The Turnover Kinetics of Major Histocompatibility Complex Peptides of Human Cancer Cells*

Elena Milner‡, Eilon Barnea‡, Ilan Beer§, and Arie Admon‡¶

Peptides presented by the major histocompatibility complex (MHC) are derived from the degradation of cellular proteins. Thus, the repertoire of these peptides (the MHC peptidome) should correlate better with the cellular protein degradation scheme (the degradome) than with the cellular proteome. To test the validity of this statement and to determine whether the majority of MHC peptides are derived from short lived proteins, from defective ribosome products, or from regular long lived cellular proteins we analyzed in parallel the turnover kinetics of both MHC peptides and cellular proteins in the same cancer cells. The analysis was performed by pulse-chase experiments based on stable isotope labeling in tissue culture followed by capillary chromatography and tandem mass spectrometry. Indeed only a limited correlation was observed between the proteome and the MHC peptidome observed in the same cells. Moreover a detailed analysis of the turnover kinetics of the MHC peptides helped to assign their origin to normal, to short lived or long lived proteins, or to the defective ribosome products. Furthermore the analysis of the MHC peptides turnover kinetics helped to direct attention to abnormalities in the degradation schemes of their source proteins. These observations can be extended to search for cancer-related abnormalities in protein degradation, including those that lead to loss of tumor suppressors and cell cycle regulatory proteins. ***Molecular & Cellular Proteomics 5:357–365, 2006.***

MHC[1] peptides are thought to be degradation products of the majority of cellular proteins, including both normal and disease-related proteins. Of particular interest are peptides expressed exclusively in cancer cells, derived from pathogen proteins, or involved in the induction of autoimmune reactions. Indeed most of the effort invested in MHC peptide studies has focused on their therapeutic potential. Because MHC peptides are presented only following the degradation of their proteins of origin and because proteins degrade at vastly differing rates, it can be assumed that the MHC peptidome of the cells correlates better with the pattern of protein degradation (the degradome) rather than with the proteome of the cells. Therefore, analysis of the MHC peptidome can be approached for its value as a source of information on the cellular protein synthesis and degradation schemes (both the normal and disease-related), thus offering a novel point of view onto the transient cellular proteome (for a review, see Ref. 1).

The relative amounts of the different MHC peptides presented at the cell surface are influenced by the rate of degradation of their source proteins, the efficiencies of their transport into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) by the subsequent loading onto the awaiting MHC molecules, and the binding affinities of the individual peptides to the presenting MHC molecules (for reviews, see Refs. 2–4). It is conceivable that some of the more abundant MHC peptides are derived from proteins that are relatively rare in the cells due to rapid degradation. On the other hand, some of the less abundant MHC peptides are possibly derived from ubiquitous stable cellular proteins that degrade very slowly and therefore contribute only marginally to the MHC peptidome. Another significant source of MHC peptides is defective ribosome products (DRiPs) that are normally rapidly disposed of by the proteasomes. DRiPs are proteins that are destabilized by mutations, mRNA or protein synthesis errors, premature termination, or deletion of residues. The proteasomes rapidly degrade these DRiPs to prevent their possible interference with normal cellular functions. Moreover futile folding, excess expression of protein subunits not assembled into multisubunit complexes, and errors in intracellular sorting directing the proteins to wrong compartments also contribute to rapid degradation of newly synthesized proteins. It is thought that some of each of the newly synthesized protein molecules are actually DRiPs. Therefore, in the cells some molecules of each protein have a normal lifetime, whereas other molecules of the same proteins are DRiPs, which degrade rapidly (for a review, see Ref. 5). A distinction should be made between DRiPs and short lived proteins (SLiPs), which are normal cellular proteins whose turnover is normally rapid and therefore also contribute a significant portion of peptides to the MHC peptide pool (6–9).

From the ‡Department of Biology, Technion-Israel Institute of Technology, 32000 Haifa, Israel and §IBM Research Laboratory, Haifa University Campus, Haifa 31905, Israel

Received, August 1, 2005, and in revised form, October 19, 2005
Published, MCP Papers in Press, November 4, 2005, DOI 10.1074/mcp.M500241-MCP200

[1] The abbreviations used are: MHC, major histocompatibility complex; sMHC, soluble MHC molecules; SILAC, stable isotope labeling by amino acids in cell culture; SLiP, short lived protein; DRiP, defective ribosome product; MudPIT, multidimensional protein identification technology.

Identifying individual DRiPs or SLiPs, both of which are rapidly degrading proteins, and assigning them to one of these categories is technically challenging due to the scarcity of these protein molecules and their peptide degradation products. DRiPs and SLiPs are degraded rapidly in the cytoplasm all the way to free amino acids and therefore may not accumulate to detectable levels even by the modern high sensitivity analytical tools. However, some of their peptide degradation products are stabilized for relatively long periods of time while bound to the MHC molecules and thus accumulate and become amenable to analysis. Identifying those MHC peptides derived from rapidly degrading proteins is interesting not only for their potential as immunotherapeutics but also as a way of detecting defects in important regulatory cellular components, such as dysfunctional tumor suppressors or cell cycle regulatory proteins. The abnormally rapid proteolysis of such proteins is known to be among contributing causes to the carcinogenic transformation of the cells (for example, p53 (10)).

