

Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal noncanonical amino acid tagging (BONCAT)

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In both normal and pathological states, cells respond rapidly to environmental cues by synthesizing new proteins. The selective identification of a newly synthesized proteome has been hindered by the basic fact that all proteins, new and old, share the same pool of amino acids and thus are chemically indistinguishable. We describe here a technology, based on the cotranslational introduction of azide groups into proteins and the chemoselective tagging of azide-labeled proteins with an alkyne affinity tag, to separate and identify, specifically, the newly synthesized proteins in mammalian cells. Incorporation of the azide-bearing amino acid azidohomoalanine is unbiased, not toxic, and does not increase protein degradation. As a first demonstration of the method, we report the selective purification and identification of 195 metabolically labeled proteins with multidimensional liquid chromatography in-line with tandem MS. Furthermore, in combination with leucine-based mass tagging, candidates were immediately validated as newly synthesized proteins. The identified proteins, synthesized in a 2-h window, possess a broad range of biochemical properties and span most functional gene ontology categories. This technology makes it possible to address the temporal and spatial characteristics of newly synthesized proteomes in any cell type.

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The proteome is a dynamic entity, tightly regulated by protein synthesis and degradation to maintain homeostasis in cells, tissues, and organisms. Many biological processes, including cell growth, differentiation, metabolism, and even learning and memory, are regulated by translational control of protein synthesis. mRNA expression data from a variety of systems indicate that the transcriptome responds dynamically to different cellular states, but because of an independent layer of translational regulation, the relationship between transcriptome and proteome is not generally predictable.

Differential proteomic approaches have been developed to compare the protein expression profiles of cells in different states; such methods include differential 2D gel electrophoresis (1, 2), isotope-coded affinity tags (3) or isobaric tags for relative and absolute quantification (4), quantitative proteomic analysis using samples from cells grown in ¹⁴N or ¹⁵N media (5, 6), and stable isotope labeling by amino acids in cell culture (7, 8). These methods have successfully elucidated complex processes such as nuclear protein dynamics (7, 9), responses to cytokines (10), and mechanisms of neurodegenerative diseases (11, 12), as well as promoting biomarker discovery (13–15). More recently, approaches focusing on posttranslational modifications have emerged to specifically explore the “phosphoproteome” (16–18) and “glycoproteome” (19–22) of a cell. Additionally, the constituents of protein complexes can be determined by means of tandem affinity purification of tagged cellular proteins followed by MS analysis (23).

Studies of proteome dynamics would be facilitated by methods that enable separation of newly synthesized proteins from the

preexisting protein pool. Enrichment of newly synthesized proteins has two advantages: (i) it permits an analysis of the primary protein synthesis response to internal and external cues, and (ii) it decreases the complexity of the sample, potentially enabling the identification of proteins expressed at low levels.

In recent years, small bioorthogonal functional groups, most notably azides and ketones, have been exploited to tag proteins (24–27), glycans (28–31), and lipids (32) in cells. These groups are incorporated by the cell’s own biosynthetic machinery; the subsequent ligation with reactive probes allows their detection (for a recent review, see ref. 33). The selective chemistry of the azide group has proven especially useful in the labeling of proteins (34–36) and cell surface glycans (37). Here we describe the use of azidohomoalanine (AHA) to tag newly synthesized proteins in mammalian cells. Tagged proteins are then separated from the preexisting proteome by affinity purification and subjected to identification by tandem MS. We demonstrate the power of the method, which we call bioorthogonal noncanonical amino acid tagging (BONCAT), by identifying 195 proteins synthesized in a 2-h time window in human embryonic kidney (HEK)293 cells.

