murine ERp60). HLA-B7

molecules (reactive with MEM-E/06 and HC10) differ from HLA-E in their electrophoretic mobility.

Figure S4

Characterization of β_2m-associated HLA-E heavy chains. The indicated cell lines and transfectants were metabolically labeled, lysed, immunoprecipitated, and electrophoresed on an IEF slab as described in the legend to figure 2. Overexpression of β_2m in FO-1-β_2m transfectants (Martayan et al., *Br. J. Cancer* 80:639, 1999) was expected to be particularly favorable for HLA-E assembly, since HLA-E has been reported to have a low affinity for β_2m (Ulbrecht et al., *Eur. J. Immunol.* 29:537, 1999). Likewise, HLA-E assembly was expected to be efficient in 221.G1 and 221.Cw1, due to the generation of an adequate supply of peptide ligands from the signal sequences of permissive class I alleles (Braud et al., *Curr. Biol.* 8:1, 1998). In contrast, parental 221 and its partially isogenic 220 mutant (defective in tapasin but retaining HLA-Cw1 expression) either lack permissive alleles, or cannot efficiently load HLA-E with its ligands (Greenwood et al., *J. Immunol.* 153:5525, 1664; Braud et al. ibidem). Therefore, they were expected to provide HLA-E with poor assembly conditions. However, contrary to expectations, IEF analysis of W6/32 and Namb-1 immunoprecipitates from cells metabolically labeled at similar specific activities revealed a strong and preferential reactivity of HLA-E with Namb-1 in all cases (lanes 2, 3, 5, 6, 8 9, 12, 13, 16 and 17), whereas 5 of the other heavy chains (HLA-F, HLA-A25, HLA-B8, HLA-Cw7, and HLA-Cw1) were preferentially reactive with W6/32 (lanes 2, 3, 8, 9, 12, 13, 16 and 17), and one (HLA-G1) was similarly reactive with the two antibodies (lanes 5 and 6). Thus, HLA-E is a particularly good β_2m assembler even in the absence of canonical peptides. Impaired peptide loading and assembly in

220 as compared to 221.Cw1 cells is documented by an inversion in the ratios of β_2m-free to β_2m-associated HLA-Cw1 heavy chains, the former recognized by L31, the latter recognized by W6/32 and Namb-1 (lanes 11-13 and 15-17).

Figure S5

Sequential immunoprecipitation/immunodepletion of HLA-E molecules. An NP40 lysate of 221 cells (1 mg) was incubated 4 consecutive times with excess amounts of immunoadsorbents, e.g. 100 μl of depletion beads preloaded with 100 μg of W6/32, Namb-1 or control (mock) antibodies of irrelevant specificity for each depletion round. Aliquots (10 μl) of the depletion beads were removed from the immunoadsorbents at the indicated depletion rounds, eluted, run on an SDS-PAGE, and electroblotted to a nitrocellulose filter (lanes 1-6 and 14-19). Immunodepleted supernatants containing residual (if any) HLA-E heavy chains and β_2m were immunoprecipitated by W6/32 and Namb-1, and electrophoresed (lanes 7-13 and 20-26) side by side with the eluates from depletion beads. All depletion and immunoprecipitation steps were carried out at 4°C. An aliquot of the total 221 lysate (100 μg) was run in parallel (lanes 13 and 26). HLA-E and β_2m were revealed by staining filters with MEM-E/02 (lanes 1-13) and Namb-1 (lanes 14-26), respectively. In agreement with a good reactivity of HLA-E with Namb-1, we observed that >90% and >95% of HLA-E and β_2m, respectively, had already been removed by the anti β_2m Namb-1 antibody at the end of the second immunodepletion round, and essentially no residual heavy or light chains were left by the end of the fourth round (lanes 4 and 6 compared to lane 2; lanes 17 and 19 compared to lane 15). In contrast, depletion with W6/32 did not result in either a clearly detectable depletion of HLA-E heavy chains (lanes 3 and 5 compared to lane 1) or a significant recovery of β_2m

(lanes 14, 16, and 18) throughout the 4 depletion rounds. Accordingly, when depleted lysates were tested for residual HLA-E heavy chains, Namb-1 was found to have entirely immunodepleted the Namb-1-reactive HLA-E and β_2m components (compare lanes 12 to 8, and 25 to 21) and to have largely, although not completely, depleted W6/32-reactive HLA-E (lane 11 compared to 7; appreciable upon overexposure), whereas W6/32 depletions were largely unsuccessful (lanes 9-10 compared to lanes 7-8, and lane 23 compared to 21), and co-immunoprecipitation of β_2m was confirmed to be very poor (lanes 20 and 22). Because the best estimate of pool overlapping can be obtained by immunodepletion with the high affinity Namb-1 antibody, the W6/32 molecular pool is not completely contained within that recognized by Namb-1. Thus, W6/32 is likely to bind some β_2m-free HLA-E heavy chains.

