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Measurement of protein synthesis using heavy water labeling and peptide mass spectrometry: Discrimination between major histocompatibility complex allotypes

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Abstract

Methodological limitations have hampered the use of heavy water (2H2O), a convenient, universal biosynthetic label, for measuring protein synthesis. Analyses of 2H-labeled amino acids are sensitive to contamination; labeling of peptides has been measured for a few serum proteins, but this approach awaits full validation. Here we describe a method for quantifying protein synthesis by peptide mass spectrometry (MS) after 2H2O labeling, as applied to various proteins of the major histocompatibility complex (MHC). Human and murine antigen-presenting cells were cultured in medium containing 5% 2H2O; class I and class II MHC proteins were immunoprecipitated, bands were excised, and Ala-/Gly-rich, allele-specific tryptic peptides were identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Mass isotopomer distributions were quantified precisely by LC–MS and shifted markedly on 2H2O labeling. Experimental data agreed closely with models obtained by mass isotopomer distribution analysis (MIDA) and were consistent with contributions from Ala, Gly, and other amino acids to labeling. Estimates of fractional protein synthesis from peptides of the same protein were precise and internally consistent. The method was capable of discriminating between MHC isotypes and alleles, applicable to primary cells, and readily extendable to other proteins. It simplifies measurements of protein synthesis, enabling novel applications in physiology, in genotype/phenotype interactions, and potentially in kinetic proteomics.

Keywords: Peptide liquid chromatography/mass spectrometry; Major histocompatibility complex proteins; Allelic polymorphism; Mass isotopomer distribution analysis; Protein synthesis.
rate-limiting step for $^2$H incorporation (Fig. 1A). Body water turns over slowly [12], and a fixed enrichment is maintained indefinitely by regular oral $^2$H$_2$O intake [8]. This enables extended labeling protocols for quantification of slow protein turnover rates. The analytical approach used in most studies to quantify label incorporation involves total hydrolysis of proteins of interest to single amino acids and quantification of $^2$H incorporation into NEAA (most often Ala) by gas chromatography–mass spectrometry (GC–MS) [5–7, 13–15]. This approach is precise and sensitive, but it can easily be confounded by contamination with extraneous proteins or free amino acids; in practice, applications have been limited to relatively abundant, easily purified proteins. Analysis of $^2$H-labeled tryptic protein fragments [16, 17] is less easily confounded, but studies have been limited to rodent serum albumin and contaminants of albumin preparations; methods for estimating fractional synthesis from experimental data are somewhat cumbersome, and validation of model assumptions underlying this methodology remains incomplete.

Here we wished to examine the utility of the SINEW approach, combined with tryptic peptide analysis by liquid chromatography (LC)–MS (Fig. 1B), for quantifying the synthesis of major histocompatibility complex (MHC) proteins. These membrane glycoproteins, abundant on immune cells, present antigens to T lymphocytes of the adaptive immune system; their extensive genetic polymorphism is important in acute allogeneic transplant rejection and autoimmune disease susceptibility. The posttranslational regulation of their fate and function is of great interest in immunology and may be affected by polymorphism. Here we have developed a peptide LC–MS method for measuring the fractional synthesis of MHC protein allomorphs in cell culture by SINEW.
The resultant innovations greatly simplify the analysis and enhance the versatility of this approach.

Materials and methods

Cell lines

The human Epstein–Barr virus (EBV)-transformed B cell line, Priess, was used to quantify synthesis of human leukocyte antigen (HLA)-DR. Priess cells are homozygous at the HLA-DR locus, expressing the DRB1*0401 allele along with the nearly monomorphic DRA*0101 gene product [18]. Monocyte-derived dendritic cells were differentiated with GM-CSF (50 ng/ml) and IL4 (1000 U/ml) for 7 days after immunomagnetic bead isolation of CD14+ monocytes from peripheral blood mononuclear cells. LCL721 cells were used to quantify the synthesis of HLA-B*08 and B*51 MHC class I heavy chain gene products [19]. A20 murine B lymphoma cells, derived from Balb/c mice (H-2d), or splenic B cells from Balb/c mice were used as a source of murine H2-A\textsuperscript{-A}\textsuperscript{d}\textsuperscript{I} (A\textsuperscript{-d}\textsuperscript{I} heterodimer) MHC class II molecules [20]. Expression of MHC molecules was verified by immunofluorescence staining followed by flow cytometric analysis (data not shown).

For analysis of murine H2-A\textsuperscript{d} MHC class II molecules, M12.NOD cells that were derived from the M12.C3 cell line by transfection with a genomic construct coding for A\textsuperscript{-d} \beta-chains (the A\textsuperscript{-d}\textsuperscript{I}–chain, identical to that in the g7 haplotype, is endogenously expressed) were used. A 4.3-kbp BamHI–KpnI fragment, containing part of the 5’ flanking sequences of the Ab\textsuperscript{A} promoter [21], was cloned into pTCF. The entire Ab\textsuperscript{A} gene was subcloned from the cosmid -Abb-TCF described above. For transfection, the extended polyclones were adjusted to approximately 5% \textsuperscript{2H}\textsubscript{2}O enrichment in medium (>24 h), cells were counted and split daily with the addition of medium containing approximately 5% \textsuperscript{2H}\textsubscript{2}O. Labeling experiments were performed for at least five doublings to ensure nearly complete (>97%) labeling. Medium and culture supernatants were analyzed for \textsuperscript{2H}\textsubscript{2}O enrichment using isotope ratio mass spectrometry (IRMS) after gravimetric dilution (MRC–HNH [Human Nutrition Research], Widdowson Laboratories, Fulbourn, Cambridge, UK) and equilibration with H\textsubscript{2} gas [29].