Results

Metabolic Labeling of Newly Synthesized Proteins with AHA. To assess the suitability of AHA for labeling newly synthesized proteins in mammalian cells, we first examined its potential toxicity in a mammalian cell line and in neurons. Incubation with methionine was used as a control, because AHA serves as a surrogate for methionine. After 2 h, propidium iodide staining revealed no differences in cell viability between AHA- and methionine-treated cells (Fig. 1A). We also examined toxicity in a less robust and more fragile mammalian cell type, primary cultured postnatal neurons. Hippocampal neurons were infected with a destabilized and myristoylated form of the fluorescent protein GFP (38) to visualize their morphology and incubated for 2 h with either AHA or methionine. We found that AHA was not toxic to neurons, as indicated by healthy neuronal processes and the absence of abnormal varicosities in the dendrites (Fig. 1B).

To determine whether AHA is incorporated into mammalian proteins, we tagged lysates prepared from AHA-treated HEK293 cells with the alkyne linker biotin-PEO-propargylamide (26). Subsequent Western blot analysis with a biotin antibody revealed the successful incorporation of AHA into a wide variety of cellular proteins (Fig. 2A). To determine the specificity of

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Abbreviations: AHA, azidohomoalanine; HEK, human embryonic kidney; BONCAT, bioorthogonal noncanonical amino acid tagging; HA, hemagglutinin.

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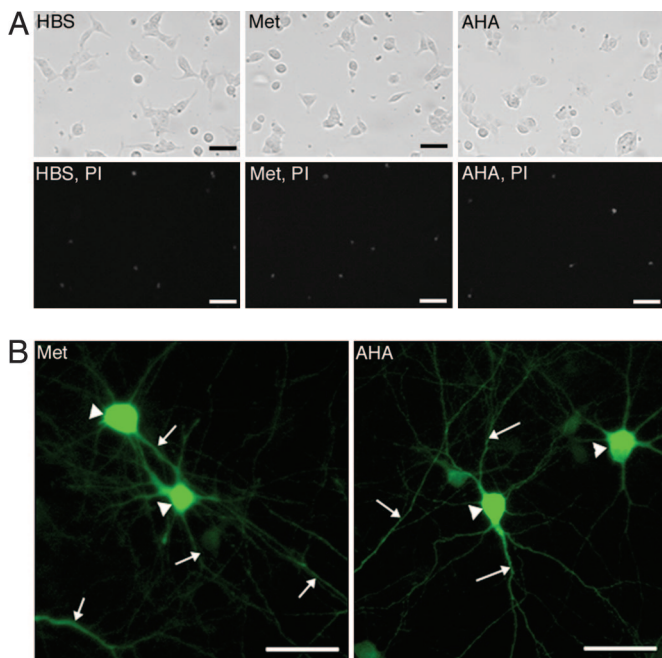


Fig. 1. AHA is not toxic to mammalian cells. (A) HEK293 cells were incubated for 2 h with HBS, 4 mM methionine in HBS, or 4 mM AHA in HBS and then incubated with propidium iodide (PI) to stain for dead cells. Shown are Nomarski (*Upper*) and PI (*Lower*) signals. (Scale bar = 100 μm .) (B) Dissociated hippocampal cultured neurons (12 days *in vitro*) infected with a destabilized and myristoylated variant of GFP were incubated for 2 h with equimolar concentrations of AHA or methionine. Images show representative neurons expressing the GFP reporter indicating no change in the gross morphology of AHA-treated neurons compared to methionine controls. Arrows indicate dendrites, and arrowheads point to somata. (Scale bar, 50 μm .)

AHA incorporation into newly synthesized proteins, we added the protein synthesis inhibitors anisomycin and cycloheximide to HEK293 cultures along with AHA. Although abundant signal was detected in cells treated with AHA, no signal was detected in either the methionine or the protein synthesis inhibitor lanes of the Western blot (Fig. 2A), confirming that this procedure labels newly synthesized proteins with high specificity. Furthermore, metabolic colabeling with [^{35}S]cysteine revealed no differences in the total radioactive signal intensity or changes in the migration pattern of total protein samples from AHA- vs. methionine-labeled HEK293 cells after 2 h of labeling with the amino acids (Fig. 2B and Fig. 5A, which is published as supporting information on the PNAS web site). Thus there is no evidence that AHA exposure alters global protein synthesis rates under the conditions used here.