Figure S6

Flow cytometry analysis of 221 transfectants with the MEM antibodies. Parental 221 cells and their single class I allele transfectants were stained with the MEM antibodies, with W6/32 as a control, and with an isotype-matched (IgG1), irrelevant antibody, and analyzed on a FACScan (Becton & Dickinson). The binding of the MEM antibodies is clearly detectable only on the surface of 221.AEH. Similar results were obtained with 3D12 and 4D12 (not shown).

Figure S7

Expression of β_2m-associated and β_2m-free HLA-E heavy chains on the cell surface. The indicated cells were surface labeled with either ^{125}I (A) or biotin (B). Soluble NP40 extracts were immunoprecipitated with the indicated antibodies, and immunoadsorbents were eluted and run on an IEF slab. The gels were either dried and

autoradiographed (A), or blotted onto nitrocellulose filters for chemiluminescence detection (B). Non-specific bands are indicated (n.s.). MEM-E/06 detected surface HLA-E heavy chains on 221.AEH but not 221.B7 cells (lanes 3 and 7). Likewise, MEM-E/08 did not appreciably react with 221, 221.G1 and FO-1-β_2m cells (lanes 10, 13, and 15). Even when surface-expressed, as in 221.AEH, MEM-reactive heavy chains were largely free of β_2m (lane 3), despite β_2m-associated HLA-E (as well as non-HLA-E) heavy chains were clearly detectable on the surface of all the tested cells by W6/32 and Namb-1 (lanes 1, 4, 5, 8, 11, 14, and 17). Cell surface-specific labeling was confirmed by the presence of the expected, neuraminidase-sensitive HLA-B8 heavy chains, whereas the major HLA-E*0101 band was neuraminidase-insensitive (lanes17-18), indicating that HLA-E can be surface expressed in the absence of sialylation. HLA-E was twice less abundant than HLA-B8 and slightly less abundant than HLA-A25 on the cell surface (compare neuraminidase-digested bands in lane 18).

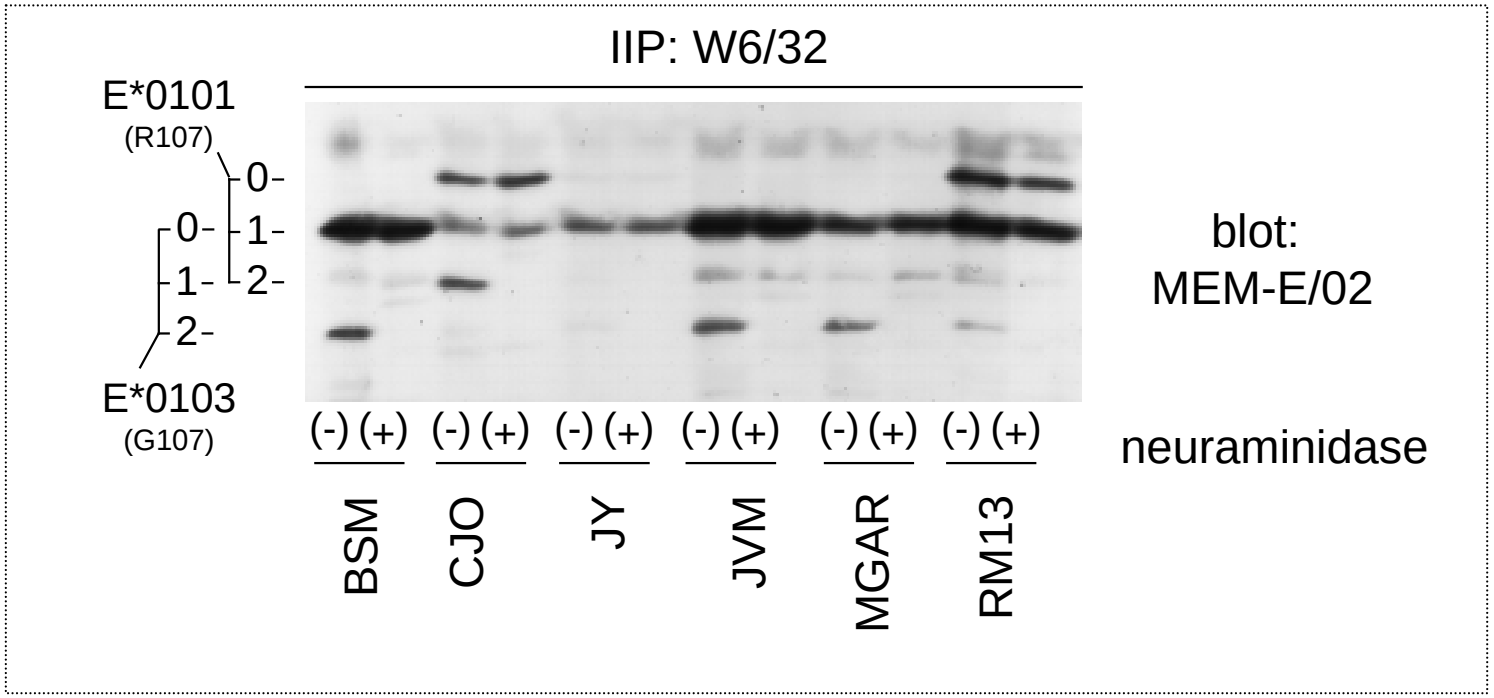
IIP: W6/32
E*0101
(R107)
0
0
1
1
2
2
E*0103
(G107)
(-) (+) (-) (+) (-) (+) (-) (+) (-) (+) (-) (+)
BSM
CJO
JY
JVM
MGAR
RM13
blot:
MEM-E/02
neuraminidase

