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Expression of Endoplasmic Reticulum Aminopeptidases in EBV-B Cell Lines from Healthy Donors and in Leukemia/Lymphoma, Carcinoma, and Melanoma Cell Lines[1]

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Peptide trimming in the endoplasmic reticulum (ER), the final step required for the generation of most HLA class I-binding peptides, implicates the concerted action of two aminopeptidases, ERAP1 and ERAP2. Because defects in the expression of these peptidases could lead to aberrant surface HLA class I expression in tumor cells, we quantitatively assayed 14 EBV-B cell lines and 35 human tumor cell lines of various lineages for: 1) expression and enzymatic activities of ERAP1 and ERAP2; 2) ER peptide-trimming activity in microsomes; 3) expression of HLA class I H chains and TAP1; and 4) surface HLA class I expression. ERAP1 and ERAP2 expression was detectable in all of the EBV-B and tumor cell lines, but in the latter it was extremely variable, sometimes barely detectable, and not coordinated. The expression of the two aminopeptidases corresponded well to the respective enzymatic activities in most cell lines. A peptide-trimming assay in microsomes revealed additional enzymatic activities, presumably contributed by other unidentified aminopeptidases sharing substrate specificity with ERAP2. Interestingly, surface HLA class I expression showed significant correlation with ERAP1 activity, but not with the activity of either ERAP2 or other unidentified aminopeptidases. Transfection with ERAP1 or ERAP2 of two tumor cell lines selected for simultaneous low expression of the two aminopeptidases resulted in the expected, moderate increases of class I surface expression. Thus, low and/or imbalanced expression of ERAP1 and probably ERAP2 may cause improper Ag processing and favor tumor escape from the immune surveillance. *The Journal of Immunology,* **2006, 176: 4869–4879.**

Aminopeptidases trim N-terminal amino acid residues of precursor polypeptides, playing important roles in various biological processes such as the maturation, degradation, modulation, and activation of proteins (1).

This N-terminal peptide trimming has been found to play a very important role also in MHC class I Ag processing (2). It customizes precursor peptides to fit them to the binding groove of MHC class I molecules. Precursor peptides produced from self- and non-self-proteins in the cytosol through the ubiquitin-proteasome degradation pathway are translocated into the endoplasmic reticulum (ER)[5] by the TAP heterodimer, trimmed to an optimal size, i.e., 8–10 residues, and then loaded onto HLA class I molecules. Although cytosolic aminopeptidases have been implicated in trimming (3–5), ER aminopeptidases are involved in the final and crucial step of the generation of MHC class I-binding peptides. Only optimally trimmed peptides with a defined binding motif are preferentially loaded onto MHC class I H chain/β_2-microglobulin (β_2m) dimers and then transported to the cell surface for recognition by CTLs. Thus, peptide trimming by ER aminopeptidases is a crucial link between Ag processing and peptide loading/Ag presentation.

Microsomes have been shown to contain peptide-trimming activity involved in the generation of MHC class I-binding peptides from larger precursors (6, 7). This trimming activity is thought to be mediated by a group of aminopeptidases. So far, two of them have been defined with respect to primary structure and substrate specificity. One was named endoplasmic reticulum aminopeptidase associated with Ag processing (ERAAP) in the mouse (8) and endoplasmic reticulum aminopeptidase 1 (ERAP1) in the rat and human (9). The latter denomination will be adopted throughout this study. ERAP1 corresponds to an aminopeptidase previously found in normal tissues of both human and murine origin, designated adipocyte-derived leucine aminopeptidase (A-LAP) (10), puromycin-insensitive leucyl-specific aminopeptidase (11), or aminopeptidase regulator of TNFR1 shedding 1 (12). Another aminopeptidase has been identified in humans and called leukocyte-derived arginine aminopeptidase (L-RAP) (13). This peptidase has high structural homology with ERAP1. Like ERAP1, it is detected in the ER lumen, enhanced by IFN-γ, and can trim precursors of

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[5] Abbreviations used in this paper: ER, endoplasmic reticulum; β_2m, β_2-microglobulin; ERAP, ER aminopeptidase; ERAAP, ER aminopeptidase associated with Ag processing; A-LAP, adipocyte-derived leucine aminopeptidase; L-RAP, leukocyte-derived arginine peptidase; AMC, aminoacyl-aminomethylcoumarin; pK, protein kinase; FI, fluorescence intensity; MFI, mean FI.

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MHC class I ligands in vitro. Because of these similarities, this aminopeptidase is also called ERAP2 (14). These two peptidases display distinct specificities when tested with aminoacyl-aminomethylcoumarin (aminoacyl-AMC) substrates: ERAP1 preferentially hydrolyzes Leu-AMC, while ERAP2 displays a preference for Arg-AMC and Lys-AMC. Recent detailed analysis indicates that these aminopeptidases have distinct specificities, particularly on certain polypeptides, and act in concert, in vivo, to remove some longer extensions from the precursor peptides (14). Previously, TLC was used to detect peptide trimming of TAP-translocated peptides in the ER and in the cytosol (15). A similar assay was used by us to quantify N-terminal trimming activity that converts a radioiodinated peptide substrate R-SLYNTVATL (abbreviated R-S9L or R10L) to SLYNTVATL (S9L) in the microsome vescicles (6). Recently, we found that this R-S9L-trimming assay detects several aminopeptidases in an EBV-B cell line that include ERAP2 and yet unidentified ER aminopeptidases and, furthermore, that ERAP2 is the main trimming enzyme among these aminopeptidases (14).

Suppression of ERAP1 expression by small interfering RNA was originally shown to partially reduce the surface MHC class I expression in mouse and human systems (16). We have subsequently confirmed this effect and demonstrated that partial suppression of surface HLA class I expression can also be obtained by suppression of ERAP2 expression by RNA interference (14). These data indicated a key role of ERAP1 and ERAP2 (possibly in combination with other ER aminopeptidases) in regulating the surface expression of HLA class I molecules.

Tumor cells can evade immune recognition by host CTLs by down-regulating MHC class I expression (17). Such down-regulated abnormal MHC class I expression has been found to be associated with defects in the genes encoding HLA-A, -B, and -C H chains, β_2m, TAP, and other components of the Ag-processing and presentation machinery. It is conceivable that abnormal MHC class I expression may result from defects in the expression of ER aminopeptidases, including ERAP1 and also ERAP2, leading to impaired peptide trimming in the ER.

In this study, in an attempt to place ER peptide trimming within the framework of our current understanding of Ag processing and presentation, we have expanded our studies on ER aminopeptidases to a large panel of EBV-transformed B cell lines from healthy blood donors and tumor cell lines derived from lymphoid and nonlymphoid cell lineages and characterized them for: 1) expression of ER aminopeptidases that include ERAP1, ERAP2, and as yet unidentified ER aminopeptidases and 2) expression of HLA class I H chains and TAP1. We have analyzed the correlation between surface HLA class I molecules and expression of the components involved in assembly of HLA class I Ags to explore the role of ER aminopeptidases in tumor cells. Finally, we have reconstituted the expression of ERAP1 and ERAP2 in two tumor cell lines selected because of their low expression of both aminopeptidases and analyzed their surface expression of HLA class I molecules.

Materials and Methods

Cell lines

The cell lines tested were composed of 15 EBV-B lymphoblastoid cell lines (EBV-B cell lines) from healthy donors; 5 leukemia-lymphoma cell lines, DAUDI (Burkitt's lymphoma), K562 (erythroid leukemia), MOLT4 (T lymphoblastoid), U937, (myelomonocytic leukemia), and WI-L2 (B lymphoblastoid); 15 carcinoma cell lines from breast (BT20, MCF7, and SK-BR3), colon (HT-29, LoVo, and LS174T), lung (A549 and Calu1), chorion (JAR and JEG-3), epidermoid, prostate, cervix, kidney and bladder (A431, H494, HeLa, KJ29, and T24, respectively); and 17 melanoma cell lines (501, 1102, 1182, 1290-2, 1704, Colo38, FO-1, HO-1, IR8, M10, M14, MEL249, MRN-1, SK-MEL37, SK-MEL93, SP6.5, and STP-1). In addition, 721.221 and 721.220 cell lines which lack the expression of HLA-A, -B, and -C molecules and tapasin, respectively, and T2 that lacks TAP were tested. T1, the parental cell line used to generate T2, was also included in this study. Cells were grown in RPMI 1640 supplemented with 10% FCS.

DNA constructs and transfection

Full-length cDNAs encoding human ERAP1 (accession number AF106037) and ERAP2 (accession number AB109031) (13) were *Eco*RI and *Bam*HI-*Xho*I cloned into the pCI-neo (Promega) (18) and pcDNA3-Hygro (Invitrogen Life Technologies) vectors, respectively. BT20 and HeLa cell lines were transfected with these cDNAs or the corresponding empty vectors using LipofectAMINE 2000 according to the manufacturer's instructions (Invitrogen Life Technologies). BT20 stable transfectants were selected with either 150 μg/ml G418 (Invitrogen Life Technologies) or 40 μg/ml hygromycin B (Sigma-Aldrich). HeLa transient transfectants were tested 48 h after transfection.