Next, we examined whether AHA might promote or alter the ubiquitin-mediated degradation of preexisting and newly synthesized proteins. To examine the ubiquitination of newly synthesized proteins, we compared the population of newly synthesized and ubiquitinated proteins in AHA and methionine-treated cells. HEK293 cells were incubated for 2 h with [^{35}S]cysteine and AHA or methionine in the presence of a proteasome inhibitor to inhibit the degradation of ubiquitinated species. No increase in radioactive signal or in ubiquitination of newly synthesized proteins was observed in AHA-treated cells when compared to buffer or methionine controls (Fig. 3), indicating that AHA incorporation does not cause severe protein misfolding or degradation when cells are challenged for 2 h. Furthermore, no increase in the ubiquitination of total protein was observed in AHA-treated cells when compared to buffer or methionine controls (see Fig. 5A and B).

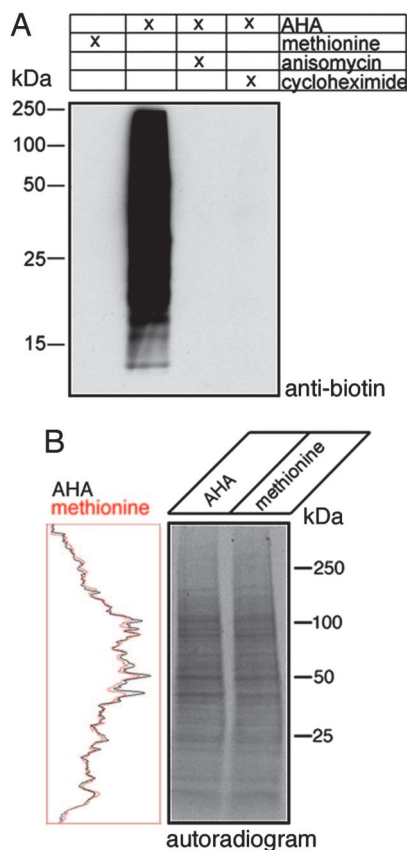


Fig. 2. Protein synthesis-dependent incorporation of AHA and analysis of global protein synthesis levels in the presence of AHA. (A) Western blot analysis for biotinylated AHA-labeled proteins in cell lysates from HEK293 cells incubated with AHA in the absence or presence of the protein synthesis inhibitors anisomycin or cycloheximide as compared to methionine control. Biotin immunoreactivity depends completely on protein synthesis and the presence of the AHA. (B) Line scan of radioactivity-labeled newly synthesized proteins transferred onto a nitrocellulose membrane from cells treated with AHA (black line) or methionine (red line) for 2 h.

Identification of Newly Synthesized Proteins by BONCAT. The BONCAT procedure is facilitated by direct proteolysis of affinity-purified proteins on the matrix, bypassing the need for an elution step. We therefore designed the alkyne affinity tag 1, which carries an N-terminal biocytin residue, two trypsin cleavage sites, a FLAG-antibody epitope, and a C-terminal propargylglycylamide (Fig. 4A). Affinity tag 1 and on-matrix proteolysis were used in all of the experiments described below. Because sequence coverage for the majority of proteins identified in shotgun proteomic approaches such as the multidimensional protein identification technique, MudPIT (see refs. 39 and 40), is lower than 40% (41), HEK293 cells were treated with AHA and with d_{10} -leucine (d_{10} -Leu) to provide an independent marker for newly synthesized proteins.