MHC peptides can be identified by the direct biochemical approach originally developed by Hunt *et al.* (11) and reviewed recently in Ref. 12. This approach is based on detergent solubilization of the membranes of the cell followed by immunoaffinity isolation of the MHC molecules with their bound peptide cargo. The peptides are separated from the MHC molecules by denaturation and ultrafiltration and identified by tandem mass spectrometry (11) (for reviews, see Refs. 1 and 12–15). A recent modification to this method involves the recombinant expression of soluble, secreted MHC (sMHC) molecules by the cells. These are collected with their bound peptides from the growth medium of the cells by immunoaffinity isolation resulting in large amounts of peptides free of contaminating cellular debris or detergents (16–19). In this way, peptides presented by any polymorphic MHC molecule can be recovered from the desired model cell lines regardless of their genetic MHC background.

The use of stable isotope labeling by amino acids in cell culture (SILAC) was developed to facilitate simultaneous determination of the relative amounts of different cellular proteins in cultured cells (20). SILAC is based on *in vivo* incorporation of heavy isotope-labeled amino acids into cellular proteins. The relative amounts of the labeled *versus* the unlabeled proteins or peptides are then determined by comparing the peak height of their mass spectrometry signals. The method is compatible with most cell culture protocols and does not interfere with normal cellular functions (Refs. 20 and 21; for reviews, see Refs. 22–24). This method of metabolic stable isotope labeling was further extended to enable the determination of protein turnover rates by Pratt *et al.* (25).

In this work, the turnover kinetics of both MHC peptides and their proteins of origin were examined simultaneously in cultured human cancer cells using a pulse-chase extension of the SILAC method. It facilitated the simultaneous analysis of the turnover kinetics of large numbers of MHC peptides leading to identification of rapidly degrading proteins, some of which possibly degrade so rapidly that they go undetected by the proteomic technologies used. Interestingly rapid turnover of some of these MHC peptides may help to direct attention to the rapid degradation of their source proteins, which are possibly of significance to the carcinogenic transformation of the cells.

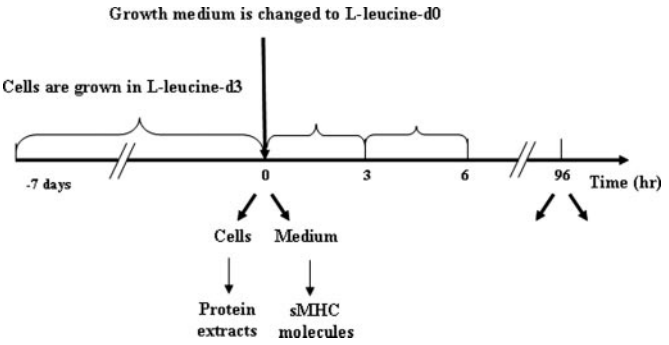

FIG. 1. **Flow chart of the experiment.**

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—The human cancer cell line UCI-107 was obtained from the ATCC. The cells were transfected (19) with the expression vector for the soluble HLA-A2.1/Q10^b (26) (a generous donation from David Margulies, National Institutes of Health). The cells were maintained in Dulbecco's modified Eagle's medium with 2 mM L-glutamine, 5% FCS, 1% penicillin-streptomycin, and 1% glutamine. The anti-HLA class I (W6/32) and the anti-HLA-A2 (BB7.2) hybridomas were obtained from the ATCC. The monoclonal antibodies were affinity-purified from mouse ascites fluid using Protein A-Sepharose 4 Fast Flow (Amersham Biosciences).

Labeling of the Cellular Proteins and HLA Peptides with Stable Isotopes—The cells were grown in Eagle's minimum essential medium supplemented with 5% dialyzed FCS, 1% penicillin-streptomycin, and 1% glutamine lacking leucine (Biological Industries, Beit Haemek, Israel). L-Leucine (Sigma) or deuterated L-leucine-5,5,5,-d_3 (99 atom %, Isotec) were prepared as 100X stock solutions of 5.2 g/liter in sterile filtered PBS. The UCI-107/A2Q10^b cells were grown in four Cell Factories (Nunc, about 3.2 liters) for at least seven cell divisions in heavy isotope medium containing d_3-leucine at a final concentration of 52 mg/liter with three to four changes of the growth medium. At time 0, the heavy isotope medium was changed into normal Dulbecco's modified Eagle's medium containing an excess of unlabeled leucine (104 mg/liter). The growth medium collected immediately before the change from the labeled medium to the unlabeled and media collected at 3, 6, 9, 12, 18, 24, 48, and 96 h after this change were stored at −20 °C until the affinity purifications of the sMHC molecules with their bound peptides (Fig. 1).