RT-PCR and sequencing

Total RNA was isolated with TRIzol (Invitrogen Life Technologies) from the CNC cell line according to the manufacturer's instructions and was retrotranscribed by random priming (Roche). The following primers were used to RT-PCR amplify an ~3000-bp region that includes the entire *ERAP2* gene, forward (Fw) 1: 5′-ATCTAAGCTTATGTTAAAAACATTCAACATGC-3′; reverse (Rv) 1, 5′-CCCTTCAAAGCCATCACCTA-3′; Fw2, 5′-CCACCCTTCAGTCAGAGGAA-3′, Rv2, 5′-TCATCAAATTGCAGCTCTGG-3′; Fw3, 5′-TGGCTTAATGAGGGTTTTGC-3′, Rv3, 5′-GCTGCTTGTTTCATGTTGGA-3′; Fw4, 5′-GTCATGGATGGGACCAACTC-3′, Rv4 5′TGAAGGAGAGCTGCCAAGTT-3′; and Fw5, 5′-TTTGTCAACGAGCAAGCATC-3′, Rv5, 5′TTAAGTATTAACCATTAGC-3′). The fragments were sequenced in a CEQ2000 automated sequencer (Beckman Coulter).

Antibodies

Rabbit polyclonal Ab and mouse mAb were used for detection or isolation of ERAP1 or ERAP2 (14, 18). Rabbit polyclonal Ab A-LAP was raised against recombinant A-LAP/ERAP1 (18). Mouse mAb 4D2 was raised against recombinant human ERAP1 and recognizes native, but not denatured ERAP1 (14). Mouse mAb 3F5 was raised against recombinant ERAP2 and recognizes both native and denatured ERAP2 (14). Mouse mAbs HCA-2 and HC10 were used for detection of HLA class I H chains. HCA-2 recognizes β_2m-free HLA-A (excluding -A24), -B7301, and -G H chains (19, 20), and HC10 recognizes β_2m-free HLA-B (excluding -B2702, -B5804, and -B73) H chains and a limited set of HLA-A H chains such as HLA-A3, -A10, -A28, -A29, -A30, -A31, -A32, and -A33 (19, 20). The mouse mAb W6/32, which recognizes β_2m-associated HLA-A, -B, -C, -E, and -G H chains (21, 22), was used to quantify cell surface expression of HLA class I Ags by flow cytometry. In addition, rabbit polyclonal Abs specific to TAP1 and ERp57 were used (23).

Western blot analysis

The procedures used have been described previously (23). In brief, cells were washed and lysed with 500 μl of a buffered saline (50 mM Tris (pH 7.5) and 150 mM NaCl) containing 1% Nonidet P-40 in the presence of a mixture of protease inhibitors (Sigma-Aldrich). Lysates clarified by high-speed centrifugation were assayed for protein concentration by the BCA assay kit (Pierce). Equal amounts of cell lysate (100 μg/lane) were subjected to SDS-PAGE on an 8% slab gel. After electrophoresis, the separated components were electroblotted onto a nitrocellulose membrane at 25 V overnight using 10 mM 3-(cyclohexylamino)-1 propanesulfonic acid (Sigma-Aldrich) buffer containing 10% methanol. Blots were incubated with purified polyclonal rabbit Abs or mouse mAbs, and then with a peroxidase-coupled secondary Ab and developed using an ECL detection kit (Amersham). Films were analyzed with the Scion Image Analysis software, and the intensity of each band was recorded and expressed as the fraction (percent) of the highest value obtained for each test component. Even sample loading was checked by staining with Ponceau S and with an anti-ERp57 polyclonal Ab (23).

ERAP1 and ERAP2 activity assay

ERAP1 and ERAP2 were isolated from cell lysates by using specific immunoabsorbents and assayed for cleavage activity against aminoacyl-AMCs. Briefly, cells were lysed at 4°C in 500 μl of a 1% Triton X-100 buffer (50 mM Tris (pH 7.5) and 150 mM NaCl). After high-speed centrifugation, clarified lysates containing 500 μg of proteins were incubated

overnight at 4°C with protein G-Sepharose beads coated with preimmune serum and then for 2 h at 4°C with protein G-Sepharose beads precoated with 10 μg of mAb 4D2 for ERAP1 or mAb 3F5 for ERAP2. ERAP1- or ERAP2-bound Sepharose beads were washed three times and suspended in 100 μl of a Tris-HCl buffer (50 mM Tris (pH 7.5) and 1 mM DTT) containing 40 μM Leu-AMC for ERAP1 and 40 μM Arg-AMC for ERAP2. After 1 h at 37°C, the reaction was terminated by addition of 1% SDS and the fluorescence intensity was measured by an LS50B fluorometer (PerkinElmer) with excitation at 380 nm and emission at 440 nm. The assays were run under Ab excess conditions that were determined by the preliminary experiments. A Lys-AMC substrate preferentially cleaved by ERAP2 was also used in some cases, as indicated. To determine the purity of the proteins bound to Ab-coated beads, these beads were suspended in 100 μl of gel-loading SDS buffer and subjected to Western blot analysis as described above.

TLC assay of ER aminopeptidase activity

This assay was used to measure peptide-trimming activity of isolated microsomes as described previously (6). Microsomes were isolated from 10^9 cells by sucrose gradient centrifugation, adjusted to equal protein concentrations, snap-frozen, and stored at −80°C. The preparations were usable up to 60 days without loss in peptide-trimming activity. Microsomes (50 μl) were treated with 0.4 mg/ml proteinase K (pK) for 30 min at 4°C, followed by washing and incubation with 2 mM PMSF for 30 min at 4°C and then lysed in PBS with 1% CHAPS (Sigma-Aldrich). Microsomes thus treated were incubated for 3 min with ^{125}I-radiolabeled peptide RSLYN TVATL (R-S9L) (132 nM; sp. act., 60 cpm/fmol) at 37°C. The reaction was terminated by the addition of an equal volume of phenol. Untreated microsomes and pK-treated microsomes were included in the assay as controls. Four microliters of the organic phase was subjected to TLC on silica gel 60 plates (Merck) using a mixture of *N*-butanol/pyridine/acetic acid/water (97:75:15:60) as described elsewhere (15). Spot radioactivities were quantified using the ImageQuant 5.0 software (Molecular Dynamics).

HLA class I assay by flow cytometry

Cell surface expression of HLA class I molecules was determined by indirect immunofluorescence staining. Cells (0.5×10^6) were washed with PBS and then stained with an excess of HLA class I-specific mAb W6/32 for 40 min on ice and then incubated with a FITC-conjugated goat anti-mouse IgG (BD Biosciences). FACS analysis was done on a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis

All raw assay data were normalized as the fraction (percent) of the highest values obtained for each assay. Normalized values were analyzed for correlation by the regression analysis using StatView software (SAS Institute).

Results

To explore the role of ER aminopeptidases in HLA class I Ag expression, a large panel of human cell lines that include EBV-B cell lines established from healthy blood donors and tumor cell lines derived from various organs and tissues, were assayed for expression of ERAP1 and ERAP2, HLA class I:H chains, and TAP1 by Western blot analysis. Western blot patterns of EBV-B cell lines and control cell lines are shown in Fig. 1*A* and those of melanoma cell lines and other lymphoid and nonlymphoid cell lines in Fig. 1, *B* and *C*. The densitometry data are given along with the normalized values, i.e., the percentage of the highest value obtained in each assay component, in Table I. These cell lines were also assayed for surface HLA class I expression, ER peptide-trimming activity, and ERAP1 and ERAP2 peptidase activity. These data are given, along with the normalized values, in Table II.

Expression of ERAP1 and ERAP2 in EBV-B cell lines

Fifteen EBV-B cell lines established from healthy blood donors were assayed for expression of ER aminopeptidases, ERAP1 and ERAP2. These two peptidases have been shown to be implicated in the generation of optimal HLA class I-binding peptides (8, 14, 16). In parallel, expression of HLA class I H chains and TAP1 was also determined.

The expression of ERAP1 and ERAP2 was first measured at the protein level by Western blotting using rabbit Ab A-LAP specific to ERAP1 (18) and mouse mAb 3F5 specific to ERAP2 (14). Lymphoid cell lines T1, T2, 721.220, and 721.221 that carry well-defined defects of specific components of the HLA class I Ag presentation pathway were included as controls for Abs used in the detection of HLA class I H chains and TAP1. T2 is devoid of TAP, 721.221 of HLA-A, -B, and -C H chains and 721.220 of tapasin (24–26).