Fig. S1

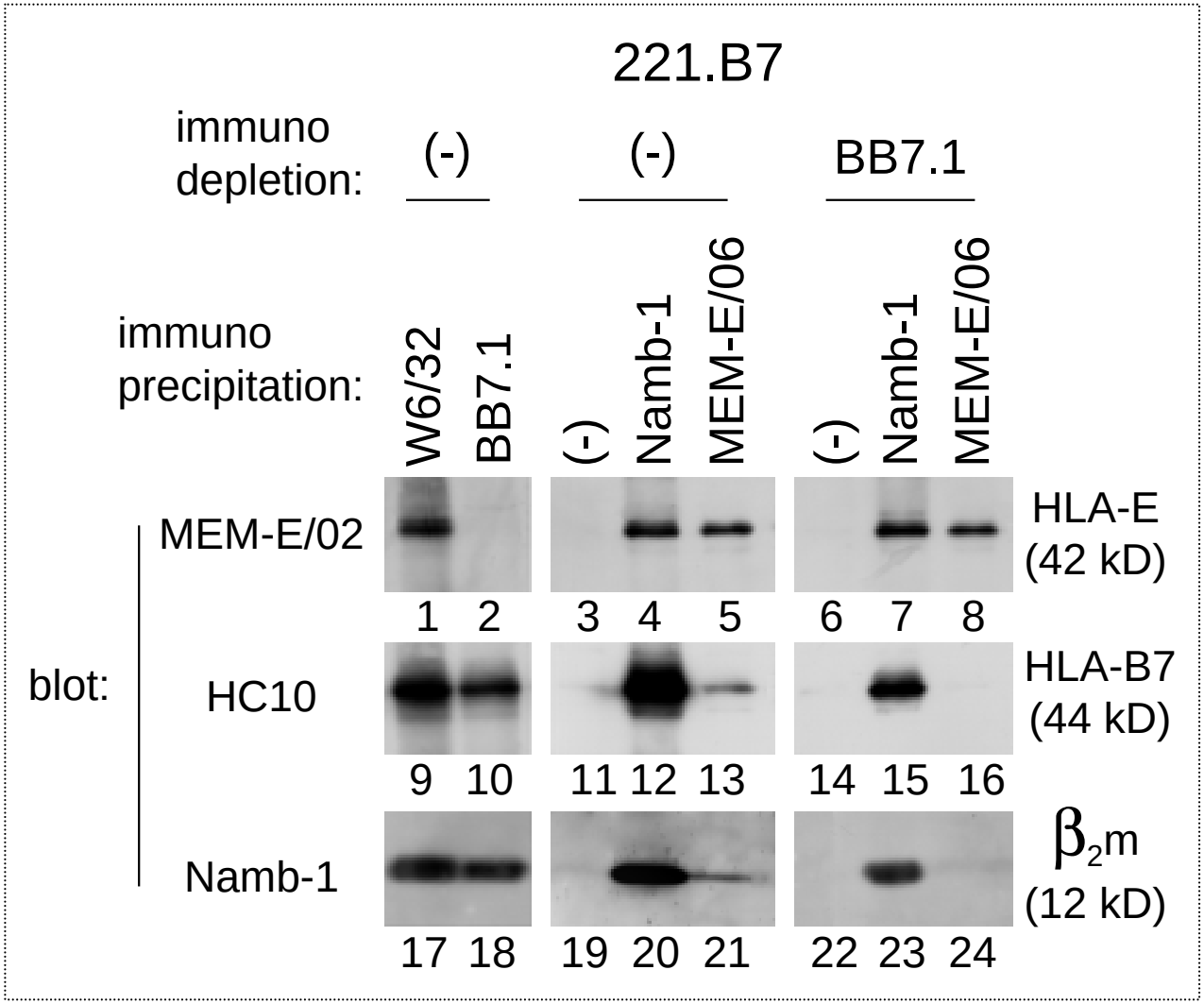
221.B7
immuno depletion:
(-)
(-)
BB7.1
immuno precipitation:
W6/32
BB7.1
(-)
Namb-1
MEM-E/06
(-)
Namb-1
MEM-E/06
blot:
MEM-E/02
HC10
Namb-1
HLA-E (42 kD)
HLA-B7 (44 kD)
β2m (12 kD)
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
17 18 19 20 21 22 23 24