MHC Class I Peptide Affinity Purification and Analysis—The sMHC molecules were recovered from the growth medium of the cells by immunoaffinity chromatography using the W6/32 antibody covalently bound to Protein A-Sepharose 4 Fast Flow (Amersham Biosciences) as in Refs. 19 and 27. The NaCl concentration of the collected conditioned medium was raised to 150 mM, thimerosal was added to 0.01% to prevent bacterial growth in the collected conditioned medium, and the pH was adjusted to 8.0. The flow rate for the column was set to about 50 ml/h. The column was washed with 10 column volumes of 150 mM NaCl, 20 mM Tris-HCl followed by 10 column volumes of 400 mM NaCl, 20 mM Tris-HCl, 10 volumes of 150 mM NaCl, 20 mM Tris-HCl, and finally with 7 column volumes of

20 mM Tris-HCl, pH 8.0. The sMHC molecules were eluted at room temperature with 0.1 N acetic acid adjusted to pH 3.0. The peptides were recovered from the purified sMHC molecules by denaturation with 10% acetic acid and 95 °C heat treatment for 5 min. The denatured subunits of the MHC molecules and some of the contaminating antibody molecules were separated from the peptides using ultrafiltration through 3-kDa-cutoff Microcon centrifugation filters (Millipore). The peptide pools were concentrated by vacuum evaporation, and the samples were resuspended and stored in 0.1 N acetic acid at 4 °C until use (19).

Preparation and Analysis of Cytoplasmic Protein Extracts—Cellular protein extracts were prepared in parallel for each of the time points of MHC peptide collection. An aliquot of the cells was washed with PBS followed by resuspension in lysis buffer (PBS containing 2 mM PMSF). After three repetitive freezing and thawing cycles, debris were pelleted at 4 °C, and the supernatant was collected. Protein concentrations were determined with the Bradford assay (Bio-Rad). The proteins were denatured, reduced, and carboxymethylated by incubation at 60 °C for 30 min with 8 M urea, 100 mM ammonium bicarbonate, and 10 mM DTT. This was followed by adding iodoacetamide to a final concentration of 10 mM and incubation for 30 min at room temperature in the dark. The protein samples were diluted 4-fold with water to bring the urea concentration to 2 M, and the proteins were digested with trypsin overnight at 37 °C. The tryptic peptides were analyzed by MudPIT using off-line strong cation exchange followed by capillary reversed-phase chromatography. After loading the sample, the strong cation exchange column was washed with 2% ACN and 0.1% acetic acid, and the tryptic peptides were eluted with increasing concentrations of ammonium acetate (0, 10, 20, 40, 60, 80, 100, 150, 250, 400, and 500 mM), 2% ACN, and 0.1% formic acid.

Mass Spectrometry Analysis—Both the HLA and the tryptic peptides were resolved by reversed-phase HPLC using homemade 0.1-mm-inner diameter fused silica capillaries packed with POROS R2 10-μm beads (Applied Biosystems). A 125-min linear gradient from 5 to 50% ACN containing 0.1% acetic acid was used at flow rates of less than 1 μl/min and electrosprayed into an ion trap mass spectrometer (LCQ DecaXP, ThermoElectron) through a 35-gauge stainless needle. The peptides were identified using Sequest (28) and Pep-Miner (29) with the National Center for Biotechnology Information non-redundant (NCBInr) data bank. Each full MS spectrum was followed by three MS/MS spectra, and individual peptide fragmentations were limited to two times by the dynamic exclusion feature of the Xcalibur control software of the mass spectrometer.

Detecting Pairs of Peptide Isotopes and Measuring Their Ratios—Related raw MS/MS spectra were clustered by Pep-Miner as described in Ref. 29. Pep-Miner then detected pairs of clusters where each pair represented the labeled and unlabeled forms of a peptide. The precursor ion of the labeled form is heavier than the unlabeled one by multiplicities of 3 amu according to the number of leucines in the peptide. In addition, in the MS/MS spectrum of the labeled form, each leucine contributes a shift of 3 amu to the fragment ions that contain the heavy amino acids. The HLA-A2 peptides studied here were usually singly charged, most likely at their amino termini, and often contained a leucine as their second and carboxyl-terminal amino acids. Pairs that share a cluster, where the heavier cluster of one is also the lighter cluster of the other, were concatenated into groups of three or more clusters, ordered by decreasing number of isotopic leucines. For each cluster-pair of labeled and unlabeled peptides, Pep-Miner provided pointers to the MS/MS spectra contained in the two clusters. This information directed us to the MS spectra containing the precursor ions of the peptides. The relative amounts of the labeled and unlabeled forms of the peptides were determined by summing up the peak heights of each of the two forms along the MS spectra in which they appeared and comparing the two sums.

RESULTS

Determining the Turnover Kinetics of MHC Peptides and Proteins—The use of high sensitivity proteomic technologies and recovery of large amounts of sMHC molecules with their peptide cargo from conditioned medium of the cells facilitated the identifications of a large numbers of MHC peptides in cell cultures. The stable isotope pulse-chase method described by Pratt *et al.* (25), modified for this research, also enabled the independent determination of the turnover kinetics of many of these MHC peptides and of the cellular proteins. The data in Table I were gathered from about 100 LC-MS/MS analyses of three different pulse-chase experiments. The vast majority of MHC peptides and proteins were fully labeled with the heavy isotope after growing the cells in the heavy isotope medium for a minimum of 7 days. Following the transfer of the cells to the growth medium containing the unlabeled leucine, the molecular masses of the peptides shifted gradually from the heavy to the light forms. This was observed as reductions of 3, 6, and 12 atomic mass units in the isotopic peaks of the peptides according to the number of leucines in each of the peptides. Fig. 2 displays a typical mass spectrum of an HLA-A2 peptide containing one leucine. As expected, the precursor mass and some of the fragment ion masses were heavier by 3 amu relative to the unlabeled molecules. Because the heavy and light peptide forms eluted from the column with only a small difference in their retention times, the spectra of the precursor ions could be summed across the duration of their elution time, and the sums of the peak height were compared. The ratio between the sums of each isotope-labeled form of the peptide was used as an indication of the molar ratio between the light and heavy forms. The half-times of the transition of the heavy to the light form are listed only for those MHC peptides that were detected with a clear enough signal and at enough time points so that relatively accurate turnover kinetics could be established (Table I).