As seen in Fig. 1*A*, ERAP1 migrated as a single band of the expected molecular size, ~115 kDa, and was seen in all of the

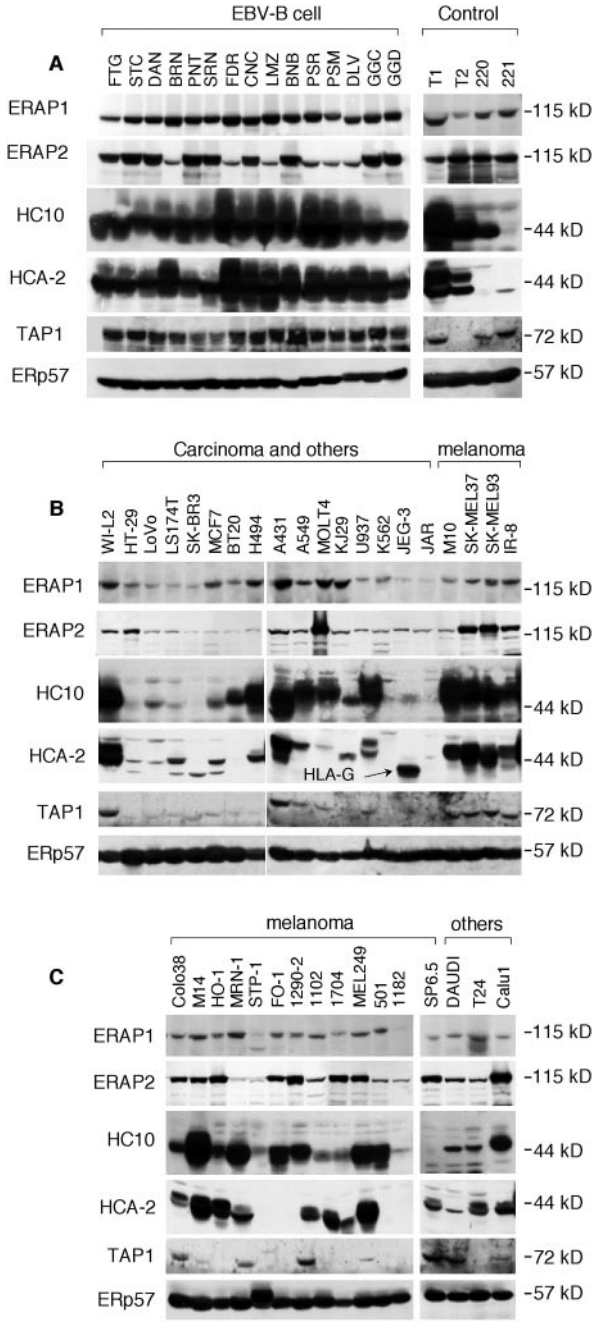

FIGURE 1. Immunoblot analysis of EBV-B and tumor cell lines. *A–C,* Equal amounts of non-ionic detergent cell lysates (100 μg/lane) from EBV-B cell lines, control mutant cells, and tumor cell lines were resolved on an 8% SDS-PAGE and probed by incubation with the indicated purified rabbit antiserum specific for ERAP1 (A-LAP), TAP1, and ERp57, and mAbs specific for ERAP2 (3F5) and HLA class I H chains (HC10 and HCA-2). A cross-reaction of HCA-2 with HLA-G is noted. A representative experiment is shown of four performed.

Table I. *Expression of HLA class I H chains, ERAP1, ERAP2, and TAP1 in EBV-B cell lines and tumor cell lines*

	HLA Class I (HCA2)		HLA Class I (HC10)		ERAP1 (ALAP)		ERAP2 (3F5)		TAP1	
Cell Lines	Raw data	%[a]	Raw data	%[a]	Raw	%[a]	Raw data	%[a]	Raw data	%[a]
BNB	3526	92	2579	52	740	89	924	87	1732	100
BRN	3246	84	4084	82	801	97	185	18	1063	61
CNC	3851	100	4342	87	755	91	581	55	1214	70
DAN	2000	52	3418	68	640	77	854	81	1098	63
DLV	2918	76	2359	47	580	70	199	19	1038	60
FDR	3234	84	4924	98	829	100	158	15	1038	60
FRT	1980	51	3012	60	476	57	556	53	1242	72
GGC	2875	75	2095	42	730	88	943	89	1180	68
GGD	1892	49	2080	42	750	90	843	80	794	46
LMZ	3756	98	4538	91	830	100	160	15	1139	66
PNT	2071	54	4150	83	680	82	1016	96	874	50
PSM	3029	79	2585	52	494	60	126	12	905	52
PSR	2934	76	2844	57	770	93	259	25	1118	65
SRN	2099	55	4142	83	675	81	855	81	786	45
STC	1931	50	3754	75	590	71	953	90	1400	81
DAUDI[b]	250	6	1342	27	180	22	319	30	870	50
K562[c]	22	1	238	5	260	31	128	12	32	2
MOLT4	150	4	3238	65	639	77	1057	100	385	22
U937	1403	36	3600	72	149	18	107	10	410	24
WI-L2	3422	89	4300	86	695	84	250	24	1010	58
A431	2950	77	4704	94	766	92	399	38	911	53
A549	786	20	4091	82	356	43	264	25	422	24
BT20	32	1	2048	41	176	21	22	2	100	6
Calu1	1250	32	2350	47	420	51	1057	100	185	11
JAR[c]	62	2	147	3	59	7	114	11	50	3
JEG-3[c]	22	1	408	8	80	10	135	13	195	11
H494	1073	28	3428	69	580	70	36	3	40	2
HeLa	3849	100	5031	100	252	30	67	6	ND	—
HT-29	191	5	252	5	275	33	369	35	87	5
KJ29[b]	465	12	1693	34	615	74	241	23	200	12
LoVo	175	5	747	15	104	13	110	10	200	12
LS174T	675	18	361	7	81	10	86	8	120	7
MCF7	533	14	1134	23	481	58	47	4	200	12
SK-BR3	27	1	29	1	80	10	24	2	250	14
T24	1100	29	1363	27	400	48	249	24	24	1
501	21	1	2085	42	333	40	152	14	23	1
1102	1759	46	767	15	323	39	236	22	57	3
1182	31	1	250	5	20	2	120	11	18	1
1290–2	1605	42	2058	41	296	36	738	70	1255	72
1704	600	16	701	14	120	14	620	59	25	1
COLO38	900	23	1149	23	348	42	415	39	567	33
FO-1[b]	27	1	2187	44	289	35	500	47	20	1
HO-1	2600	68	987	20	296	36	597	56	20	1
IR-8	1403	36	1900	38	422	51	488	46	634	37
M10	1743	45	4700	94	240	29	117	11	654	38
M14	2950	77	3980	80	544	66	444	42	140	8
MEL249	3300	86	2315	46	344	41	520	49	185	11
MRN-1	1531	40	2614	52	660	80	80	7	494	29
SK-MEL37	3300	86	4200	84	283	34	666	63	693	40
SK-MEL93	2700	70	3500	70	373	45	921	87	734	42
SP6.5	800	21	101	2	110	13	610	58	900	52
STP-1	24	1	645	13	74	9	73	7	20	1

[a] Data are normalized as the percentage of the highest values detected for each parameter.
[b] Cell lines defective in β_2m expression (27, 30–32).
[c] Cell lines defective in HLA-A, B, C H chain expression (28, 29).

tested cell lines, including the control cell lines. No additional bands were detected. ERAP2 was also detected in all of the tested cell lines but, unlike ERAP1, it gave two distinct bands of ~115 and ~60 kDa. Although the band of ~115 kDa corresponds to full-length ERAP2, the band of ~60 kDa likely corresponds to L-RAPs, a truncated form of L-RAP/ERAP2 previously observed in the human T cell line Jurkat (13). Because L-RAP is known to have no peptidase activity (13), only the ~115-kDa band was considered in quantifications of active ERAP2 by Western blot. No EBV-B cell lines that completely lack ERAP1 and/or ERAP2 were found.

As shown in Table I, the expression level of ERAP1 quantified by densitometry was high and fairly invariable in all cell lines, whereas that of ERAP2 was variable, either high or low. To facilitate visual comparison, the densitometry data were normalized as percentages of the highest density value obtained for ERAP1 and ERAP2, and the percent expression is depicted side-by-side for each cell line in Fig. 2*A*. The levels of expression of these two peptidases were comparable in nine cell lines, but in six cell lines (BRN, FDR, LMZ, PSR, PSM, and DLV) high ERAP1 expression was associated with low expression of ERAP2. It thus appeared that there are two different patterns of expression of ERAP1 and

Table II. *ER peptide trimming and ERAP1 and ERAP2 activity in EBV-B cell lines and tumor cell lines*

Cell Line	% R-S9L Trimming		ERAP1 Activity		ERAP2 Activity		HLA Class I Surface	
	Raw data	%[a]	FI	%[a]	FI	%[a]	MFI[b]	%[a]
BNB	ND	—	567	100	2050	57	1586	74
BRN	66	72	392	69	227	6	1732	81
CNC[c]	48	52	567	100	**64**	**2**	1729	81
DAN	72	78	281	50	1943	54	1319	62
DLV	ND	—	272	48	167	5	1652	77
FDR	56	61	425	75	254	7	1660	78
FRT	74	80	292	51	2032	57	1602	75
GGC	ND	—	252	44	3182	89	1246	58
GGD	ND	—	235	41	1931	54	1399	65
PNT	46	50	503	89	3099	86	1910	89
PSM	ND	—	245	43	191	5	1330	62
PSR[c]	ND	—	438	77	**338**	**9**	1599	75
SRN	44	48	402	71	2585	72	1513	71
STC	64	70	486	86	3143	87	1419	67
DAUDI[c,d]	16	17	210	37	**42**	**1**	1	0
K562	28	30	**40**	**7**	31	1	2	0
MOLT4[c,e]	54	59	352	62	3330	93	189	9
U937	14	15	123	22	62	2	123	6
WI-L2[c]	44	48	487	86	**134**	**4**	2137	100
A431[c]	50	54	194	34	**86**	**2**	961	45
A549[c]	4	4	**70**	**12**	**72**	**2**	446	21
BT20[c]	4	4	**16**	**3**	98	3	189	9
Calu1	ND	—	353	62	1420	40	420	20
JAR[e]	20	22	32	6	11	0	1	0
JEG-3[e]	22	24	24	4	61	2	33	2
H494	8	9	432	76	224	6	466	22
HeLa	ND	—	19	3	90	3	200	9
HT-29	92	100	133	23	1210	34	139	7
KJ29[c]	20	22	452	80	**201**	**6**	1	0
LoVo[c]	6	7	56	10	121	3	1	0
LS174T	28	30	78	14	296	8	70	3
MCF7	40	43	362	64	121	3	165	8
SK-BR3	4	4	4	1	28	1	1	0
T24[c]	34	37	180	32	**98**	**3**	356	17
501[c]	24	26	**72**	**13**	51	1	393	18
1102[c]	30	33	240	39	**0**	**0**	176	8
1182	1	(1)	2	(0)	62	(2)	1	(0)
1290–2	30	(33)	167	(29)	1690	(47)	344	(16)
1704	46	(50)	132	(23)	3594	(100)	88	(4)
COLO38	56	(61)	169	(30)	1476	(41)	402	(19)
FO-1[c,d]	54	(59)	165	(29)	**70**	(2)	1	(0)
HO-1	80	(87)	192	(34)	1337	(37)	207	(10)
IR-8	60	(65)	301	(53)	747	(21)	420	(20)
M10[c]	ND	—	**65**	(11)	23	(1)	256	(12)
M14	44	(48)	303	(53)	1151	(32)	131	(6)
MEL249[c]	62	(67)	293	(43)	**458**	(13)	1	(0)
MRN-1	16	(17)	224	(40)	208	(6)	366	(17)
SK-MEL37	44	(48)	191	(34)	2037	(57)	1091	(51)
SK-MEL93	ND	—	195	(34)	1843	(51)	204	(9)
SP6.5[c]	46	(50)	193	(34)	**252**	(7)	219	(10)
STP-1	10	(11)	17	(3)	67	(2)	1	(0)