Affinity-purified proteins were identified by MudPIT (39, 40). A set of stringent search constraints including fully tryptic status of each peptide (42); a minimum of two valid peptides per locus; minimum crosscorrelation coefficients of 1.8, 2.5, and 3.5 for singly, doubly, and triply charged ions, respectively; and a minimum of one peptide containing an AHA-derived or d_{10} -Leu modification were required to ensure that identified proteins were translated during the AHA-labeling step. After perfect tag cleavage, the mass gain of tagged AHA over methionine is 107 atomic mass units. In the case of imperfect tag cleavage, the resulting mass gain of tagged AHA over methionine would be

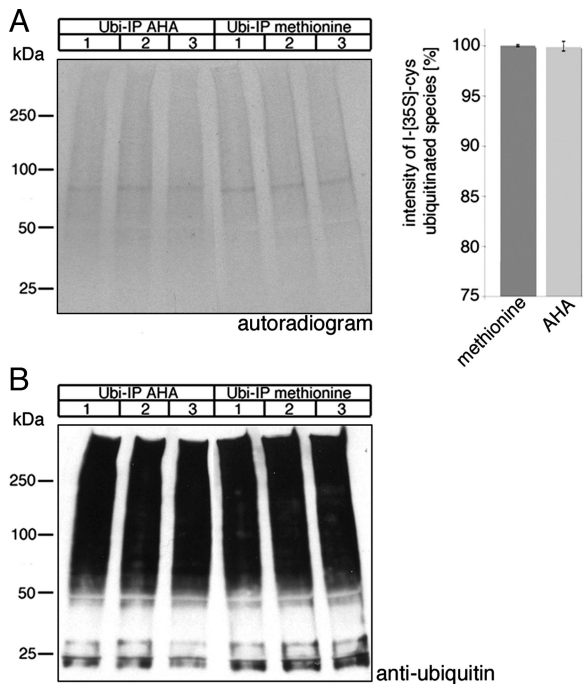


Fig. 3. Newly synthesized AHA-labeled proteins are not subject to increased protein degradation. (A) Autoradiogram analysis of immunoprecipitated ubiquitinated newly synthesized proteins of cell extracts from AHA-treated HEK293 cells as compared to methionine control samples (2-h treatment in the presence of [³⁵S]cysteine). Quantification of radioactive signals of immunoprecipitates is shown in the graph. *n* = 11 in four independent experimental sets. (B) Corresponding Western blot analysis for ubiquitinated proteins of immunoprecipitates in AHA-treated HEK293 cells as compared to methionine control samples.

695.6 atomic mass units (with no further fragmentation of the tag moiety required), whereas in the event of failed tagging (unligated AHA) the mass loss of AHA over methionine would be 5.1 atomic mass units. A detailed list of all SEQUEST and DTASELECT (The Scripps Research Institute) search constraints can be found in *Supporting Text*, which is published as supporting information on the PNAS web site. Four independent experiments were run.

To test our ability to detect newly synthesized proteins, we examined AHA incorporation in the endogenous proteome of HEK293 cells. In addition, we transiently expressed a hemagglutinin- (HA) tagged version of the brain-specific (43, 44) mammalian huntingtin-associated protein 1A (HAP1A) in HEK293 cells. Proteins were purified from HEK293 cells expressing HA-HAP1A treated with either AHA or methionine for 2 h. Although abundant biotin signal was detected in lysates prepared from AHA-treated cells, negligible biotin signal was detected in the lysates prepared from methionine-treated cells (Fig. 4B). In addition, HA-HAP1A was exclusively present in the Neutravidin affinity matrix bound fraction of AHA-treated lysates (Fig. 4B) and absent after trypsinization. Given that HA-HAP1A contains 14 methionine residues, a complete exchange of methionine with tagged AHA would lead to a mass gain of 23 kDa. We observed tagged HA-HAP1A migrating as a “smear” ≈7 kDa higher than untagged HA-HAP1A, indicating successful incorporation and tagging of up to four AHA residues. This incomplete exchange of methionine fits with the observation of decreased efficiency of AHA activation by methionyl-tRNA synthetases made by Kiick *et al.* (45).