MHC protein isolation

Immunoprecipitation was performed using modifications of established protocols [30]. Unlabeled and \textsuperscript{2H}\textsubscript{2}O-labeled cell pellets were lysed for 1 h at 4 °C in 1% CHAPS (Sigma–Aldrich) or 1% IGEPAL CA-630 (NP40) (Sigma) in 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), and protease inhibitors (Roche Complete Protease Inhibitors used per manufacturer’s instructions) at a ratio of up to 10\textsuperscript{6} cells/ml lysis buffer. Nuclei and debris were removed by centrifugation (10,000g in a microcentrifuge, 30 min, 4 °C). After preclaring with protein A or protein G Sepharose (20 μl packed beads, Sigma–Aldrich), immunoprecipitation was performed using protein A or protein G Sepharose and 10–30 μg of the appropriate mAb (1 h to overnight, 4 °C). Negative controls included the last preclaring step, irrelevant control antibody, and precipitation from a cell lacking the protein of interest. Pellets were washed repeatedly in lysis buffer with 0.1% detergent. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) was performed on 10% acrylamide gels under nonreducing conditions. Gels were fixed and stained with Coomassie blue or silver, and bands were excised and stored at or below −20 °C.

Sample preparation and peptide MS

SDS–PAGE gel pieces were thawed and transferred into 96-well polymerase chain reaction (PCR) plates for automated sample preparation in a Mass Prep Station (Waters). Briefly, the gel bands were destained, reduced (dithiothreitol [DTT]), alkylated (iodoacetamide), and digested with trypsin overnight at 37 °C. Thereafter, 20 μl of supernatant was pipetted into a sample vial and loaded onto an autosampler for LC–tandem mass spectrometry (MS/MS) analysis.

All LC–MS/MS and LC–MS experiments were performed using an Eksigent NanoLC-1D Plus (Eksigent Technologies, Dublin, CA, USA) high-performance liquid chromatography (HPLC) system and an LTQ Orbitrap mass spectrometer (ThermoFisher, Waltham, MA, USA). Peptides were separated by reverse-phase chromatography using a flow rate of 300 nl/min and an LC Packings PepMap 100 C18 column (75 μm i.d. × 150 mm, 3 μm particle size, Dionex, Sunnyvale, CA, USA). Peptides were loaded onto a precolumn (Dionex Acclaim PepMap 100 C18, 5 μm particle size, 100 Å pore size, 300 μm i.d. × 5 mm) from the autosampler with 0.1% formic acid for 5 min at a flow rate of 10 μl/min. The 10-port valve was then switched to allow elution of peptides from the precolumn onto the analytical column. Solvent A was 0.1% aqueous formic acid, and solvent B was acetonitrile + 0.1% formic acid. The gradient employed was 5–50% B in 40 min. The LC eluate was sprayed into the mass spectrometer by means of a New Objective nanospray source. For MS/MS experiments, all m/z values of eluting ions were measured in the Orbitrap mass analyzer set at a resolution of 7500.
Peptide ions with charge states of 2+ and 3+ were then isolated and fragmented in the LTQ linear ion trap by collision-induced dissociation, and MS/MS spectra were acquired.

For peptide identification, the data were processed using Bioworks Browser (version 3.2.1 SP1, Thermo Fisher Scientific). MS/MS data were converted to dta (text) files using the Sequest Batch Search tool (within Bioworks). The data files were converted to a single mgf file using an SSH script in the SSH Secure Shell Client program (version 3.2.9, build 283, SSH Communications). These combined files were then submitted to the Mascot search algorithm (Matrix Science, London, UK) and searched against the mouse or human NCBI database, as appropriate, using a fixed modification of carbamidomethyl (for cysteines) and a variable modification of oxidation (for methionines).

The peptide identifications from the Mascot search were matched to the M0 precursor peak in the chromatograms through careful scrutiny of the MS/MS data contained within the Mascot search result and the MS/MS spectra from the raw data. Once the MS/MS data for individual unlabeled peptides had been matched, the retention times of the peptides were established and this information was used for the subsequent integrations (see below). For quantification of mass isotopomer distributions, selected ion monitoring (SIM) experiments, in which limited m/z regions were exclusively scanned for specific unlabeled or labeled ions, were performed. Peak integration was performed using Xcalibur QualBrowser software (version 2.0, Thermo Fisher Scientific). Briefly, peaks were smoothed within QualBrowser (Boxcar, 7 points) to remove any spikes within the peaks before reconstructed ion chromatograms were generated for each mass isotopomer and the areas under the peaks were measured. The reconstructed ion chromatograms posed no difficulties with respect to baseline drift or variable peak shapes. Peak contamination was rarely seen and was readily detectable by visual inspection of chromatograms when present; the data shown were from uncontaminated chromatograms. Each peak was integrated across the same retention time range to ensure that the areas of the peaks were consistent for each mass isotopomer. Measured peak areas were copied into Microsoft Excel spreadsheets for further analysis. The same peak integration procedure was used for unlabeled, partially labeled, and fully labeled peptides.