Comparisons between Turnover Kinetics of MHC Peptides and Proteins—The vast majority of MHC peptides were derived from proteins that were not detected through their tryptic peptides by the MudPIT analysis. Likewise most of the proteins observed through their tryptic peptides did not contribute to the detected MHC peptide pool. Of special interest to this research were those cases when the turnover kinetics could be determined with sufficient certainty for both the HLA peptides and their source protein (for example, fatty-acid synthase; Fig. 3) for which a good correlation was observed between the turnover kinetics of its MHC peptides and tryptic peptides. As expected, all of the tryptic peptides belonging to one protein displayed similar turnover time courses. Likewise in the very few cases when two MHC peptides derived from one protein were observed, both displayed the same turnover kinetics (data not shown).

Examples for MHC Peptides and Proteins with Different Turnover Rates—Here we identified 100 MHC peptides with

TABLE I

HLA-A2Q10^b peptides purified from UCI-107 cells that were identified with high certainty

The MHC peptides with biphasic turnover kinetics are in bold. *amu*, atomic mass units.

No.	Mass	Sequence	Protein	Complete turnover time	$t_{1/2}$
	amu			*h*	*h*
1	1210.7	LLLDVPTAAVQA	γ-Interferon-inducible protein IP-30 precursor	6	3
2	1011.5	LLLDVPTAAV	γ-Interferon-inducible protein IP-30 precursor	6	3
3	951.6	LLGPRLVLA	TMP21; transmembrane trafficking protein	6	3
4	965.6	ALATLIHQV	COP9 complex subunit 7a	6	5
5	913.6	GLLGTLVQL	Catenin β1	6	5
6	868.5	LLIPGLATA	NADH dehydrogenase (ubiquinone) 1α subcomplex	6	5
7	861.5	ILGPTFTL	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	6	5
8	1037.7	KLLEPVLLL	Similar to 40 S ribosomal protein S16	6	5
9	1034.6	FVFPGELLL	Solute carrier family 1 (neutral amino acid transporter), member 5	6	4
10	910.5	ALPPVLTTV	Unnamed protein product	9	?
11	946.5	SLVEEDALA	Hypothetical protein FLJ30668	9	3
12	1079.7	VLLKARLVPA	NPD019	9	4
13	947.5	ALYVAVVNV	Seven-transmembrane domain protein	9	5
14	1121.5	TLWVDPYEV	B-cell translocation protein 1	9	5
15	959.5	SLFPGQVVI	Polymerase (DNA-directed), α (70 kDa)	9	8
16	974.6	AILPTSIFL	SKB1 homolog	9	4
17	846.5	ALSRITSV	Unknown (protein for MGC:14124)	9	5
18	855.5	ALLGGLVNV	Progestin and adipoQ receptor family member IV	9	6
19	900.5	ALFPGVALL	Protein-disulfide isomerase mER60 precursor	12	8
20	1015.5	FQDPVPLTV	Transcription intermediary factor 1	18	12
21	1032.5	ALPEIFTEL	Similar to eukaryotic translation initiation factor 2, 26 subunit 3γ, 52 kDa	24	4
22	1094.6	SLLPPDALVGL	Sec23 protein	24	11
23	1360.7	ALWDIETGQQTV	Guanine nucleotide-binding protein, β-2 subunit	24	12
24	1115.5	SLFEGTWYL	Hydroxymethylglutaryl-CoA synthase	24	15
25	969.6	VIAEILRGV	Nucleolar protein 5A	24	20
26	**984.5**	**ALMPVLNQV**	**Homolog of yeast mRNA transport regulator 3**	**48**	**6**
27	908.5	NLDTSVFI	Similar to RIKEN cDNA 2610003J06	48	12
28	1258.6	FLFDGSPTYVL	Fatty-acid synthase	48	15
29	989.5	ILGGSLFGLL	ET putative translation product	48	23
30	968.6	SLLDPVPEV	Similar to RIKEN cDNA G431004K08	48	24
31	**1020.6**	**FLSSVIQNL**	**Proteasome 26 S non-ATPase subunit 1**	**96**	**6**
32	**1038.6**	**YLLPAIVHI**	**DEAD box polypeptide 17 isoform p82; probable RNA-dependent helicase p72**	**96**	**11**
33	929.5	SLLDKIIGA	Polymerase I and transcript release factor	96	14
34	1049.6	VLMQDLAFL	Unnamed protein product	96	52
35	786.4	SLAGGILGV	Protein similar to heterogeneous nuclear ribonucleoprotein K	?	24
36	867.5	Unidentified sequence	Unidentified protein source	6	3
37	918.5	Unidentified sequence	Unidentified protein source	6	3
38	969.6	Unidentified sequence	Unidentified protein source	6	5
39	906.5	Unidentified sequence	Unidentified protein source	6	5
40	915.5	Unidentified sequence	Unidentified protein source	9	6
41	1000.6	Unidentified sequence	Unidentified protein source	9	6
42	1050.6	Unidentified sequence	Unidentified protein source	9	7
43	1159.7	Unidentified sequence	Unidentified protein source	12	5
44	930.4	Unidentified sequence	Unidentified protein source	12	6
45	1061.6	Unidentified sequence	Unidentified protein source	18	12
46	909.4	Unidentified sequence	Unidentified protein source	24	?
47	929.5	Unidentified sequence	Unidentified protein source	24	6
48	1037.5	Unidentified sequence	Unidentified protein source	24	18
49	1014.7	Unidentified sequence	Unidentified protein source	24	21
50	825.5	Unidentified sequence	Unidentified protein source	48	11
51	972.6	Unidentified sequence	Unidentified protein source	96	24