[a] Data are normalized as the percentage of the highest values detected for each parameter.
[b] Data are expressed as specific MFI by staining with W6/32 minus MFI of an isotype-matched irrelevant Ab.
[c] Cell lines with impaired ERAP1 and/or ERAP2 activity are in bold.
[d] Cell lines defective in β_2m expression (27, 30–32).
[e] Cell lines defective in HLA-A, -B, and -C H chain expression (28, 29).

ERAP2 in EBV cell lines: 1) ERAP1^{high}/ERAP2^{high} and 2) ERAP1^{high}/ERAP2^{low}.

Because the production of HLA class I-binding peptides is directly linked to the activity of trimming peptidases and Western blotting may detect both functionally active and inactive enzymes, next we determined the enzymatic activity of ERAP1 and ERAP2. The peptidases were isolated from cell lysates with Triton X-100 by the use of protein G-Sepharose beads coated with mouse mAb 4D2 specific to ERAP1 or mouse mAb 3F5 specific to ERAP2, and the isolated peptidases were tested for their cleavage activity against their preferred fluorogenic substrates, Leu-AMC and Arg-AMC. As shown in Fig. 3*A*, Western blot analysis indicated that mAb 4D2 beads and mAb 3F5 beads isolate specifically ~115-kDa ERAP1 and ERAP2, respectively. The mAb 3F5 has been found to bind native ~115-kDa ERAP2, but not native ~60-kDa ERAP2 (data not shown). In the activity assay, as shown in Fig. 3*B*, ERAP1 and ERAP2 were reasonably specific for Leu-AMC and Arg-AMC: ERAP1 gave fluorescence intensity (FI) of 420 for

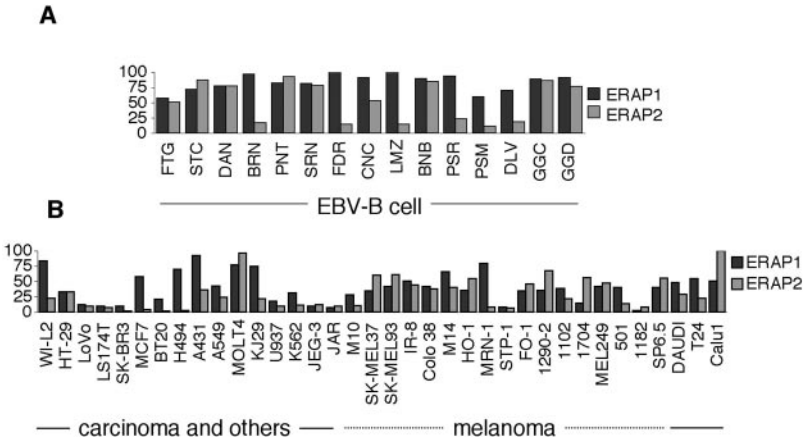

FIGURE 2. Quantitative analysis of ERAP1 and ERAP2 expression in EBV and tumor cell lines. Densitometric values from the Western blotting data of ERAP1 and ERAP2 in EBV (*A*) and tumor cell lines (*B*) are expressed as percentage of the highest value obtained for each enzyme.

Leu-AMC and an FI of 53 for Arg-AMC, whereas ERAP2 gave an FI of 26 for Leu-AMC and an FI of 625 for Arg-AMC. Substrate cleavage reflected the amounts of the aminopeptidases visualized in the blots, indicating a similar sensitivity of the two assays.

Using these cleavage assays, the activity of ERAP1 and ERAP2 was detected in 14 EBV-B cell lines. The FI values obtained were normalized as percentages of the highest FI value obtained for both ERAP1 and ERAP2 (Table II) and depicted side-by-side with the normalized Western blotting data in Fig. 3*C*. In most EBV-B cell lines tested, the measured ERAP1 activity (assessed using Leu-AMC) and ERAP2 activity (assessed using Arg-AMC) correlated well with their respective expression at the protein level. The only discrepancy between expression and activity (low Arg-AMC hydrolysis and high ERAP2 expression) was found in the CNC cell line. ERAP2 cDNA from CNC cells was amplified by RT-PCR and sequenced, but no nucleotide variations (as compared with accession number AB109031) were detected that could account for an impaired function. Except for this cell line, the Western blot data are indicative of the expression level of enzymatically active ~115-kDa ERAP1 and ERAP2.

Cleavage activity by unidentified ER aminopeptidases in EBV-B cell lines

A TLC assay that quantifies N-terminal trimming of the labeled peptide R-S9L (also designated as R10L) was used to determine aminopeptidase activity inside microsome vesicles (6). Recently, this R-S9L-trimming assay was found to detect a group of aminopeptidases that include ERAP2 and several as yet unidentified aminopeptidases (14).

The R-S9L-trimming assay was performed for eight EBV-B cell lines. Microsomes were purified by differential centrifugation from

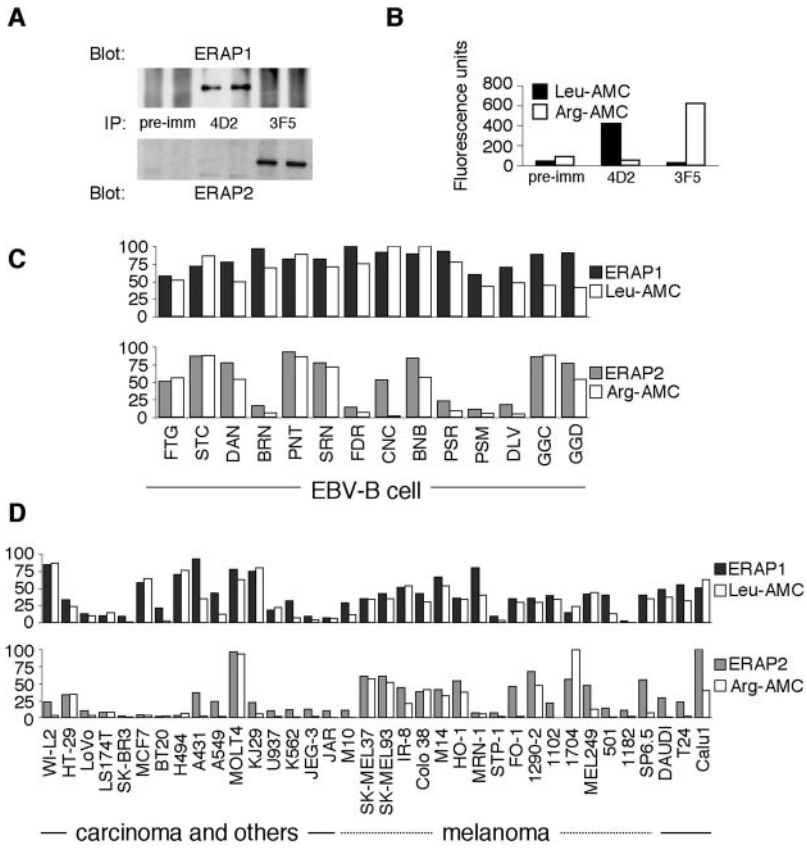

FIGURE 3. ERAP1 and ERAP2 activity in EBV-B and tumor cell lines. *A,* ERAP1 and ERAP2 were isolated (in duplicate) from Triton X-100 cell lysates (see *Materials and Methods*) using protein G beads coated with specific Abs (4D2 and 3F5, respectively) or preimmune serum (pre-imm). One set was eluted, run on SDS-PAGE, Western blotted, and the specific 115-kDa bands were identified by Abs to ERAP1 (A-LAP) and ERAP2 (3F5). *B,* The remaining half was tested for enzymatic activity with specific fluorogenic substrates: Leu-AMC (■) and Arg-AMC substrates (□) (see *Materials and Methods*). The results are reported in fluorescence units. *C* and *D,* ERAP1 and ERAP2 were isolated as above from the indicated cell lines and tested for Leu-AMC and Arg-AMC cleavage, respectively. The fluorescence units were normalized as percentages of the highest value obtained for both ERAP1 and ERAP2 (□) and depicted side-by-side with the respective Western blotting densitometric values (■ and ▨) for EBV-B cell lines (*C*) and tumor cell lines (*D*). Data are representative of two experiments.