Calculations

Cell growth

Log-transformed cumulative cell counts (cell density per milliliter × volume, corrected for removal of cell samples or dilution since the start of the experiment) were plotted as a function of time. Doubling times were calculated from the slope of the best-fitting straight line according to the relationship:

\[ t_2 = \ln(2)/\text{slope}. \]

Fractional protein synthesis

Standard nomenclature for mass isotopomer distribution analysis (MIDA) calculations was used [31]. For each peptide, the mass species corresponding to the monoisotopic molecular weight was defined as M0. This species comprises only the lowest atomic weight isotopes of each element (i.e., all \(^{12}\text{C}^1\text{H}^{16}\text{O}^{14}\text{N}^{32}\text{S}\)). Replacement of \(^1\text{H}\) by \(^2\text{H}\), replacement of \(^{12}\text{C}\) by \(^{13}\text{C}\), and so on at any one position adds 1 Da, and the collection of these isotopomers (isotopic isomer) is referred to as the M1 mass isotopomer. Any combination of two of these substitutions, or replacement of \(^{18}\text{O}\) by \(^{16}\text{O}\), adds 2 Da, yielding M2 and so on.

Integrated LC peak intensities (designated \(I\)) measured across the first-dimension MS mass envelope of the peptide of interest were converted to fractional molar abundances (designated \(A\)) by dividing the peak intensity of the mass isotopomer \(M_i\) \((i = 0, 1, 2, \ldots, N)\) by the sum of the peak intensities of all \((N + 1)\) quantifiable mass isotopomers in the peptide mass envelope:

\[ A_i = I_i / \sum_{i=0}^{N} I_i. \]

To measure the changes in mass isotopomer distributions over time during a continuous \(^2\text{H}_2\text{O}\) labeling experiment, the fractional molar abundance of each mass isotopomer at baseline was subtracted:

\[ \Delta A_i(t) = A_i(t) - A_i(t = 0). \]

Replicate measurements of \(A_i\) and \(\Delta A_i\) were averaged, and standard deviations were calculated.

Fractional protein synthesis \((f)\) was calculated from the fractional abundance of each mass isotopomer using the relationship

\[ f(t) = \Delta A_i(t)/\Delta A_i^\infty, \]

where \(\Delta A_i^\infty\) represents the maximal shift in fractional molar abundance of the mass isotopomer from baseline, obtained empirically from fully labeled samples. Estimates of \(f\) from different mass isotopomers within one peptide were averaged, and standard deviations were calculated. Mass isotopomers with low values of \(\Delta A_i^\infty\) gave substantially noisier estimates of \(f\) (see Results) and were excluded from the analysis.

Mass isotopomer distribution analysis

MIDA uses combinatorial probability algorithms to calculate the theoretical mass isotopomer distribution for any biomolecule both at baseline (i.e., with all isotopes present at their natural abundance) and after stable isotope labeling. MIDA algorithms have been described exhaustively elsewhere [31] and were used in a software implementation kindly provided by R.A. Neese and M.K. Hellerstein.

The expected baseline mass isotopomer distributions, \(A_i^\text{baseline}\), were calculated from the elemental composition of each peptide, which was determined from its sequence and corrected for added protons (+1/charge acquired) and for carbamidomethylation of any Cys residues present. In addition, the mass isotopomer distributions of fully labeled peptides were modeled. In the simplest model, used throughout the main text, the measured \(^2\text{H}_2\text{O}\) enrichment in medium was taken to represent the precursor pool enrichment (designated \(p\) in the standard nomenclature used in MIDA calculations [31]). Mass isotopomer distributions were then calculated, using MIDA software, for different numbers of labeling sites (designated \(n\)). Alternative models (see Supplementary material) invoking uniform label dilution were calculated with MIDA software using the indicated values of \(n\) and \(p\). A nonuniform dilution model was assembled in two steps. First, the baseline mass isotopomer distribution and the contribution of the fully labeled hydrogens were modeled using MIDA software. Second, a spreadsheet program was used to adjust for the contribution of the partially labeled hydrogen atoms using Eq. (A3) of Ref. [31].

\(\Delta A_i\) values can be determined accurately only for those mass isotopomers that can be quantified in both the unlabeled and labeled samples [31]. To allow comparison of MIDA calculations to experiment, the predicted distributions were truncated to include only the \((N + 1)\) mass isotopomers that were experimentally quantifiable and normalized to add up to 100%:

\[ A_i^\text{NORMAL} = A_i^\text{MIDA} / \sum_{i=0}^{N} A_i^\text{MIDA}. \]

The truncation perturbs the linear relationship between measured mass isotopomer abundances and \(f\) [31]; however, this bias is negligible when the measured mass isotopomer distributions account for more than 90% of the total mass envelope [31], as was the case here (data not shown). The goodness of fit of the observed mass iso-
topomer distributions, $A_{\text{obs}}$ was assessed by calculating root mean square deviations (RMSDs) from the truncated, normalized MIDA values:

$$\text{RMSD} = \sqrt{\sum_{i=0}^{N} (A_{\text{obs}} - A_{\text{NORM}})^2 / (N + 1)},$$

where the summation was performed over all $(N+1)$ observed mass isotopomers.