As expected, 11 different valid peptides for HA-HAP1A (27.3% sequence coverage) were identified in four AHA MS experiments (Fig. 6, which is published as supporting informa-

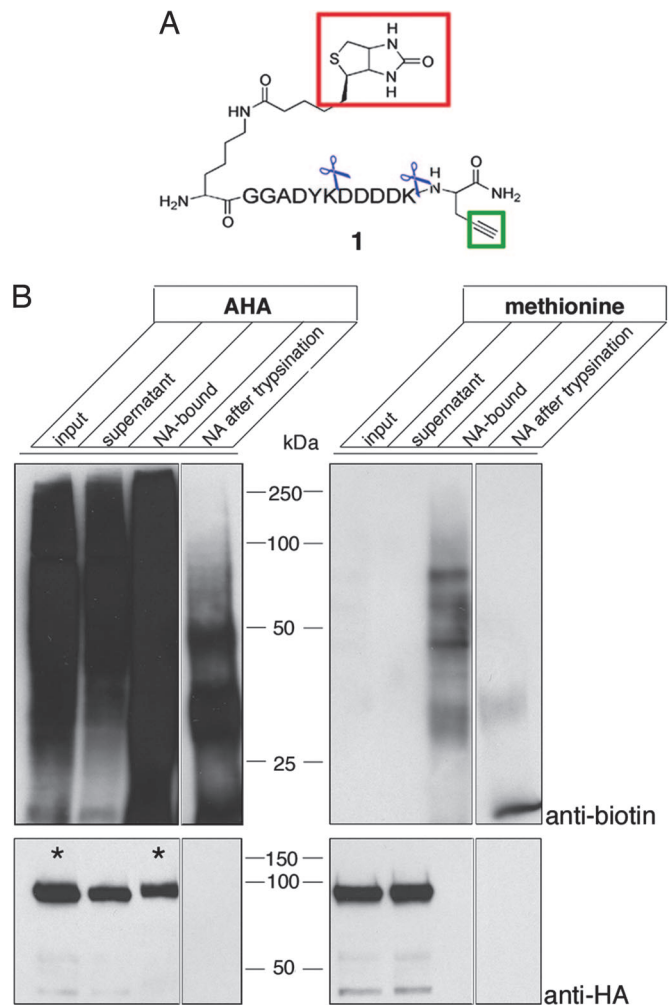


Fig. 4. Purification of AHA-labeled proteins after azide-alkyne ligation with a biotin-FLAG-alkyne tag. (A) Structure of the tryptic-cleavable biotin-FLAG-alkyne tag 1. Biotin (red rectangle), alkyne (green rectangle), and the tryptic cleavage sites (blue scissors) are indicated. The FLAG epitope DYKDDDDK is separated from the biotin moiety by a short linker (GGA). (B) Western blot analysis for affinity-purified biotinylated proteins using the biotin-FLAG-alkyne tag. Cell lysates from both AHA and methionine-treated HEK293 cells were subjected to [3 + 2] cycloaddition with the biotin-FLAG-alkyne tag and subsequently purified by using Neutravidin affinity matrix. Except for the nonspecific protein staining of samples containing Neutravidin affinity resin (Neutravidin affinity-matrix bound), control samples show no biotin signal. Note the higher migration level of alkyne-tagged HA-HAP1A protein (lanes indicated by asterisks) compared to the untagged protein in the methionine control and in the supernatant of the AHA sample. Sizes of marker proteins are indicated. NA, Neutravidin.

tion on the PNAS web site online), but no single peptide was recovered in control samples treated with methionine. Representative fragmentation spectra for HA-HAP1A peptides without modifications, with several d₁₀-Leu substitutions, or with an AHA substitution show that modifications can be reliably detected using AHA/d₁₀-Leu mass tagging (Fig. 6). Six identified peptides contained at least one modification due to either an AHA-derived or a d₁₀-Leu modification. This suggests that the combination of affinity purification of alkyne-tagged AHA-labeled proteins and d₁₀-Leu mass tagging allows secure validation of newly synthesized proteins.