Fig. S2

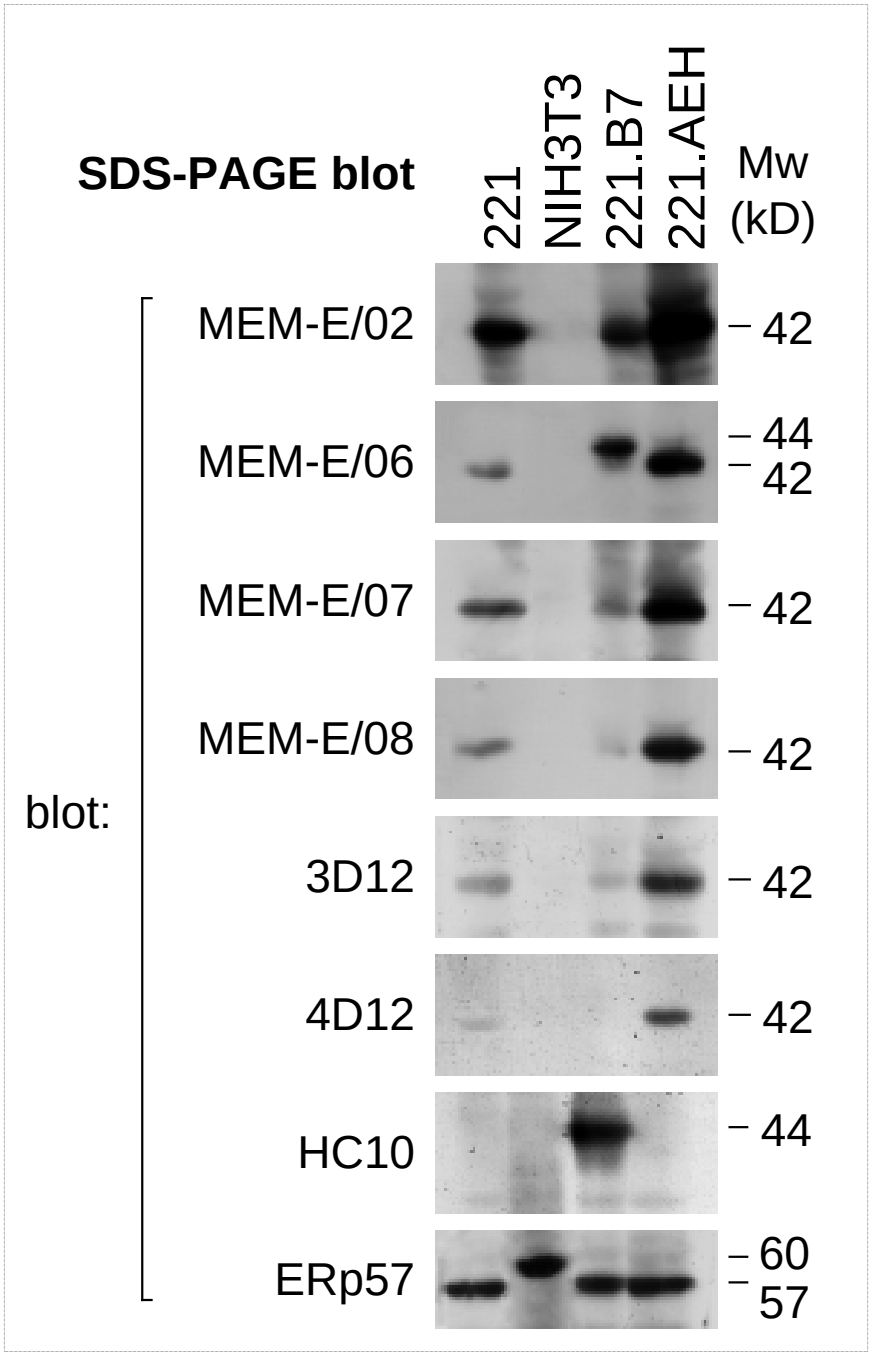
SDS-PAGE blot
221
NIH3T3
221.B7
221.AEH
Mw
(kD)
blot:
MEM-E/02
42
MEM-E/06
44
42
MEM-E/07
42
MEM-E/08
42
3D12
42
4D12
42
HC10
44
ERp57
60
57