**Results**

**Approach and peptide identification**

Initially, the synthesis of HLA-DR0401 molecules in Priess cells was analyzed using the SINEW approach with peptide LC–MS analysis (Fig. 1B). Cells grew indistinguishably in the presence and absence of 4.5% $^2\text{H}_2\text{O}$ in medium (Fig. 1C); the $^2\text{H}_2\text{O}$ enrichment remained constant throughout the culture period (Fig. 1D). HLA-DR αβ heterodimers were immunoprecipitated and visualized by Coomassie blue staining after SDS–PAGE analysis (Fig. 1E). To identify tryptic peptides suitable for kinetic analysis, unlabeled α- and β-chain bands were excised, digested with trypsin, and analyzed by LC–MS/MS for peptide sequencing. Matches to the DRα and DR0401β protein sequences were identified using the Mascot search algorithm and are shown in Fig. 2A and B. Peptides rich in Ala and/or Gly, two of the amino acids that are nearly fully labeled from $^2\text{H}_2\text{O}$ in mice [6], were identified in both chains (Table 1; underlined in Fig. 2A and B). The quantification of mass isotopomer distributions in the relevant $m/z$ range is shown for the DRα (51–67) peptide in Fig. 2C and D. A single LC peak of the expected $m/z$ ratio was observed for the lowest (M0) mass isotopomer of the peptide, comprising only the lowest atomic weight isotopes of each element (Fig. 2C). Even in the absence of $^2\text{H}_2\text{O}$ labeling, a series of mass isotopomers (M0–M4) was present at this retention time (Fig. 2D) owing to the presence of stable isotopes in nature (e.g., 1.09% $^{13}\text{C}$, 0.02% $^{2}\text{H}$). The individual mass peaks were separated by 0.5 $m/z$ units due to the $z=2$ charge state of this peptide. The identity of this peptide was confirmed by comparing its MS/MS spectrum, extracted from raw data, with the MS/MS spectrum embedded in the Mascot search results (data not shown).

**Fig. 2.** Identification of peptides suitable for $^2\text{H}_2\text{O}$ labeling studies. (A and B) Identification of Ala-/Gly-containing HLA-DR4-derived tryptic peptides. DRα (A) and DRβ (B) bands excised from SDS gels of L243 immunoprecipitates from Priess cell extracts were reduced, carbamidomethylated, digested with trypsin, and analyzed by LC–MS/MS. Alignments of tryptic fragments to the sequence of their parent polypeptides are shown. Peptides selected for analysis are shown in bold type and underlined. (C and D) LC–MS analysis of mass isotopomer distributions for the DRα peptide, FASFEAQGALANIAVDK. (C) LC chromatogram of a DRα digest, with SIM for $m/z = 876.45$, corresponding to the M0 mass isotopomer of the doubly charged peptide. Integration of the principal LC peak for this mass isotopomer, and for higher order mass isotopomers, was used to quantify mass isotopomer distributions. (D) Mass spectrum for the LC peak of the intact DRα peptide showing a single, well-resolved set of mass isotopomers.
Analysis of mass isotopomer distributions

The LC peak of each of the mass isotopomers of the unlabeled DRx peptide was integrated, and its abundance was expressed as a percentage of the total abundance of the M0 to M4 mass isotopomers (Fig. 3A). Standard deviations (SDs) of six replicate injections ranged from 0.08% to 0.43% for different mass isotopomers, indicating high analytical precision. MIDA allows baseline fractional molar abundances to be predicted from the elemental composition of the peptide; the combinatorial probability algorithms used in MIDA have been reviewed [31]. The data agreed well with MIDA predictions (Fig. 3A) within experimental error; the RMSD between MIDA prediction and experiment was 0.15% for the average of the replicates and ranged from 0.08% to 0.43% for different mass isotopomers; RMSD was 0.43% for the average of five replicates, and RMSDs for individual determinations ranged from 0.08% to 0.43% for different mass isotopomers. The shape of the mass isotopomer distribution was reproducible, conforming to the isotope enrichment in the precursor pool (p). During 2H2O labeling, 2H atoms enter the peptide individually at an unknown number of biosynthetic labeling sites, at each of which 2H is biosynthetically incorporated from 2H2O without label dilution from competing sources (i.e., at p = 4.5% 2H enrichment in this experiment). This model gave excellent agreement with the labeled mass isotopomer distributions when the value of p was adjusted for optimal fit to the data (n = 22–23 in several independent determinations, RMSD = 0.2–0.3%) (Fig. 3C and D, and Table 2). The optimal value of n was well matched to the total number of C=H bonds [22] nominally attributable to the side chains of Ala (n = 4/residue) and Gly (n = 2). Thus, 2H incorporation into the DRx peptide under these culture conditions is almost entirely accounted for by labeling of Ala and Gly, although contributions from other amino acids, and label dilution in Ala and Gly, were not ruled out by our data.

The DR0401β (73–80) peptide was also analyzed. Its unlabeled mass isotopomer distribution was reproducible, conforming to MIDA predictions (Figs. 3E and S1C). SDs ranged from 0.09% to 0.43% for different mass isotopomers; RMSD was 0.43% for the average of five replicates, and RMSDs for individual determinations ranged between 0.15% and 0.71%. For the labeled samples, the best model fit to the data was obtained assuming n = 8 or 9 labeling sites labeled at p = 4.5% 2H enrichment (Fig. 3F–H and Table 2). This labeling pattern is well matched to the eight labeling sites attributable to the two Ala residues in this peptide. MIDA algorithms were used to model the effects of 2H2O labeling on the mass isotopomer distributions [31]. The shape of the labeled distributions depends on the number of labeling sites (n) and the isotope enrichment in the precursor pool (p). During 2H2O labeling, 2H atoms enter the peptide individually at an unknown number of labeling sites within the NEAAs present; the precursor/product relationship depends on the amino acid composition of the peptide. The simplest model involves a fixed number (n) of biosynthetic labeling sites, at each of which 2H is biosynthetically incorporated from 2H2O without label dilution from competing sources (i.e., at p = 4.5% 2H enrichment in this experiment). This model gave excellent agreement with the labeled mass isotopomer distributions when the value of n was adjusted for optimal fit to the data (n = 22–23 in several independent determinations, RMSD = 0.2–0.3%) (Fig. 3C and D, and Table 2). The optimal value of n was well matched to the total number of C=H bonds [22] nominally attributable to the side chains of Ala (n = 4/residue) and Gly (n = 2). Thus, 2H incorporation into the DRx peptide under these culture conditions is almost entirely accounted for by labeling of Ala and Gly, although contributions from other amino acids, and label dilution in Ala and Gly, were not ruled out by our data.