We next explored the identity and representation of all HEK293 cell proteins synthesized in a 2-h time window. A total of 195 unique proteins were identified in AHA samples (Table

Table 1. Overview of identified proteins in four independent experiments in HEK293 cells

Proteins identified in	Number of proteins	Percentage, %
Four AHA exp.	30	15.2
Three AHA exp.	23	11.6
Two AHA exp.	55	27.8
One AHA exp.	87	43.9
Two AHA and one Met exp.	1	0.5
One Met exp.	2	1.0

Shown are the numbers and percentages of proteins identified in the four independent experiments. Listed counts for identified proteins were acquired under the following criteria: minimum of two valid peptides and one modified peptide per locus, fully tryptic status required and minimal crosscorrelation coefficient and DeltCN (normalized difference of crosscorrelation coefficients between the best sequence and each of the other sequences), as indicated in *Material and Methods*. Proteins that are subsets of each other (a subset protein is one for which all peptides are found in another protein) were combined and counted as a single identification in the report. exp., experiment(s).

1), with 108 proteins (54.6%) identified in more than one experiment. In control samples, only two proteins were identified containing a d₁₀-Leu modification in a single experiment (Cytokeratin type II and Keratin 9); no single protein was identified in more than one experiment. One identified protein was shared by AHA and control samples (splice isoform 1 of DNA-dependent protein kinase catalytic subunit). The entire list of the 195 newly synthesized proteins, as well as the list of nonredundant peptides identified in the four independent experiments, is provided in Tables 2 and 3, which are published as supporting information on the PNAS web site, respectively. A Western blot analysis for EF2 and histone proteins confirmed the identification of newly synthesized proteins as well as their proper subcellular localization (for histones) in AHA-treated HEK293 cells with BONCAT (Fig. 7, which is published as supporting information on the PNAS web site).

In all four experiments, a total of 1,028 nonredundant peptides accounted for the 195 proteins identified using the above-stated search constraints for SEQUEST and DTASELECT. Of the 1,028 identified peptides, 331 contained at least one modification (assessed by using a Python script FINDMODDTASELECT) due to either d₁₀-Leu or AHA, 295 peptides contained at least one d₁₀-Leu modification, 28 peptides were identified with at least one unligated AHA residue, and one peptide was identified with a successfully tagged and perfectly cleaved AHA residue. No AHA-ligated imperfectly digested peptide was identified. Oxidized methionine was observed in 41 of the peptides, with 10 of these bearing at least one d₁₀-Leu modification. The majority of d₁₀-Leu-containing peptides showed a complete exchange of the deuterated amino acid. Five peptides were identified as a mixed population of deuterated and native leucine. That the majority of modified peptides contain a d₁₀-Leu modification is most likely because of the high abundance of this amino acid in proteins (9.89% in the database indicated above). The remaining 697 (of 1,028) nonredundant peptides contained neither d₁₀-Leu-/AHA modifications nor an oxidized methionine but are included in the list because a peptide that contained a d₁₀-Leu or AHA modification was identified from the same protein.

It is worth noting that 257 additional proteins, which passed the search constraints of a minimum of two valid peptides per locus and minimum crosscorrelation coefficients (for values, see above) but did not pass the AHA or d₁₀-Leu modification criteria, were identified in AHA-treated samples. One hundred nine of these nonmodified proteins were also identified in the methionine-treated samples, among them proteins such as the

natively biotinylated protein pyruvate carboxylase (46) or keratin family proteins; the latter is a common contaminant in MS experiments. Only 15 proteins without any metabolic modifications were identified in methionine-treated samples.

To assess the false-positive rate, we modified a protocol developed by Peng *et al.* (47). On the single-peptide level, we found three single false-positive peptide identifications among the AHA samples (of a total of 2,840 nonredundant peptides) compared with seven false-positive identifications of a total of 19 peptides for control samples, when a fully tryptic status (42) and a single modified peptide were required (Table 4, which is published as supporting information on the PNAS web site). Note that none of these false-positive peptides passed our filter criteria requiring a minimum of two peptides for valid protein identification.