Fig. S3

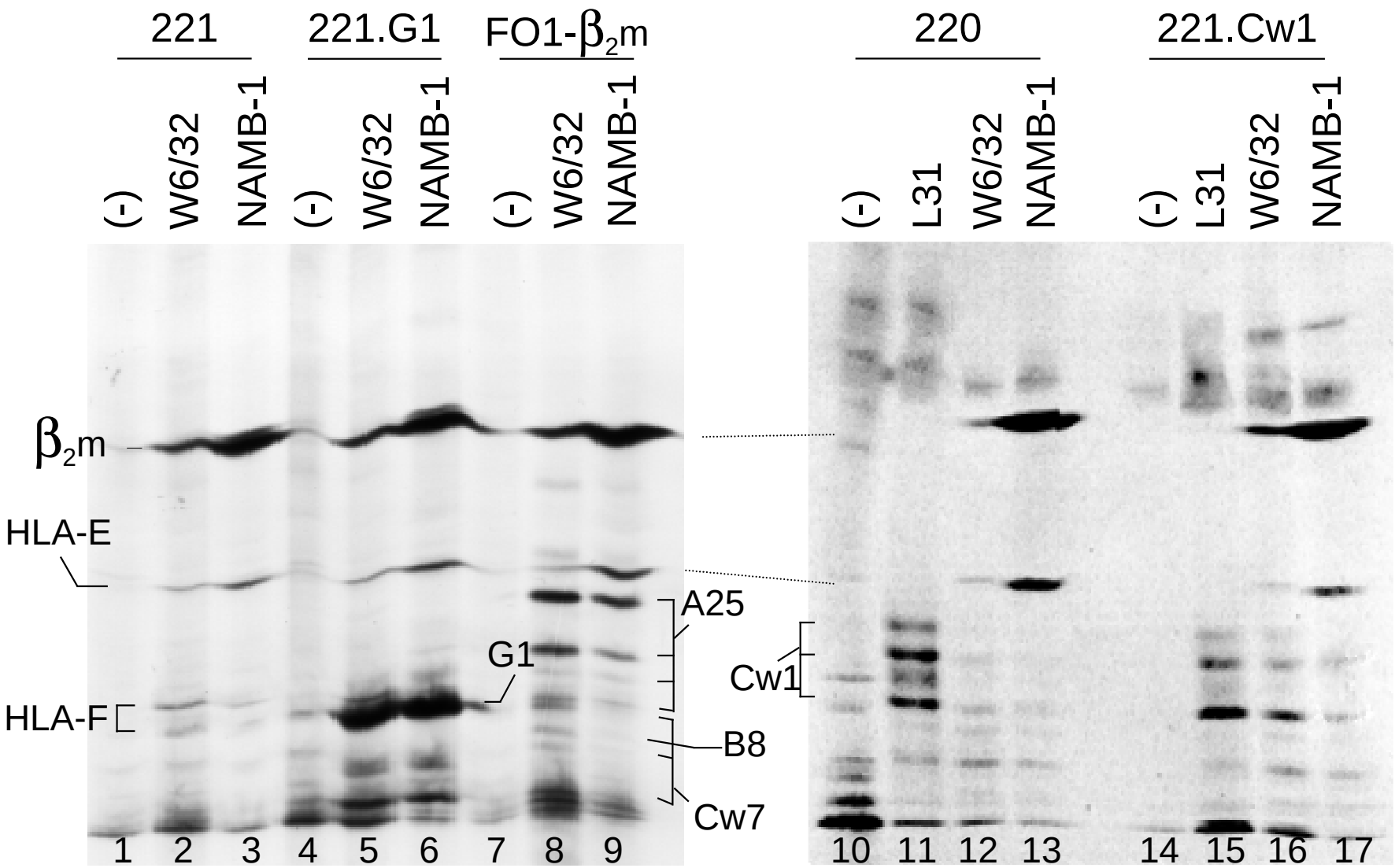
221
221.G1
FO1-β2m
220
221.Cw1
(-)
W6/32
NAMB-1
L31
β2m
HLA-E
HLA-F
G1
A25
Cw1
B8
Cw7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Fig. S4