The DR0401β (73–80) peptide was also analyzed. Its unlabeled mass isotopomer distribution was reproducible, conforming to MIDA predictions (Figs. 3E and S1C). SDs ranged from 0.09% to 0.43% for different mass isotopomers; RMSD was 0.43% for the average of five replicates, and RMSDs for individual determinations ranged between 0.15% and 0.71%. For the labeled samples, the best model fit to the data was obtained assuming n = 8 or 9 labeling sites labeled at p = 4.5% 2H enrichment (Fig. 3F–H and Table 2). This labeling pattern is well matched to the eight labeling sites attributable to the two Ala residues in this peptide.

We wished to examine whether the MHC-derived peptides also incorporate the 2H label at exchangeable sites, such as solvent-exposed O=H and N=H bonds [32], which would confound the analysis of biosynthetic label incorporation at nonexchangeable C=H bonds. Thus, unlabeled Press cells were lysed in 2H2O-enriched lysis buffer, immunoprecipitated, and washed in unenriched buffer.

Table 1  
MHC protein-derived peptides used for analysis of 2H2O labeling.  

| Cell | mAb | Polypeptide | AAa | Sequence | m0/p| 2H |
|------|-----|-------------|-----|----------|-----|-----|
| Priess | L243 | DRx | 51–67 | FASTFAGALANIAVK | 876.46 | 2
| | | DR0401β | 73–80 | ΔDYTDYCR | 478.22 | 2
| LCL721* | W6/32 | BB | 132–145 | SWTAADTAOITQR | 760.28 | 2
| | | BS1 | 122–145 | DYATLNEKSSWTAADTAOITQR | 1327.48 | 2
| | | | 317–338 | GCSYSQAEADSQGDSVSLTA | 1023.72 | 2
| A20 | MKD6 | αx | 43–65 | LPEFQULIFEPGGIQNAK | 838.13 | 3
| M12.NOD | OX-6 | Aα | 73–80 | ΔEYDYCR | 461.21 | 2
| A20 | MKD6 | Aαβ | 73–80 | ΔEYDYCR | 468.22 | 2

* Mascot searches identified multiple peptides from the indicated HLA or H-2 polypeptides following specific immunoprecipitation using cells and antibodies as shown. Peptides were selected on the basis of their Ala/Cys content and polymorphism.

** Amino acid sequence numbering refers to the mature protein.

*** Amino acids in single-letter code. Ala/Gly residues (whose side chain C=H bonds are known to be accessible to labeling from 2H2O) are underlined, and relevant polymorphisms are in bold.

**** Mass-to-charge ratios experimentally determined by LC–MS, where m0 mass represents the lowest mass isotopomer.

***** Charge determined by the spacing of peaks in the MS mass envelope.

****** The full set of HLA-DR0401-derived tryptic peptides is shown in Fig. 2A and B. No peptides were found to match the DRB4*0101-encoded DRw53 3-chain, which is coexpressed in the DR4 haplotype and pairs with DRx; thus, DRw53 might not have been coisolated in substantial amounts.

******* LCL721 is heterozygous in the MHC class I region; allele-specific HLA-B peptides were selected for analysis. Both polymorphisms within the peptide and adjacent polymorphisms affecting tryptic cleavage can be exploited; the latter is illustrated here by the Ser131 polymorphism in the B51 (122–145) peptide, which destroys a tryptic cleavage site present in B8.

******** The α-chains of Aα and Aαβ are identical; the peptide shown was found in MKD6 immunoprecipitates from A20 cells (Aα) and in OX-6 immunoprecipitates from M12.NOD (Aαβ).

********* The z = 3 triply charged peptide species were also present, albeit at lower abundance.
LC–MS analysis revealed no distortion of the unlabeled mass isotopomer distributions due to \( ^2\text{H}_2\text{O} \) exposure post lysis (Fig. S1d). Thus, label retention at exchangeable sites does not confound measurements of protein synthesis.