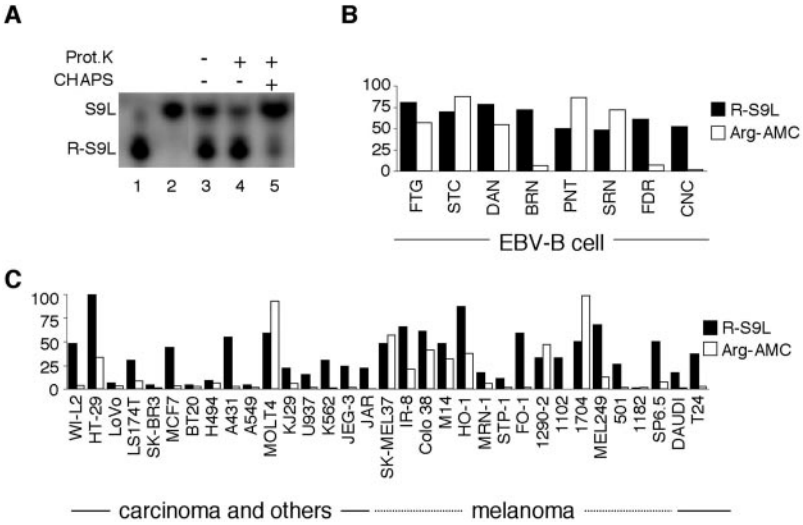

FIGURE 4. Comparison between ERAP2 activity and R-S9L trimming in EBV-B and tumor cell lines. *A,* Representative TLC analysis of an EBV-B cell line. Microsomes purified from a EBV-B cell line were left untreated (*lane 3*), treated with pK to remove extraluminal peptidases (*lane 4*), or treated with pK and lysed in PBS with 1% CHAPS (*lane 5*) and then incubated 3 min with 3 μl of ^{125}I-labeled R-S9L. Undigested labeled peptides R-S9L and S9L were included for TLC calibration (*lanes 1* and *2*). *B* and *C,* R-S9L trimming from a panel of cell lines is expressed as the percentage of the highest value and shown side-by-side with the ERAP2 activity for EBV-B cell lines (*B*) and tumor cell lines (*C*). Data are representative of three experiments.

cell homogenates and treated with pK and then CHAPS. Untreated, pK-treated and pK/CHAPS-treated microsomes were tested for their aminopeptidase activity that trims R-S9L to S9L by the TLC assay. Representative TLC patterns are shown for the EBV-B cell line JY in Fig. 4*A*. The difference in radioactivity of the S9L spot between pK-treated microsomes and pK/CHAPS-treated microsomes was taken to be indicative of luminal ER aminopeptidase activity. The difference, expressed as percentage of the input R-S9L, was used as a measure of R-S9L-trimming activity.

Assay data of R-S9L-trimming activity is presented in Table II. The R-S9L-trimming activity is also shown side-by-side with the ERAP2 activity for each cell line in Fig. 4*B*. At a glance, it is evident that high R-S9L-trimming activity was associated with very low ERAP2 activity in three cell lines (BRN, FDR, and CNC). This indicates that, in these cells, unidentified aminopeptidases are involved predominantly in the R-S9L trimming.

Correlation of ERAP1 and ERAP2 expression with HLA class I expression in EBV-B cell lines

To evaluate the implication of ERAP1 and ERAP2 in HLA class I surface expression, EBV-B cell lines were measured for surface HLA class I expression by indirect immunofluorescence staining using mAb W6/32 that identifies all classical class I HLA-A, -B, and -C H chains and nonclassical class I HLA-E and -G H chains. The mean fluorescence intensity (MFI) was determined by flow cytometry. MFI and the percentage values of the highest MFI observed are presented in Table II. All EBV-B cell lines displayed high and fairly similar surface HLA class I expression >1200 MFI. The normalized percentage of HLA class I surface expression is plotted side-by-side with the normalized ERAP1 activity (labeled Leu-AMC) and ERAP2 activity (labeled Arg-AMC) in Fig. 5*A*.

Surface HLA class I expression in these EBV-B cell lines correlated fairly well with ERAP1 activity, but not with ERAP2 activity. In six cell lines, BRN, FDR, CNC, PSR, PSM, and DLV, high surface HLA class I expression was associated with very low ERAP2 activity. Thus, ERAP1 activity correlates with surface HLA class I expression better than ERAP2 activity.

Expression of ERAP1 and ERAP2 in tumor cell lines

Next, a panel of tumor cell lines, including 5 leukemia/lymphoma cell lines, 17 melanoma cell lines, and 15 carcinoma cell lines, was assayed for the expression of ERAP1, ERAP2, HLA class I H chains, and TAP1 by Western blotting, followed by densitometry, and the data were processed and presented as described above for EBV-B cell lines.

As shown in Fig. 1, *B* and *C*, essentially all tumor cell lines were found to express ~115-kDa ERAP1 and ~115-kDa ERAP2 to some extent. Truncated ERAP2 of ~60 kDa was also detected in all cell lines (data not shown). Unlike EBV-B cell lines, the level of expression in tumor cell lines was highly variable for both

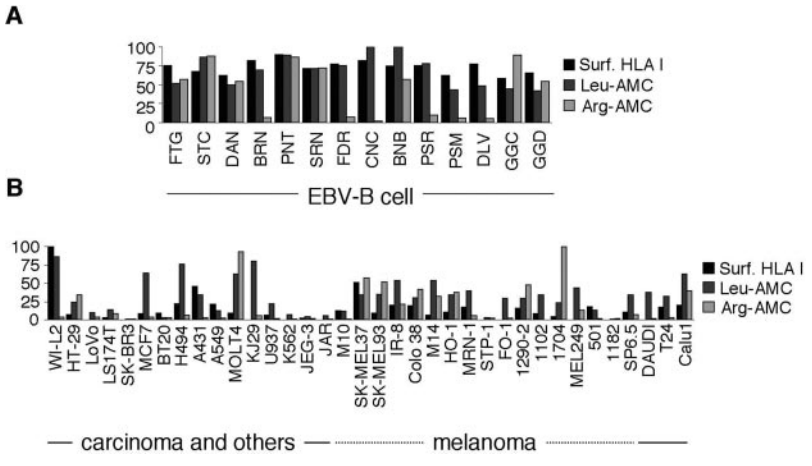

FIGURE 5. Correlation between ERAP1 and ERAP2 activity with HLA class I surface expression. Surface HLA class I expression and activity data of ERAP1 and ERAP2 are expressed as percentage of the highest value obtained for each enzyme and plotted against each other for EBV (*A*) and tumor cell lines (*B*).

ERAP1 and ERAP2. As seen in Table I, the expression level quantified by densitometry, i.e., the percent expression, ranged between 2 and 92% for ERAP1 and between 2 and 100% for ERAP2. Cell line 1182 was barely positive for ERAP1 at a longer exposure of the chromatograph shown in Fig. 1*C*. The cell lines BT20, H494, HeLa, MCF7, and SK-BR3 were only weakly positive for ERAP2.

The normalized expression of ERAP1 and ERAP2 is presented side-by-side for each cell line for direct comparison in Fig. 2*B*. These data, along with those for EBV-B cell lines (Fig. 2*A*), were evaluated statistically for correlation by regression analysis. The regression plot with R^2 and p values is shown in Fig. 6*A*. No significant correlation between ERAP1 and ERAP2 expression was found, $R^2 = 0.13$ and $p = 0.009$. The statistical values obtained for EBV-B cell lines were $R^2 = 0.016$ and $p = 0.65$, and those for tumor cell lines were $R^2 = 0.08$ and $p = 0.10$. Thus, it is clear that the expression of ERAP1 and ERAP2 is independent and not coordinated.

Tumor cell lines were then assayed for the aminopeptidase activity of ERAP1 and ERAP2. ERAP1 and ERAP2 were isolated from each tested cell line with specific Ab-coated Sepharose beads and assayed for the cleavage activity against aminoacyl-AMC as described for EBV-B cells.

For direct comparison, the FI values were normalized as the percentage of the highest FI value obtained in each of ERAP1 and ERAP2 activity assays (Table II). These normalized values are depicted side-by-side with the respective normalized Western blotting data for each cell line in Fig. 3*D*.

In many cell lines, the enzymatic activities (cleavage of Leu-AMC and Arg-AMC) appeared to be proportional to ERAP1 and ERAP2 expression, estimated by Western blotting, over a wide range of expression levels, suggesting a similar sensitivity of the two assays. However, as shown in bold in Table II, low enzymatic activities (≤15%) were associated with relatively high (≥20%) ERAP1 or ERAP2 expression in 5 and 12 tumor cell lines, respectively. The cell lines CNC, A431, and FO-1 also displayed poor or undetectable cleavage of a second ERAP2 substrate (Lys-AMC). Like the EBV-B cell line CNC, these tumor cell lines express functionally impaired ERAP proteins.