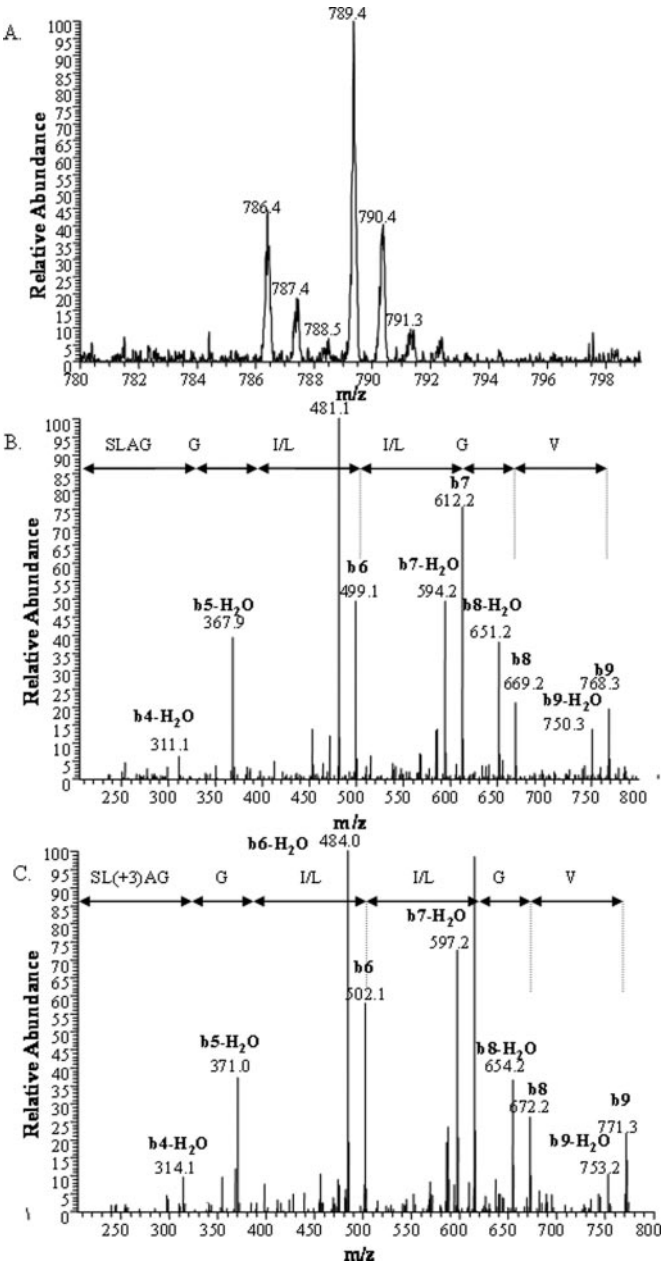

FIG. 2. **A fraction containing the unlabeled (786.4-Da) and the labeled (789.4-Da) forms of the MHC peptide SLAGGILGV from protein similar to heterogeneous nuclear ribonucleoprotein K.** A zoom scan (MS) spectrum of the peptide is displayed in *A* with the MS/MS spectra of the unlabeled and labeled forms shown in *B* and *C*, respectively.

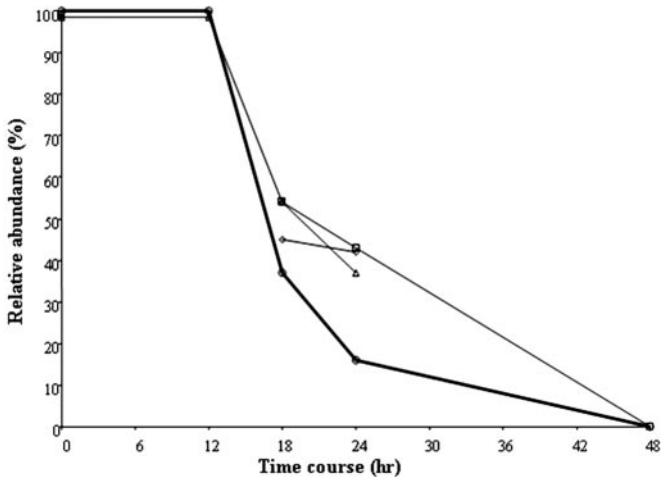

FIG. 3. **The time course of turnover of HLA peptide and tryptic peptides from the protein fatty-acid synthase.** ◇, tryptic peptide LHLSGIDANPNALFPPVEFPAPR; □, tryptic peptide TLLEGSGLESIISI-IHSSLAEPR; △, tryptic peptide HSQDLAFLSMLNDIAAVPATAMPFR; ○, HLA peptide FLFDGSPTYVL.