The analysis was extended to other MHC proteins. Human HLA-A, -B, and -C class I molecules were isolated from EBV-transformed B cells, and murine H2-A\(^{\alpha}\) class II molecules were isolated from M12.NOD transfectants (Fig. 1F and G). H2-A\(^{\alpha}\) molecules, isolated from A20 B lymphoma cells, were obtained in lower abundance and detected by silver staining (not shown). LC–MS/MS analyses identified suitable peptides within H2-A\(^{\alpha}\) and H2-A\(^{\alpha}\) \( \alpha \)- and \( \beta \)-chains (Table 1; note that both alleles share

![Graphs showing mass isotopomer distributions](image-url)
Ala and Gly, implying contributions from other amino acids to labeling. Quantification of fractional protein synthesis

In metabolic labeling experiments, the enrichment in the isotopically labeled precursor pool is generally kept constant, so that newly synthesized molecules incorporate the label to the same extent regardless of when they are synthesized. In the SINEW approach, however, the NEAA tRNAs used for protein synthesis are labeled indirectly from $^2\text{H}_2\text{O}$. At early time points, label equilibration might be incomplete, so that $p$ or $n$ might increase over time. This would confound the estimation of fractional protein synthesis ($f$) from $^2\text{H}_2\text{O}$ labeling data. Previous work has shown that C–H bonds of Ala and Gly precursors are rapidly labeled from $^2\text{H}_2\text{O}$ [5–7,10], but considerable label dilution has been noted in Pro [15] and data for $^2\text{H}_2\text{O}$ labeling of other NEAAs in cell culture are unavailable. Thus, the possibility of delayed label entry was a concern, particularly for peptides whose label incorporation patterns were only partially accounted for by their Ala and Gly content.

Therefore, we considered the behavior of mass isotopomer distributions during a labeling time course assuming that there is no change in $p$ or $n$. If this assumption is correct, old molecules, which retain the baseline mass isotopomer distribution, will gradually be replaced (during protein turnover) or diluted (due to cell growth) with new molecules, all of which possess a mass isotopomer distribution identical to that of fully labeled molecules. Thus, the mass isotopomer distribution, $A_i(t)$, of a partially turned-over protein pool (i.e., the fractional molar abundance, $A_i$ of mass isotopomer $i$ at time $t$) is the weighted average of the unlabeled $[A_i(0)]$ and fully labeled ($A_i^\infty$) mass isotopomer distributions; the weighting factor, $f(t)$, represents fractional protein synthesis:

$$A_i(t) = [1 - f(t)] \times A_i(0) + f(t) \times A_i^\infty.$$ 

Thus, fractional protein synthesis, $f(t)$, can be calculated as

$$f(t) = [A_i(t) - A_i(0)]/[A_i^\infty - A_i(0)] = \Delta A_i^f(t)/\Delta A_i^\infty. \quad (1)$$

Therefore, if there is no change in precursor/product relationships during the experiment, each mass isotopomer will change from its baseline to its fully labeled value at the same rate, characteristic of the rate of fractional protein synthesis, and will provide an independent estimate of $f$. In contrast, if the label equilibrates poorly...
during early time points, this will distort the mass isotopomer distributions of molecules synthesized early on; therefore, analysis of different mass isotopomers using Eq. (1) will yield systematically different estimates of $f$ (see Fig. S3 in Supplementary material).

Accordingly, we examined whether the analysis of different mass isotopomers yields convergent estimates of $f$ according to Eq. (1). Fig. 4A shows the mass isotopomer distributions of the H2-Aα (43–65) peptide from Aα7 molecules isolated after varying times of [2H2O] labeling of M12.NOD cells. All intermediate distributions intersected at a common “iso-abundant” point near M2 (arrow). This observation was consistent with the intermediate distributions representing binary mixtures of unlabeled and fully labeled molecules (analogous to the isosbestic point found in absorbance spectra of binary reactant/product mixtures that is lost in more complex mixtures). Fig. 4B shows the abundance change of each mass isotopomer over time; values of $f$, calculated from each mass isotopomer using Eq. (1), are shown in Fig. 4C. All mass isotopomers gave convergent estimates of $f$. Estimates from M2, however, were less precise because M2 changed much less than the other mass isotopomers during the experiment, resulting in a bigger impact of analytical error on estimates of $f$. A similar time course was observed for the Aβ7β (73–80) peptide, further validating this method of estimating $f$ (Fig. 4D).

The labeling kinetics of H2-Ad from A20 cells (Fig. 4E), HLA-DR0401 from Priess cells (Fig. 4F), HLA-B8 (Fig. 4G), and HLA-B51 (Fig. 4H) from LCL721 were also analyzed. In each case, convergent...
estimates of $f$ were obtained from different mass isotopomers within any one peptide. This result was consistent with the hypothesis that the precursor/product relationships for peptide labeling from $^2$H$_2$O are effectively independent of time, justifying the use of Eq. (1) to calculate $f$ from $^2$H$_2$O-labeled peptide mass isotopomer distributions. The estimates of $f$ were validated by comparison between different charge states of the same peptide, different peptides of the same polypeptide, or a peptide from a partner chain (Figs. 4D–H). The estimates were less precise for H2-Ak molecules; this was attributed to the low expression of these molecules in A20 cells, resulting in low MS abundance. The fractional synthesis rates measured in these experiments corresponded to half-lives on the order of approximately 1 day (Fig. 4D–H), with different contributions from cell growth and from replacement of turned-over proteins to fractional synthesis, depending on the MHC molecule and its cellular background (A. De Riva et al., manuscript in preparation).

Studies in primary APCs

This analytical approach also was feasible for primary antigen-presenting cells (APCs) cultured in vitro or obtained ex vivo. Unlabeled and fully labeled mass isotopomer distributions of the DRalpha peptide, obtained from immature human monocyte-derived dendritic cells, and the baseline mass isotopomer distributions of the A$^d$ $\alpha$ and $\beta$ peptides, obtained from Balb/c splenic B cells, closely conformed to MIDA predictions (Table 2). Thus, the SINEW approach will be applicable to primary APCs.