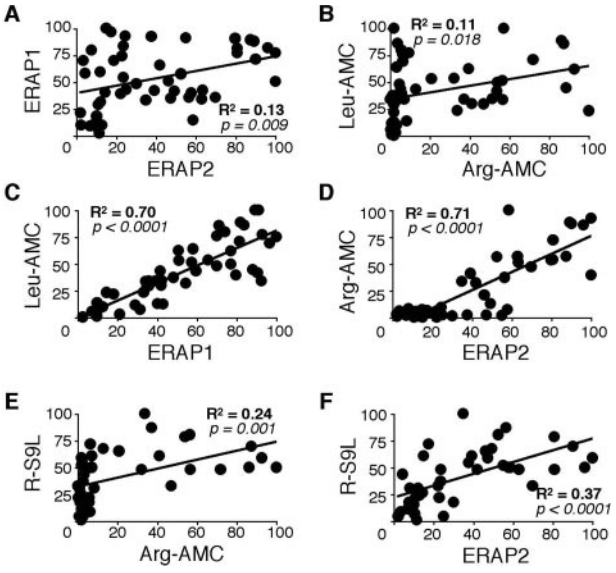

FIGURE 6. Regression analysis of ERAP1, ERAP2, and unidentified ER aminopeptidases. Densitometric analysis of Western blots and enzymatic activity data of ERAP1, ERAP2, and unidentified ER aminopeptidases of all of the tested cell lines, normalized as indicated in the previous figures, are plotted against each other. The activity data are indicated by the substrate name with which the activity was assayed: Leu-AMC for ERAP1 activity, Arg-AMC for ERAP2 activity, and R-S9L for ERAP2 plus unidentified ER aminopeptidases. *A,* Expression of ERAP1 vs expression of ERAP2 (*A*), activity of ERAP1 vs activity of ERAP2 (*B*), activity of ERAP1 vs its expression (*C*), activity of ERAP2 vs its expression (*D*), activity of ERAP2 vs R-S9L peptide trimming (*E*), expression of ERAP2 vs R-S9L peptide trimming (*F*). Regression lines and coefficients are shown.

In the regression analysis of the whole data set, including EBV-B cell and tumor cell lines, the correlation between ERAP expression and aminoacyl-AMC hydrolysis was highly significant. The R^2 value was 0.70 ($p < 0.0001$) for ERAP1 vs Leu-AMC (Fig. 6*C*) and 0.71 ($p < 0.0001$) for ERAP2 vs Arg-AMC (Fig. 6*D*). When the cell lines with impaired ERAP1 or ERAP2 cleavage were excluded, the R^2 values were not or slightly affected, in that they became 0.70 ($p < 0.0001$) for ERAP1 vs Leu-AMC, and 0.82 ($p < 0.0001$) for ERAP2 vs Arg-AMC. This indicates that in general ERAP1 and ERAP2 are enzymatically active and the levels of their expression reflect the levels of their peptidase activity. Conforming to the lack of correlation in the levels of their expression (Fig. 6*A*), ERAP1 and ERAP2 activities did not correlate significantly (Fig. 6*B*). The R^2 values were 0.11 ($p = 0.018$) for the whole data set, and 0.18 ($p = 0.012$) when the cell lines with impaired AMC substrate cleavage activity were omitted.

Cleavage activities by unidentified ER aminopeptidases in tumor cell lines

As mentioned previously, ER aminopeptidase activity detected by the use of the peptide substrate R-S9L has been found to be due to ERAP2 and one or several unknown aminopeptidases. In fact, high R-S9L-trimming activity was detected in the absence of ERAP2 activity in three of the eight EBV-B cell lines tested (Fig. 4*B*). Thus, it appeared that in these cell lines, the R-S9L-trimming activity is primarily attributable to unidentified aminopeptidases.

We, therefore, looked for a similar phenotype, i.e., low ERAP2 activity/high R-S9L trimming, in tumor cell lines. The R-S9L assay data are presented in Table II and, side-by-side with normalized ERAP2 activity data, in Fig. 4*C*. At a glance, it is clear that many cell lines, both carcinomas and melanomas, have reasonably high R-S9L-trimming activity and hardly detectable ERAP2 activity (labeled Arg-AMC). In these cell lines, unidentified aminopeptidase(s) must be involved in R-S9L-trimming activity. Indeed, as shown in Fig. 6*E*, no significant correlation was seen between R-S9L trimming and ERAP2 activity. The R^2 values were 0.24 ($p = 0.001$) for the whole set of cell lines and 0.31 ($p = 0.001$) when the impaired Arg-AMC substrate cleavage cell lines were excluded. A significant correlation was found between R-S9L-trimming and ERAP2 expression. The R^2 value was 0.37 ($p < 0.0001$; Fig. 6*F*). It thus appeared that reduced ERAP2 activity is complemented by some other, as yet unidentified, aminopeptidases.

Correlation of ERAP1 and ERAP2 expression with HLA class I expression in tumor cell lines

Surface HLA class I expression of tumor cell lines was assessed by mAb W6/32 in flow cytometry. MFI and the normalized values are presented in Table II. The level of expression was generally low (MFI <467 for all but three cell lines) and highly variable. HLA-A, -B, and -C expression was either undetectable or barely detectable in 11 cell lines that include 7 cell lines (DAUDI, K562, JAR, JEG-3, KJ-29, LoVo, and FO-1) that are known for their aberrant expression of β_2m and/or HLA class I H chains (27–32) and 4 cell lines (SK-BR3, 1182, MEL249, and STP-1) for which no information is available in the literature. The normalized HLA

class I surface expression (labeled surf. HLA I) is plotted side-by-side with the normalized ERAP1 and ERAP2 activities (labeled Leu-AMC and Arg-AMC, respectively) in Fig. 5*B*.

The correlation of surface HLA class I expression with each of expression and activity of ERAP1 and ERAP2 was estimated by regression analysis. The regression plots with R^2 and p values are shown for all cell lines, including EBV-B cell lines, in Fig. 7, *A–D*. Surface HLA class I expression correlated significantly with the expression and activity of ERAP1 ($R^2 = 0.58$ and 0.48, respectively; $p < 0.0001$ in both cases; Fig. 7, *A* and *B*), but not with the expression and activity of ERAP2 ($R^2 = 0.13$, $p = 0.01$ and $R^2 = 0.09$, $p = 0.03$, respectively; Fig. 7, *C* and *D*). The correlation of surface HLA class I expression with R-S9L-trimming activity, that detects ERAP2 plus some other aminopeptidases, was not significant, although the R^2 value was somewhat greater than that seen for the correlation with ERAP2 activity, $R^2 = 0.19$, $p = 0.004$; Fig. 7*E*).

Next, we evaluated HLA class I H chain expression for correlation with surface HLA class I expression (Fig. 7*F*), ERAP1 expression (Fig. 7*G*), and ERAP2 expression (Fig. 7*H*). The R^2 values were 0.55, 0.54, and 0.09, respectively. These data altogether suggest that ERAP1 is more closely coordinated with HLA class I H chains (total as well as surface expressed) than ERAP2.

Effect of ERAP1 and ERAP2 transfection on surface HLA class I expression in tumor cells

Finally, to determine whether the available levels of ERAP1 and ERAP2 might be limiting for surface HLA class I expression in some neoplastic cells, two cell lines (BT20 and HeLa) were selected on the basis of their low expression of both aminopeptidases (see Fig. 1*A* and data not shown) and were transfected with ERAP1 or ERAP2 cDNAs or the vector DNA alone. Transfectants were tested for ERAP1 and ERAP2 expression by Western blotting, for ERAP1 and ERAP2 activities by assessing the cleavage of specific aminoacyl-AMC substrates, and for HLA class I surface expression by flow cytometry as described above.

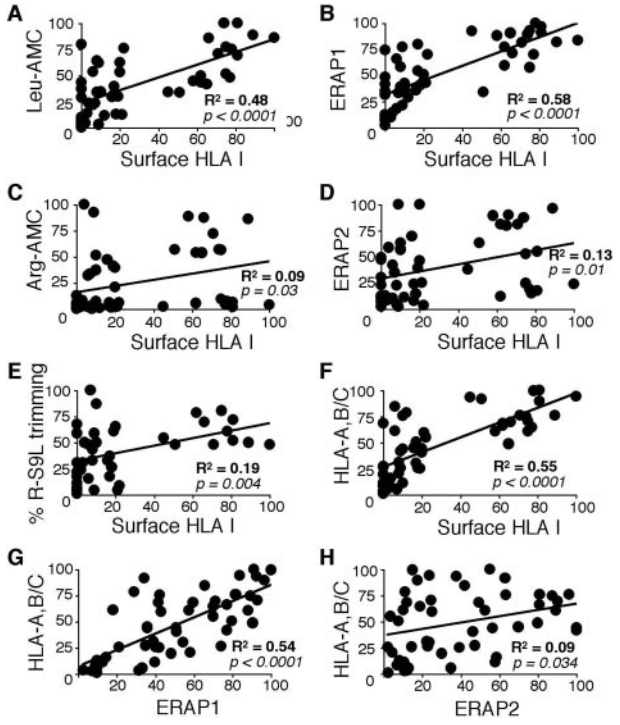

FIGURE 7. Regression analysis of HLA class I molecules against ERAP1, ERAP2, and unidentified ER aminopeptidases. Surface HLA class I expression is plotted against ERAP1 activity (*A*), ERAP1 expression (*B*), ERAP2 activity (*C*), ERAP2 expression (*D*), and R-S9L trimming (*E*) for all of the tested cell lines. HLA class I H chain is plotted against surface HLA class I expression (*F*), ERAP1 expression (*G*), and ERAP2 expression (*H*). Regression lines and coefficients are shown.