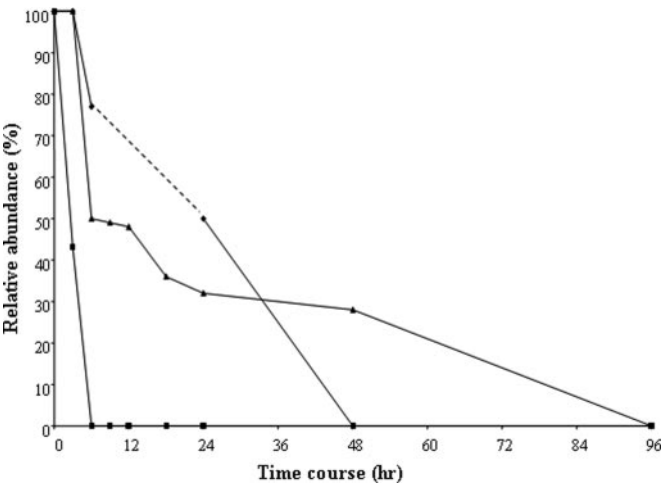

FIG. 4. **Examples for MHC peptides with fast, medium, and slow turnover kinetics.** ■, MHC peptide LLGPRLVLA from transmembrane trafficking protein, possibly a SLiP. ◆, MHC peptide SLLD-PVPEV from an unknown protein, a protein with a long turnover time. ▲, MHC peptide FLSSVIQNL from proteasome 26 S non-ATPase subunit 1, an example for an MHC peptide possibly derived from a DRiP.

high certainty for which both the heavy and the light isotope forms could be measured. Of these, relatively accurate turnover times could be determined for only 51 different peptides (an example is shown in Fig. 4, and the full listing is shown in Table I). 35 of these have the NIH-A2 score >5 (a score of matching of the sequence to the HLA-A2 consensus motif (30)) and Pep-Miner score of 80–99 (29), corresponding to Sequest Xcorr >1.4 for singly charged peptides and >2.2 for doubly charged peptides (28). Many other peptides observed with the heavy and the light isotope could be identified, but their MS signals were not resolved well enough to accurately establish turnover times. A large variability was observed in the turnover rates of the different MHC peptides (Table I). Many peptides turned over very rapidly, some even turning over with more than half of their molecules within the first 3-h collection time point. About 26% of the peptides shifted in more than 32% and up to 63% of their molecules by the 3-h time point. Other peptides turned over slower. In total, we

identified 389 MHC peptides with a NIH-A2 score >5 and with a Pep-Miner score of 80–99.

SLiPs and DRiPs—Rapidly turning over MHC peptides can be derived from normal, from SLiPs, or from abnormal, rapidly turning over proteins, many of which are possibly DRiPs. A comparison of the turnover rates between the MHC peptides and of their proteins of origin was not possible in this study for the vast majority of peptides and proteins because all of the rapidly turning over MHC peptides and many other relatively abundant MHC peptides were derived from proteins that were not detected as tryptic peptides, most likely due to their low level expression or due to the rapid degradation. Biphasic turnover was observed for some of the MHC peptides (Fig. 4; see the "Discussion"). Typically this biphasic turnover had an initial rapid phase followed by slower turnover kinetics that often lasted for many hours (Table I).

DISCUSSION

Analysis of Turnover Kinetics Based on SILAC with Heavy Leucine

The incorporation of stable isotope amino acids into proteins is becoming a method of choice for determining the relative abundances of proteins in cultured cells. This method, termed SILAC by Ong *et al.* (20), has been extended by Pratt *et al.* (25) for the determination of protein turnover kinetics. This method was adapted here to follow in parallel the turnover kinetics of MHC peptides and their proteins of origin during an extended time period. Leucine was used as the labeled amino acid because it is relatively abundant (about 10% relative abundance) in cellular proteins and is very likely to be present in the majority of the peptides presented by the HLA-A2 molecules, being part of their consensus binding motif. However, because leucine was the only amino acid used in this study, the turnover time courses could be defined only for leucine-containing peptides. The morphology of the cells was indistinguishable when grown in the normal or in the heavy isotope amino acid medium. Therefore, we could assume that the turnover kinetics of the proteins did not change when the cells were grown in the labeled medium. Furthermore several other cell lines, such as a breast cancer cell line (MDA-231) and small cell lung cancer cells (H-69AR), were grown in the d_3-labeled culture media without any noticeable change in the appearance of the cells.

The full incorporation of d_3-leucine into cellular proteins was expected to be completed after five cell doublings (20). This turned out to be an insufficient time for the cell culture used here. Preliminary experiments to evaluate whether the proteins have fully shifted to the heavy isotope labeled amino acid during 5 days indicated that a longer period was needed for full incorporation of the heavy isotope. Therefore, we extended labeling time to seven cell doublings (7 days) and recommend that such an assay should be performed in every such study.