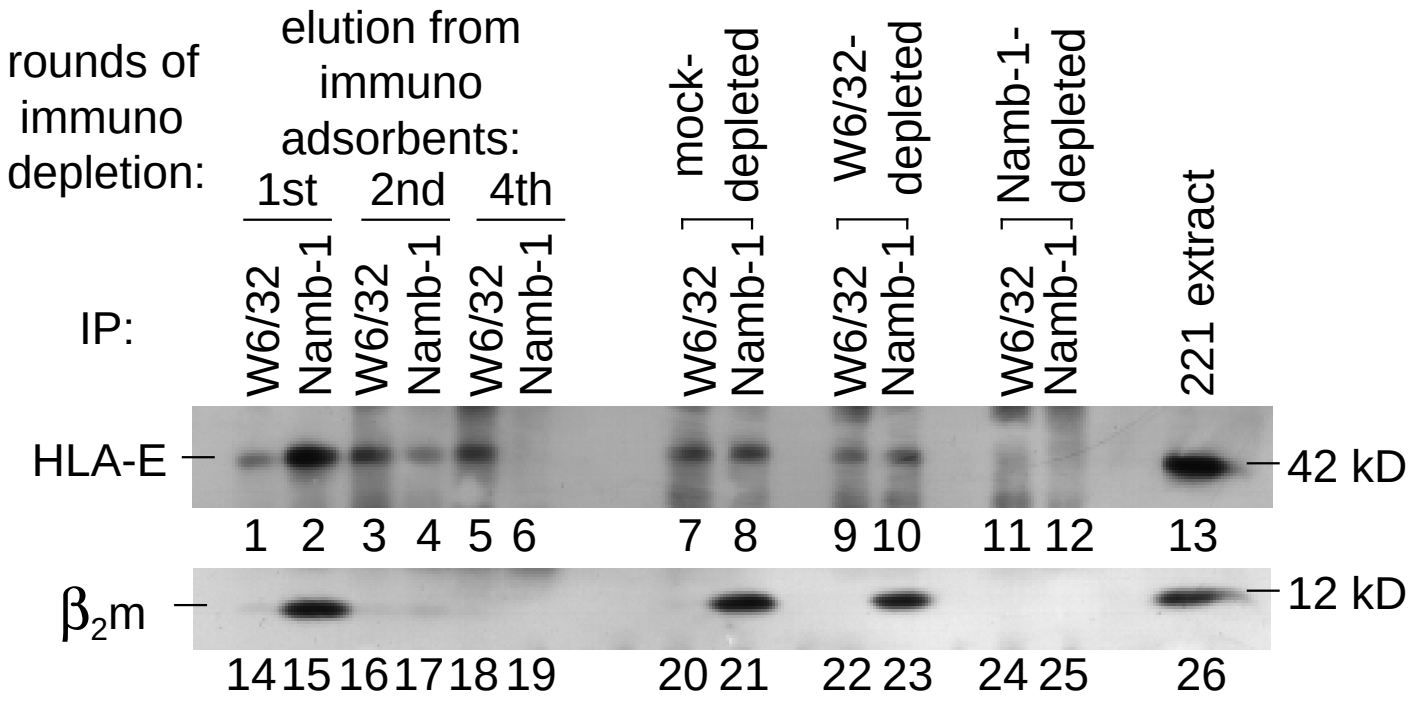
rounds of immuno depletion:
elution from immuno adsorbents:
1st
2nd
4th
mock-depleted
W6/32-depleted
Namb-1-depleted
IP:
W6/32
Namb-1
221 extract
HLA-E
42 kD
1 2 3 4 5 6 7 8 9 10 11 12 13
β2m
12 kD
14 15 16 17 18 19 20 21 22 23 24 25 26