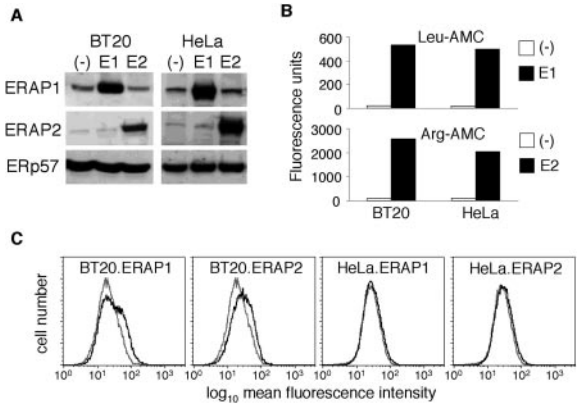

FIGURE 8. Expression of ERAP1 and ERAP2 increase HLA class I surface expression in tumor cell lines. BT20 and HeLa cells were transfected with either ERAP1 (E1) or ERAP2 (E2), or with vector alone (−). The expression of ERAP1 and ERAP2 was evaluated by Western blot (*A*). Their enzymatic activities were assessed by measuring the cleavage of the fluorogenic substrates Leu-AMC and Arg-AMC, following isolation on Ab-coated beads, as described in the legend of Fig. 3 (*B*). Surface HLA class I expression was analyzed by flow cytometry with the W6/32 Ab (*C*). Negative controls stained with isotype-matched primary Abs did not exceed a MFI of 5. ERAP-transfected and vector-transfected cell lines are represented by black and gray lines, respectively. A representative experiment is shown of three that were performed.

Whereas vector DNA had no effect, ERAP1 and ERAP2 cDNAs enhanced 7 and 20 times, respectively, the expression of the two aminopeptidases in both BT20 and HeLa (Fig. 8*A*, and densitometric data, not shown). ERAP1 and ERAP2 reached levels comparable to their endogenous expression in EBV-B cell lines (cf with Fig. 1A). These expression levels were proportional to functional aminopeptidase activities in both cells (Fig. 8*B*). However, despite functional reconstitution, surface HLA class I expression was only slightly increased. As compared with the vector DNAs, transfection with ERAP1 and ERAP2 increased W6/32 MFI values in BT20 cells from 20 to 30 and 29, respectively. In HeLa cells, MFI values increased from 26 to 29 and 30, respectively (Fig. 8*C*). These values correspond to 50 and 11% increments in BT20 and HeLa, respectively. Similar results were obtained in three separate experiments. Thus, ERAP1 and ERAP2 both affect class I expression, although to a different extent in different tumor cell lines.

Discussion

In this study, we have assayed a large panel of human lymphoid and nonlymphoid cell lines of various cell types to investigate the expression of ER aminopeptidases that include ERAP1, ERAP2, and unidentified ER aminopeptidases. These peptidases have been implicated in the generation of optimal HLA class I-binding peptides.

We have measured the expression of ERAP1 and ERAP2 by Western blotting using specific Abs and their enzymatic activities by assessing the cleavage of specific aminoacyl-AMC substrates. Our results demonstrate that ERAP1 and ERAP2 are expressed: 1) at extremely variable levels in all the tested cell lines, including HLA class I-defective cell lines; 2) independently of each other

and not coordinately; and 3) in amounts proportional to their aminopeptidase activities in most cell lines. In addition, the reduced expression of ERAP2 is complemented by the expression of unidentified aminopeptidases, and the peptidase activity of ERAP1 has significantly high correlation with HLA class I expression, but such correlation is not seen for ERAP2 and unidentified ER aminopeptidases. Transfection of ERAP1 and ERAP2 in two tumor cell lines, selected for low expression of both aminopeptidases, results in modest increases of HLA class I expression, more evident in one of the cell lines.

Imbalanced expression of ER aminopeptidases in tumor cell lines

We found that EBV-B cells from different healthy donors display limited variability in the ER aminopeptidase expression: they express similar high levels of HLA class I and ERAP1, and either high or low levels of ERAP2. Therefore, there are only two phenotypes in the EBV-B cell lines, i.e., ERAP1^{high}/ERAP2^{high} and ERAP1^{high}/ERAP2^{low}. Efficient in vitro trimming of precursors of HLA class I-binding peptides requires the removal of N-terminal extensions containing hydrophobic and basic residues through the joint action of ERAP1 and ERAP2 (14). Thus, the variation in the aminopeptidase complement of nontransformed B lymphocytes is consistent with the idea that ERAP1 and ERAP2 must be both expressed above a threshold that is necessary for concerted Ag trimming in these professional APCs.

Strikingly in contrast, tumor cell lines from all tested lineages are extremely variable in the expression of not only HLA class I molecules, as known for many years, but also in the expression and enzymatic activities of ERAP1, ERAP2, and, possibly, of additional unknown aminopeptidases (as shown by the R-S9L-trimming assay). There is nearly a two-log difference in the levels of ERAP1 and ERAP2 (proteins and enzymatic activities) between high and low expressors and, more important, ERAP1 and ERAP2 are independently regulated, i.e., their expression levels in a given cell are often discordant. All of the possible combinations from very high to very low expression of the two enzymes are represented in tumors; extreme phenotypes, i.e., ERAP1^{high}/ERAP2^{low} phenotypes, and vice versa, being rather frequent.

According to the model of concerted Ag trimming, any imbalance in ER aminopeptidases would result in the insufficient trimming of certain N-terminal extensions and excessive trimming of others. Consequently, certain epitopes would be inefficiently generated, while others might even be destroyed. In agreement with this model, the suppression of human ERAP1 by RNA interference or murine ERAAP by homologous recombination, depressed the presentation of some epitopes, and increased or left unmodified that of others (8, 16, 33). Potentially, imbalances in the expression of ERAP1 and ERAP2 may also suppress or derange the presentation of certain tumor-associated Ags. Therefore, our results indicate that tumor cells that completely lack ERAP expression are probably rare (no ERAP negatives were detected in our extensive cell panel) and that the expressed ERAP polypeptides are functional in most cells. Nevertheless, significant numbers of tumors are probably impaired in their ability to precisely trim Ag precursors, because they express extremely low activity levels of at least one ER aminopeptidase.

We have identified one EBV-B cell line and few tumor cell lines displaying high ERAP1 and/or ERAP2 expression but low enzymatic activities. At least in one case, direct gene inactivation is unlikely, since no nucleotide sequence variations were detected as compared with functional ERAP2 cDNA. An impaired enzymatic activity of a wild-type protein may result from an incorrect folding, possibly as a consequence of abnormal posttranslational modifications, such as glycosylation defects or incorrect formation of disulfide bonds, or an absence of cofactors or collaborating proteins. Alternatively, substrate cleavage in the test tube may be sensitive to nonspecific functional inactivation by unknown factors coimmunoprecipitated in some cell lines.

An imbalanced expression of ER aminopeptidases is not expected to result in a major decrease in the levels of cell surface class I molecules, as demonstrated by RNA interference with ERAP1 and ERAP2 expression (14) and, more recently, in ERAAP-deficient mice (33). In agreement with these data, surface HLA class I expression could be detected at significant levels in cells expressing imbalanced levels of ERAP1 and ERAP2 and was only moderately enhanced upon transfection with ERAP1 or ERAP2 in two cell lines, although selected from our large panel in view of their extremely low constitutive expression of these aminopeptidases.

Recent studies in ERAAP-deficient mice indicate that the cells lacking ERAAP are not deficient in peptide supply but instead are deficient in the composition of the MHC-associated peptide pool (33. Thus, complementary approaches (suppression and reconstitution of ERAP/ERAAP expression) in humans and mice concordantly indicate a subtle but possibly crucial role of peptide trimming in the optimization of class I peptide ligand-MHC interactions. In agreement with this idea, the different enhancement of class I molecules in the two cell lines tested by us may reflect the more or less strict dependence on trimming of different sets of class I alleles in different cells. Alternatively, the levels of the members of the HLA class I Ag-processing and presentation machinery (or other unknown factors) may differ in different cell lines and independently influence a limiting step (other than peptide trimming) in class I assembly. In this respect, it should be noted that the knockdown of class I expression by small interfering RNA was effective in HeLa cells treated with IFN-γ, a situation producing an increased demand for trimming in a cell line in which the constitutive functions required for trimming/processing are otherwise sufficient to sustain peptide presentation by a low number of expressed class I molecules. Thus, marginal enhancement of class I expression by overexpressing ERAP1 and ERAP2 in HeLa is not surprising and supports the idea that proper trimming requires the right balance and the concerted action of ER aminopeptidases, members of the Ag-processing machinery, and class I H chains.

Most likely, an analysis of the peptide repertoire isolated from single HLA class I allele in these cell lines may be required to identify tumors carrying qualitative defects in their HLA class I Ag repertoire. Ideally, these studies should be conducted in tumor cell lines fully characterized in their expression of all ER aminopeptidases, but this must await the availability of specific Abs to as yet unidentified ER aminopeptidases other than ERAP1 and ERAP2.