Correlation between the Proteome and the MHC Peptidome of the Same Cells

Previously we have compared the MHC peptidome with the proteome of the same cultured cells (41) and noticed a very limited correlation between them. Here we confirm and further extend the observation that only a limited correlation (about 6%) is observed in the amounts of the MHC peptides presented by cells and the relative amounts of the proteins of origin of the these peptides in the same cells. This finding supports the assumption that many of these MHC peptides are derived from the transient proteome, which is composed of fast degrading proteins (5), and that many of these MHC peptides are derived from newly synthesized molecules that degrade rapidly after synthesis (most likely SLiPs or DRiPs). As expected, many of these rapidly degrading proteins are relatively less abundant in the cells possibly due to their short lifetimes. Such rapidly degrading proteins are often left undetected by the proteomic tools currently in use unless a special effort is invested to trace them. Following the normal or abnormal degradation of these proteins, some of the resulting peptides end up as MHC peptides, which are stabilized and accumulate to relatively large amounts when bound to soluble MHC molecules. Therefore, these peptides can serve as an indication for the transient presence of their source proteins in the cells. This observation supports the claim that the MHC peptidome represents the degradome better than the proteome of the cells (1).

Distinguishing between DRiPs and Degradation Products of Normal Protein

Often some of the newly synthesized molecules of individual proteins are DRiPs and therefore degrade rapidly, whereas other newly synthesized molecules of the same proteins that fold properly are stable for their normal expected lifetimes. A distinction should therefore be made between SLiPs and DRiPs. The SLiPs are protein molecules that normally degrade rapidly and therefore contribute more to the MHC peptide pool than the more slowly degrading proteins of the same expression level. DRiPs on the other hand are those protein molecules that degrade faster than their normal rate due to errors in folding or assembly into functional complexes. They originate from rare or abundant protein molecules and from rapidly or slowly turning over molecules. Biphasic turnover kinetics for these proteins as well as for the MHC peptides derived from them can be explained by rapid degradation of part of the protein molecules and slower degradation of the rest (31). We assume that when MHC peptides were observed with biphasic turnover kinetics the initial rapid turnover indicates a DRiP origin for these MHC peptides, whereas the slower turning over MHC peptide molecules are possibly derived from the more stable, properly folded, and also possibly functional protein molecules. Thus, the method described here offers yet another way for distinguishing between DRiPs

and normally degrading proteins. MHC peptides derived from DRiPs display biphasic turnover kinetics, whereas those derived from properly folded molecules have monophasic kinetics (albeit a rapid one if they are derived from SLiPs). Interestingly many of the peptides with biphasic kinetics were derived from proteins that are listed in the data banks as subunits of multisubunit complexes. Alternatively the rapid degradation of part of the protein molecules can be explained by surplus synthesis in the cells that leads to the rapid degradation of the excess molecules. Another possible explanation for different turnover kinetics for different molecules of the same protein is the existence of subpopulations of cells in the same tissue culture, each expressing and degrading the same proteins at different rates.

The study described here supports the notion that SLiPs and DRiPs are among the most important sources for peptides presented by MHC class I molecules (5, 7, 9, 32–34). Furthermore this method can help pinpoint the nature of this pool of rapidly degrading proteins by exploiting the relatively long lifetime of some of their degradation products as MHC peptides. This method is also important as a way of determining the turnover kinetics of MHC peptides. Once the experiment is expanded to include the determination of the turnover kinetics of a larger number of proteins and MHC peptides, it will enable better correlation between the turnover times of MHC peptides and of their source proteins. MHC peptides derived from DRiPs are possibly good vaccine candidates for clinical use in immunotherapy (5, 7, 9, 32–34).

Bioinformatic Analysis of the Kinetic SILAC Data

Peptide identification was performed with Sequest and Pep-Miner using the NCBInr data bank and allowing for one or more heavy leucines in a peptide. An important new feature of Pep-Miner, developed for this study, allows for finding SILAC-related spectra even when they appear in different LC-MS/MS runs. This is in contrast to the usual use of SILAC for comparing peaks in the same spectrum or in adjacent spectra. This new feature allowed for tracing the dynamic behavior of peptides and proteins during the time course of the experiment. It also helped us compare SILAC data between different cell lines or cell treatments and between the MHC peptidome and the proteome of the cell. Moreover the dynamic behavior of peptides could be traced even for unidentified peptides because the association of the two forms with one another is done at the MS/MS spectrum level rather than at the postidentification level. This capability is especially significant for MHC peptides, which are mostly singly charged and therefore more difficult to identify than tryptic peptides, with their lysines or arginines at the carboxyl terminus. To increase somewhat the signal to noise ratio, the relative abundances of the heavy and light isotopes of a peptide were estimated by summing up the peak heights of each of the forms in the MS domain along the elution period and comparing the sums.

Once a peptide was identified, the knowledge about the locations of labeled leucines in its sequence can be used to validate, or invalidate, the SILAC analysis results because each labeled leucine contributes a mass shift of 3 amu. The use of SILAC to help improve protein identifications using peptides mass fingerprinting data has already been proposed (21).