Discussion

Here we have used peptide LC–MS, in conjunction with the SINEW approach (Fig. 1B), to quantify the fractional synthesis of various MHC proteins. In the process of validating this method, we tested key underlying assumptions, explored the generality and versatility of the method, and greatly simplified the algorithms used to calculate fractional protein synthesis from experimental data. Importantly, the method is capable of distinguishing turnover rates of closely related MHC protein allotypes present in the same cell. Collectively, these improvements substantially broaden the utility of the SINEW approach.

Specificity is achieved at several steps in the procedure. MHC proteins of interest are enriched by immunoprecipitation followed by SDS–PAGE. However, contamination with the immunoprecipitating antibody, noncovalently associated or copurified proteins, and keratin remains detectable by LC–MS/MS (data not shown). Nonetheless, suitable peptides specific for the proteins of interest are found, and uncontaminated mass envelopes are obtained, as indicated by the close match to MIDA predictions. Unique peptide masses and retention times allow discrimination between peptides derived from MHC class I and class II allelic variants even when several MHC molecules are present. In Fig. 4G and H, we analyzed two alleles encoded at the HLA-B class I MHC locus, but HLA-A and -C were coisolated using the W6/32 antibody. Preliminary studies in another lymphoblastoid cell line (not shown) suggest that the entire class I haplotype will be resolvable provided that the HLA type is available. Thus, peptide LC–MS greatly improves analytical specificity in the SINEW approach compared with the analysis of $^2$H incorporation into protein-derived NEAs after total hydrolysis. The tolerance of our method to complexity in the protein samples suggests that our approach may have applications in kinetic proteomics.

Measured mass isotopomer abundances must be both precise and accurate to enable determination of $f$ by stable isotope labeling [31]. Orbitrap MS analysis satisfies these criteria, comparing favorably with the performance of other MS modalities in previous studies [16,17]; the data are analytically precise (SDs = 0.2–0.3% for high-abundance samples) and accurate (abundance independent and consistent with MIDA predictions). Accuracy diminishes somewhat at lower analyte abundance; thus, the best results require minimization of sample losses throughout the procedure. Nonetheless, analysis of MHC molecules isolated from as few as 10$^5$ EBV-B cells was possible, comparing favorably with $^{35}$S radiolabeling. Substantial shifts in abundance are observed after complete $^2$H$_2$O labeling on the order of −20% for M0 and +10% for other mass isotopomers in each peptide. Thus, estimation of $f$ to within a few percentage points is possible, provided that mass isotopomers that show the greatest fractional abundance shifts during labeling are selected for analysis. Allowing for the contribution of cell growth to fractional synthesis measured by our approach, our measurements (Fig. 4 and data not shown) are compatible with the MHC protein half-lives measured previously by radiolabeling in immortalized B cell lines [30,32] and monocyte-derived dendritic cells [34].

The success of this approach relies on the identification of peptides that incorporate sufficient label from $^2$H$_2$O. Prospective identification of such peptides from their amino acid sequence remains a complex challenge because the efficiency with which each amino acid incorporates the $^2$H label depends on the activity of complex biosynthetic pathways and on the abundance of unlabeled amino acids present in culture medium, and this may dilute any label incorporated via endogenous amino acid synthesis to an unknown extent. Previous work showed that C–H bonds in Ala and Gly are nearly fully equilibrated with $^2$H from $^2$H$_2$O in vivo in rodents, contributing $n$ = 4 and $n = 2$ labeling sites each, respectively, to peptides containing these amino acids [6,11]. We reasoned that these amino acids should be well labeled in vitro as well based on previous studies showing good labeling of protein-derived Ala in cell culture [13] and based on the lack of Ala and a modest amount of Gly present in RPMI medium. Accordingly, we preselected peptides on the basis of Ala/Gly content. In several of the peptides examined, Ala or Gly labeling explained most or all of the labeling sites inferred from MIDA calculations, but this did not rule out contributions from other amino acids that would have been expected from previous work [11]. Indeed, in other peptides, such as the A$^d\alpha$ peptide, a substantial proportion of labeling sites must have arisen from labeling of other NEAs. Similarly, in previous studies performed in vivo, some well-labeled peptides that were not rich in Ala/Gly were found [16,17]. A preliminary analysis of 21 peptides (including data in Table 1 and unpublished work) reinforces our impression that numerous amino acids contribute to labeling in our system (data not shown).

Counting of Ala and Gly residues, therefore, will often underpredict the extent of labeling and will miss some well-labeled peptides. However, most proteins contain some Ala-/Gly-rich peptides, and other well-labeled peptides may be identified empirically if needed, so this does not appear to be a serious limitation in practice. Moreover, we observed no peptides in which the apparent amount of Gly present in RPMI medium. Accordingly, we preselected peptides on the basis of Ala/Gly content. In several of the peptides examined, Ala or Gly labeling explained most or all of the labeling sites inferred from MIDA calculations, but this did not rule out contributions from other amino acids that would have been expected from previous work [11]. Indeed, in other peptides, such as the A$^d\alpha$ peptide, a substantial proportion of labeling sites must have arisen from labeling of other NEAs. Similarly, in previous studies performed in vivo, some well-labeled peptides that were not rich in Ala/Gly were found [16,17]. A preliminary analysis of 21 peptides (including data in Table 1 and unpublished work) reinforces our impression that numerous amino acids contribute to labeling in our system (data not shown).