ERAP1, ERAP2, and HLA class I: linkage and derangement

Despite the imbalanced, variable expression of ER aminopeptidases, ERAP1 was significantly correlated with HLA class I, being invariably expressed at high levels in EBV-B cells, and conforming to the linked patterns of expression (the class I "coordinome") previously detected among the members of the Ag-processing machinery in tumor cell lines (23). In contrast, ERAP2 was less homogeneous in its expression even in EBV-B cells and extensively deviated from HLA class I in tumor cells. A possible interpretation of the closer coordination of ERAP1 with HLA class I molecules is that precursor peptides carrying hydrophobic amino acid residues at their N terminus are particularly abundant in the ER, and ERAP1 is the crucial aminopeptidase initiating their trimming. In this case, it would be necessary to postulate that TAP preferentially translocates such precursor peptides into the ER. The strong length

specificity and preference of ERAP1 for peptides with large hydrophobic C terminus (34) make this enzyme more adapted to generate efficiently the great majority of MHC class I epitopes, with the possible exception of peptides with C-terminal basic residues bound by some human class I alleles. Interestingly, mice which transport into the ER and present exclusively antigenic peptides with hydrophobic C termini express ERAAP, the closest ERAP1 homolog, but lack ERAP2 (33).

Whatever the mechanism of class I-ERAP1 coregulation, ERAP2 appears to be more loosely linked to the class I coordinome in B cells. This weak linkage may be further weakened by neoplastic transformation, explaining the extensive derangement in ERAP2 expression observed in tumor cells. It remains to be determined whether imbalanced ERAP (primarily ERAP1, but also ERAP2) expression is a novel strategy of immune evasion and, if so, whether it is particularly advantageous to tumors.

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References

1. Taylor, A. 1993. Aminopeptidases: structure and function. *FASEB J.* 7: 290–298.
2. Rock, K. L., and A. L. Goldberg. 1999. Degradation of cell proteins and the generation of MHC class I-presented peptides. *Annu. Rev. Immunol.* 17: 739–779.
3. Reits, E., J. Neijssen, C. Herberts, W. Benckhuijsen, L. Janssen, J. W. Drijfhout, and J. Neefjes. 2004. A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation. *Immunity* 20: 495–506.
4. York, I. A., A. X. Mo, K. Lemerise, W. Zeng, Y. Shen, C. R. Abraham, T. Saric, A. L. Goldberg, and K. L. Rock. 2003. The cytosolic endopeptidase, thimet oligopeptidase, destroys antigenic peptides and limits the extent of MHC class I antigen presentation. *Immunity* 18: 429–440.
5. Reits, E., A. Griekspoor, J. Neijssen, T. Groothuis, K. Jalink, P. van Veelen, H. Janssen, J. Calafat, J. W. Drijfhout, and J. Neefjes. 2003. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* 18: 97–108.
6. Fruci, D., G. Niedermann, R. H. Butler, and P. M. van Endert. 2001. Efficient MHC class I-independent amino-terminal trimming of epitope precursor peptides in the endoplasmic reticulum. *Immunity* 15: 467–476.
7. Serwold, T., S. Gaw, and N. Shastri. 2001. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. *Nat. Immunol.* 2: 644–651.
8. Serwold, T., F. Gonzalez, J. Kim, R. Jacob, and N. Shastri. 2002. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* 419: 480–483.
9. Saric, T., S. C. Chang, A. Hattori, I. A. York, S. Markant, K. L. Rock, M. Tsujimoto, and A. L. Goldberg. 2002. An IFN-γ-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nat. Immunol.* 3: 1169–1176.
10. Hattori, A., H. Matsumoto, S. Mizutani, and M. Tsujimoto. 1999. Molecular cloning of adipocyte-derived leucine aminopeptidase highly related to placental leucine aminopeptidase/oxytocinase. *J. Biochem.* 125: 931–938.
11. Schomburg, L., H. Kollmus, S. Friedrichsen, and K. Bauer. 2000. Molecular characterization of a puromycin-insensitive leucyl-specific aminopeptidase, PILS-AP. *Eur. J. Biochem.* 267: 3198–3207.
12. Cui, X., F. Hawari, S. Alsaaty, M. Lawrence, C. A. Combs, W. Geng, F. N. Rouhani, D. Miskinis, and S. J. Levine. 2002. Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding. *J. Clin. Invest.* 110: 515–526.
13. Tanioka, T., A. Hattori, S. Masuda, Y. Nomura, H. Nakayama, S. Mizutani, and M. Tsujimoto. 2003. Human leukocyte-derived arginine aminopeptidase: the third member of the oxytocinase subfamily of aminopeptidases. *J. Biol. Chem.* 278: 32275–32283.
14. Saveanu, L., Carroll, O., Lindo, V., Del Val, M., Lopez, D., Lepelletier, Y., Greer, F., Schomburg, L., Fruci, D., Niedermann, G., and van Endert, P. 2005. Human aminopeptidases ERAP1 and ERAP2 associate for concerted peptide trimming in the endoplasmic reticulum. *Nat. Immunol.* 6: 689–697.
15. Momburg, F., J. Roelse, G. J. Hammerling, and J. J. Neefjes. 1994. Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *J. Exp. Med.* 179: 1613–1623.
16. York, I. A., S. C. Chang, T. Saric, J. A. Keys, J. M. Favreau, A. L. Goldberg, and K. L. Rock. 2002. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8–9 residues. *Nat. Immunol.* 3: 1177–1184.
17. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv. Immunol.* 74: 181–273.
18. Hattori, A., K. Kitatani, H. Matsumoto, S. Miyazawa, T. Rogi, N. Tsuruoka, S. Mizutani, Y. Natori, and M. Tsujimoto. 2000. Characterization of recombinant human adipocyte-derived leucine aminopeptidase expressed in Chinese hamster ovary cells. *J. Biochem.* 128: 755–762.
19. Stam, N. J., H. Spits, and H. L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C locus products. *J. Immunol.* 137: 2299–2306.
20. Sernee, M. F., H. L. Ploegh, and D. J. Schust. 1998. Why certain antibodies cross-react with HLA-A and HLA-G: epitope mapping of two common MHC class I reagents. *Mol. Immunol.* 35: 177–188.
21. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell* 14: 9–20.
22. Kovats, S., E. K. Main, C. Librach, M. Stubblebine, S. J. Fisher, and R. DeMars. 1990. A class I antigen, HLA-G, expressed in human trophoblasts. *Science* 248: 220–223.
23. Giorda, E., L. Sibilio, A. Martayan, S. Moretti, I. Venturo, M. Mottolese, G. B. Ferrara, S. Cappellacci, L. Eibenschutz, C. Catricala, P. Grammatico, and P. Giacomini. 2003. The antigen processing machinery of class I human leukocyte antigens: linked patterns of gene expression in neoplastic cells. *Cancer Res.* 63: 4119–4127.
24. Salter, R. D., and P. Cresswell. 1986. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J.* 5: 943–949.
25. Shimizu, Y., D. E. Geraghty, B. H. Koller, H. T. Orr, and R. DeMars. 1988. Transfer and expression of three cloned human non-HLA-A,B,C class I major histocompatibility complex genes in mutant lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA* 85: 227–231.
26. Grandea, A. G. III, M. J. Androlewicz, R. S. Athwal, D. E. Geraghty, and T. Spies. 1995. Dependence of peptide binding by MHC class I molecules on their interaction with TAP. *Science* 270: 105–108.
27. Browning, M. J., J. A. Madrigal, P. Krausa, H. Kowalski, C. E. Allsopp, A. M. Little, S. Turner, E. J. Adams, K. L. Arnett, W. F. Bodmer, et al. 1995. The HLA-A,B,C genotype of the class I negative cell line Daudi reveals novel HLA-A and -B alleles. *Tissue Antigens* 45: 177–187.
28. Guillaudeux, T., A. M. Rodriguez, M. Girr, V. Mallet, S. A. Ellis, I. L. Sargent, R. Fauchet, E. Alsat, and P. Le Bouteiller. 1995. Methylation status and transcriptional expression of the MHC class I loci in human trophoblast cells from term placenta. *J. Immunol.* 154: 3283–3299.
29. Santamaria, P., A. L. Lindstrom, M. T. Boyce-Jacino, S. H. Myster, J. J. Barbosa, A. J. Faras, and S. S. Rich. 1993. HLA class I sequence-based typing. *Hum. Immunol.* 37: 39–50.
30. Delfino, L., G. Ciccarelli, D. Bini, A. Morabito, S. Pozzi, A. Martayan, E. Giorda, A. Setini, R. Fraioli, P. Giacomini, and G. B. Ferrara. 1999. HLA-A, -B, -C genotyping and expression in human nonlymphoid tumor cell lines. *J. Immunother.* 22: 7–15.
31. Pollack, M. S., S. D. Heagney, P. O. Livingston, and J. Fogh. 1981. HLA-A, B, C and DR alloantigen expression on forty-six cultured human tumor cell lines. *J. Natl. Cancer Inst.* 66: 1003–1012.
32. Martayan, A., M. Fiscella, A. Setini, G. Ciccarelli, R. Gambari, G. Feriotto, A. Beretta, A. G. Siccardi, E. Appella, and P. Giacomini. 1997. Conformation and surface expression of free HLA-CW1 heavy chains in the absence of β_2-microglobulin. *Hum. Immunol.* 53: 23–33.
33. Hammer, G. E., F. Gonzalez, M. Champsaur, D. Cado, and N. Shastri. 2006. The aminopeptidase ERAAP shapes the peptide repertoire displayed by major histocompatibility complex class I molecules. *Nat. Immunol.* 7: 103–112.
34. Chang, S. C., F. Momburg, N. Bhutani, and A. L. Goldberg. 2005. The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a "molecular ruler" mechanism. *Proc. Natl. Acad. Sci. USA* 102: 17107–17112.