Limitation of Analysis of Very Rapid Turnover Kinetics

Each time point of the experiment represents accumulation of MHC peptides secreted to the growth medium of the cells with the presenting MHC molecules during the interval from the previous collection of growth medium. In contrast, each time point of protein analysis represents the isotope content of the actual proteome at the time of collection. During the first 3 h, the MHC peptide samples did not contain any peptides fully shifted from the heavy to the light forms. This is expected due to the delay between the synthesis of proteins containing the unlabeled leucine introduced when the medium was changed and the presentation of their degradation products by MHC class I complex. It takes about 15 min for newly synthesized protein molecules to reach the proteasome and another 15 min to enter the endoplasmic reticulum (32), and an additional 15 min are needed for MHC class I molecule to reach the cells surfaces once they are loaded with peptides in the endoplasmic reticulum (35). Therefore, it can be assumed that the time lag between the protein synthesis and its MHC peptide presentation is about an hour. Thus, the MHC peptide repertoire of the first 3 h included peptides that were secreted by the cells very soon after the medium change, and these did not have sufficient time to shift completely in their isotope content from the heavy to the light. Moreover after the replacement of the labeled growth medium with the medium containing an excess of the unlabeled leucine, the cells still have some heavy amino acids in the cytoplasmic pool that they use for the synthesis of new proteins. Correlating turnover of MHC peptides and their source proteins is possible with this approach only if both the proteins and the MHC peptides are abundant enough to be traced accurately enough (fatty-acid synthase for example).

Abnormally Rapid Degradation

The loss of tumor suppressors and cell cycle checkpoint proteins due to mutations leading to misfolding or to instability, and therefore to rapid degradation of the proteins, is among the known causes for carcinogenesis (10). Moreover proteins involved with cell cycle control are normally degraded when the cell passes the specific phase in the cell cycle. Therefore the rapid turnover of these proteins is a hallmark of rapidly dividing normal cells in general and of cancer cells in particular. The presence of elevated levels of MHC peptides due to rapid degradation of such proteins can be exploited to target these cells. Identifying MHC peptides

that exhibit abnormally rapid turnover can draw attention to abnormalities in the synthesis and degradation of such proteins.

Examples for Rapidly Turning Over Peptides of Possible Interest

The following peptides are of possible significance for the cellular biology of these cancer cells. They were noticed among the rapidly turning over peptides and therefore drew our attention. These are either SLiPs or DRiPs that are difficult to distinguish due to their rapid turnover kinetics.

Transmembrane Trafficking Protein; TMP21 (LLGPRLVLA)—This MHC peptide of TMP21 displayed rapid turnover kinetics and drew our attention because this protein expression is known to be down-regulated, up to 9-fold, in cisplatin-resistant cells. Anti-cancer cisplatin treatment induces apoptosis (36). The rapid turnover kinetics may indicate that the degradation of TMP21 confers chemoresistance to these cells.

Catenin β1; CTNNB1 (GLLGTLVQL)—β-Catenin mediates adhesion between cells and regulates normal cell growth and behavior. Processes such as several stages of embryogenesis, wound healing, and tumor cell metastasis are regulated by the assembly and disassembly of adhesion proteins. Several CTNNB1 mutations that caused abnormal cell proliferation, regulation, and tumor progression have been found in colorectal cancer cell lines, primary ovarian carcinomas and other cancers (37, 38). In normal resting cells, β-catenin is localized to the adherent junctions on the cell membrane, and free cytoplasmic levels of β-catenin are very low because the protein is rapidly destroyed by ubiquitin-proteasome degradation. Although activating β-catenin mutations that stabilize the protein have been discovered in many tumors, a reduction of β-catenin bound to the cell surface has been demonstrated recently in several tumors, such as thyroid, colon, and esophagus tumors (39). The reduction is consistent with progressive deregulation of intercellular adhesion in cancer that promotes tumor detachment from the primary site and facilitates tumor spread. Both reduction of β-catenin membrane immunoreactivity and its aberrant nuclear localization parallel loss of tumor differentiation and poor prognosis. Also rapidly turning over peptides may appear as a result of high levels of expression of mutated protein products in the malignant cells or due to reduction of β-catenin bound to the cell surface (10).

B-cell Translocation Protein 1 (TLWVDPYEV)—This MHC peptide was observed in many of our large scale HLA peptide analysis projects (19) such as UCI-101, UCI-107 (ovarian cancer), and PC-3 (prostate cancer). This MHC peptide attracted our attention because the *BTG1* gene locus has been shown to be involved in a chromosomal translocation in B-cell chronic lymphocytic leukemia. This protein possesses antiproliferative activity, and thus its abnormal degradation may enhance the carcinogenic transformation of different types. *BTG1* is a member of a family of antiproliferation genes (40) and is therefore a putative tumor suppressor gene. *BTG1* expression is maximal in the G_0/G_1 phases of the cell cycle and is down-regulated when cells progress through G_1.

Acknowledgments—We are grateful to Dr. Stacy H. Shoshan for critical reading of the manuscript and Ilana Navon for help with the mass spectrometry.

* This work was supported by the Greta Koppel Small Cell Lung Carcinoma (SCLC) research fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 972-48293407; Fax: 972-48225153; E-mail: admon@tx.technion.ac.il.

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