Counting of Ala and Gly residues, therefore, will often underpredict the extent of labeling and will miss some well-labeled peptides. However, most proteins contain some Ala-/Gly-rich peptides, and other well-labeled peptides may be identified empirically if needed, so this does not appear to be a serious limitation in practice. Moreover, we observed no peptides in which the apparent number of labeling sites ($n$) was markedly less than that attributable to Ala/Gly, suggesting that the counting of Ala and Gly provides a useful lower bound on the number of labeling sites that may be expected. Importantly, this lower bound is likely to apply in vivo in rodents, where Ala and Gly are known to be well labeled. A more complete and accurate set of rules for predicting highly labeled peptides from amino acid sequence will require refinement of predictive algorithms and validation in larger data sets.

Given the potential complexity of precursor/product relationships for $^2$H$_2$O labeling of NEAs, it is interesting that a simple MIDA model, in which a fixed number of labeling sites are assumed to be labeled at the $^2$H enrichment in medium water, accounts quantitatively for the observed labeling patterns, as was also found previously for selected rodent serum proteins [17]. However, more
complex MIDA models, involving label dilution in some or all of the NEAA precursor pools, fit the data equally well (see Fig. S4 in Supplementary material). Thus, neither the experimental data nor the MIDA models are sensitive to the biochemical details that govern labeling of peptides from $^{2}$H$_{2}$O. Indeed, the calculation of fractional protein synthesis, using Eq. (1), makes no assumptions about the precursor/product relationships, as captured in MIDA calculations (values of $n$ and $p$), nor does it assume any knowledge as to the metabolic source of the label (e.g., whether $^{2}$H is introduced via Ala, Gly, or any other amino acid). For this reason, the approach outlined here permits calculation of fractional protein synthesis despite our incomplete understanding of the biochemical details underlying the observed labeling patterns.

The use of Eq. (1) to calculate $f$ represents an application of the rise-to-plateau approach [35]. This is a rigorous approach for determining fractional synthesis that is feasible when a stable isotope label can be administered continuously for extended periods, allowing empirical definition of plateau labeling. Labeling to plateau is possible in the SINEW approach both in cell culture and in living animals [6]. The only additional assumption inherent in Eq. (1) is that the precursor/product relationship remains constant on the time scale of the experiment, which was consistent with our empirical results. It should be noted that the assumption of a constant precursor/product relationship also underlies the MIDA-type algorithms used in previous work to calculate $f$ [16,17], although this assumption was not previously tested experimentally. Moreover, our model calculations (see Figs. S3 and S5 in Supplementary material) show that minor violations of these assumptions (small fluctuations in values of $n$ or $p$ or delayed label equilibration from $^{2}$H$_{2}$O into NEAA precursor pools) have only minimal impact on estimates of $f$. This argues for the robustness of our approach. Operationally, Eq. (1) greatly simplifies calculation of $f$ and of the associated experimental error.

Our validation studies focused on cultured, immortalized B cell lines, but the method also is applicable in human monocyte-derived dendritic cells and murine splenic B cells. By extrapolation from our results, in vivo studies in rodents appear to be feasible, consistent with previous work on abundant serum proteins [16,17]. $^{2}$H$_{2}$O enrichments of 5%, used in the cell culture studies presented here, are routinely used for extended continuous labeling of rodents in vivo; $^{2}$H$_{2}$O label equilibration with Ala and Gly, which are present in the peptides we selected, is rapid and nearly complete in vivo [5–7,10]. Moreover, overall label incorporation in cell culture appears to be less complete than would be expected from the data of Commerford and coworkers [11] in mice (based on data in Table 1 and unpublished observations). This is likely due to competition with unlabeled exogenous amino acids in medium and serum. These considerations suggest that the values of $n$ in vivo will not be the same as in cell culture but rather will generally be higher. This would pose no difficulty in calculating $f$ from shifts in mass isotopomer distributions in vivo and may even make it easier to analyze physiological systems. However, the precursor/product relationships (best-fit values of $n$ for the peptides of interest) would need to be determined in vivo because they cannot be simply extrapolated from results obtained in cell culture.

Further work will be required to examine whether the approach can be extended to human in vivo studies because lower $^{2}$H$_{2}$O enrichments (1–2% in plasma) are routinely attained in humans and additional sources of label dilution may be present [6]. However, MIDA calculations predict that at 1–2% $^{2}$H$_{2}$O enrichment, the shifts in mass isotopomer abundance for fully labeled HLA-B and -DR peptides would range from −4% to −13% in M0 and from +2% to +8% in the best higher order mass isotopomers. This may be sufficient to estimate $f$.

The approach can readily be extended to other proteins. A powerful feature of $^{2}$H$_{2}$O labeling is the ability to combine studies of protein dynamics with analyses of lipid synthesis [36] and cell proliferation (measured as DNA synthesis, reviewed in Ref. [8]) using the same biosynthetic labeling. The use of peptide LC–MS in SINEW experiments promises to become a convenient and powerful tool for studies of protein synthesis and turnover in a variety of biomedically relevant in vitro settings and ultimately in kinetic proteomics. The ability to distinguish biosynthetic rates of protein allomorphs, in particular, will be an invaluable tool for dissecting the role of protein dynamics in genotype/phenotype relationships. Extension to in vivo settings appears to be feasible and would open up a wide range of potential applications in which synthesis and turnover of long-lived proteins may be measured in healthy human populations and animal models and their perturbation by diseases and therapeutic interventions may be quantified.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ah.2010.04.018.

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