Fig. S5

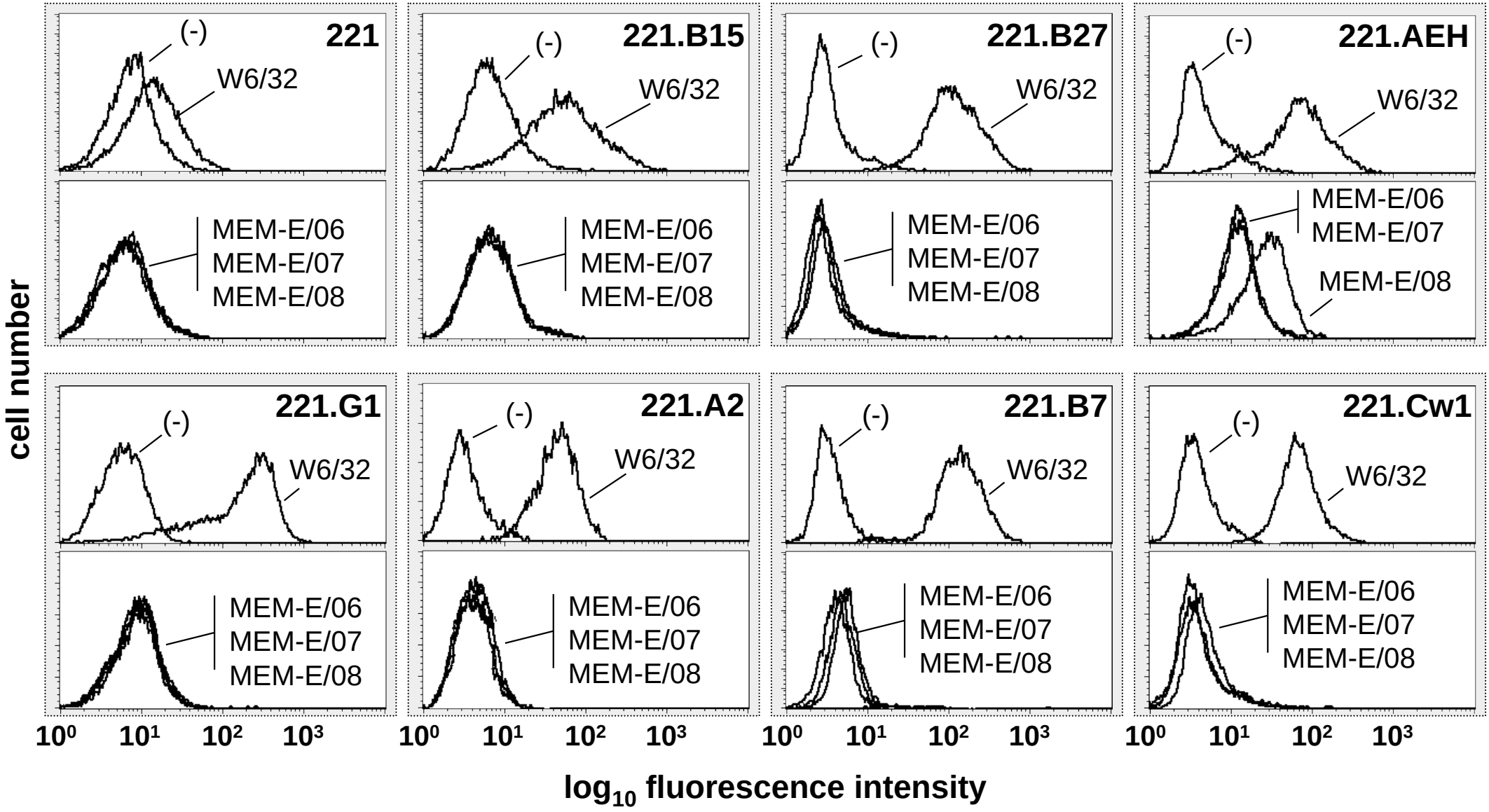
221
221.B15
221.B27
221.AEH
221.G1
221.A2
221.B7
221.Cw1
(-)
W6/32
MEM-E/06
MEM-E/07
MEM-E/08
cell number
10⁰ 10¹ 10² 10³
log10 fluorescence intensity

Fig. S6

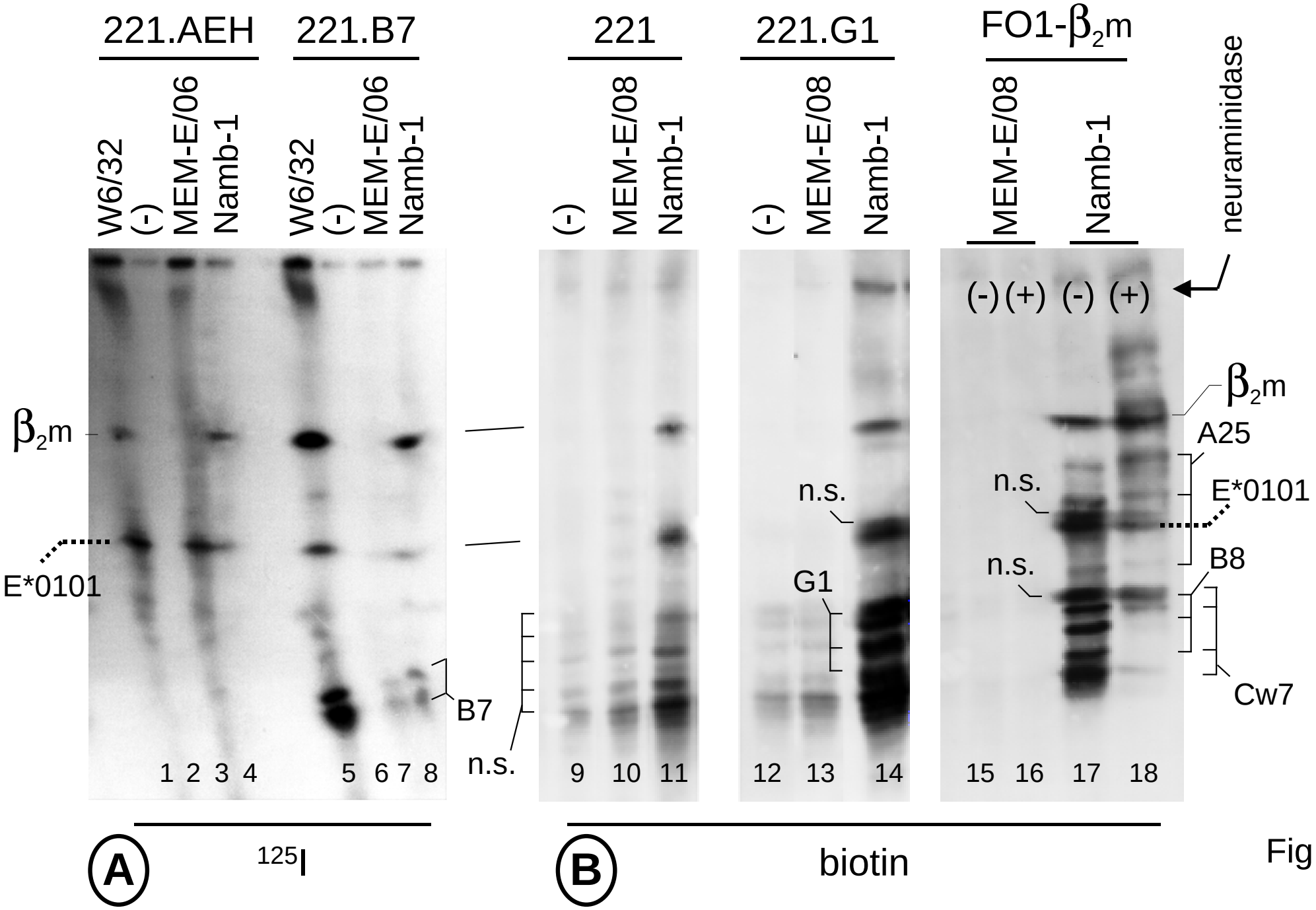
221.AEH
221.B7
221
221.G1
FO1-β2m
W6/32
(-)
MEM-E/06
Namb-1
MEM-E/08
neuraminidase
(+)
β2m
E*0101
B7
n.s.
G1
A25
B8
Cw7
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A
125I
B
biotin

Fig. S7