We observed no evident unique features of either of the modified peptides such as a preference for one ion series over the other or the in-sequence localization of the modification. Furthermore, we subjected nontrypsinized and trypsinized AHA-Biotin-Flag-tag fractions to MS analysis, which revealed the successful identification of all expected fragments, indicating that the tag can be trypsinized as anticipated, and the remaining fragments can be detected (data not shown). A reduction in sample complexity might be beneficial for the future detection of more modified peptides.

A wide range of biochemical and functional properties (indicated in Table 2) are represented among the 194 endogenous HEK293 proteins identified in these four experiments. Isoelectric points ranged from 4.6 (nucleophosmin) to 11.4 (histone H4). In addition, a wide range of molar masses, from 11 kDa (histone H4) to 466 kDa (splice isoform 2 of DNA-dependent protein kinase catalytic subunit) were also represented, showing that BONCAT can detect proteins with diverse biochemical properties. We examined the 194 candidates for their association to Gene Ontology categories using the software tool GOMINER (48). Annotations representing the majority of protein categories were assigned to 162 of the 194 proteins identified (Fig. 8, which is published as supporting information on the PNAS web site). Among the identified candidates were highly expressed housekeeping genes like pyruvate kinase M2, α enolase, members of the ribonucleoprotein family for mRNA-binding (heterogenous nuclear ribonucleoprotein K), or the structural proteins actin and β -tubulin. More importantly, the identification of generally lower abundance proteins like transcription and translation activity regulators (for example SART-1, ribosome biogenesis protein BOP1, or SWI/SNF-related matrix associated actin-dependent regulator of chromatin subfamily A member 5) demonstrate that our technology possesses sensitivity adequate to identify proteins of low expression levels. Finally, among the 194 (195 including HA-HAP1A) proteins, we identified soluble cytoplasmic proteins (actin or HA-HAP1A), membrane-associated proteins as well as insoluble proteins, e.g., the endoplasmic reticulum protein calnexin, the nuclear matrix protein matrin 3, or lamin B. It is worth noting that the average methionine content of our candidate proteins is 2.40%, only slightly higher than the methionine content of the whole database (2.13%, determined with a Python script AAEXCLUDE; Fig. 9, which is published as supporting information on the PNAS web site, provides a graph of methionine content in human proteins), indicating that BONCAT does not enrich for proteins high in methionine content. In support of this, one candidate protein identified, histone H4, possesses only two methionine residues in its sequence and was yet identified. Most mammalian proteins are subject to a variety of posttranslational modifications including N-terminal processing by methionyl aminopeptidases. In the human database used in the present study, 5.08% of all protein sequences (2,538 entries) contain only one methionine, as the initiating amino acid. The N-terminal processing and cleavage of the first (methionine) residue during protein maturation could prevent identification of these proteins. However, a recent study (49) has reestimated that N-terminal posttransla-

tional processing and acetylation is likely to occur for only 30%, rather than 80%, of all mammalian proteins. In this context, we suggest the possibility that AHA can be processed by methionyl aminopeptidases (see *Supporting Text*). Finally, we note that 511 ORFs (1.02% of a total of 49,925 ORFs) of the human database do not contain a single methionine residue and therefore could not have been detected with BONCAT. These 511 ORFs include proteins predicted to be <25 residues as well as incomplete sequences. Taken together, these data indicate that we can enrich and identify newly synthesized proteins covering a wide range of biochemical and functional properties after a 2-h window of AHA incubation.

Concluding Remarks

A thorough understanding of cellular function requires a dynamic view of the proteome. Temporal resolution is essential if one is to describe the immediate responses of cells to environmental changes, rather than responses that may be secondary or tertiary. Elucidating these immediate changes in the protein composition of a cell at a given state is a challenge requiring specialized tools, techniques specific and sensitive enough to specifically detect subtle changes in proteomic content. Taking a series of “snapshots” of cellular actions will allow us to capture the temporal dynamics of these processes. Understanding the temporal dynamics of processes such as signal transduction initiation and diversification will provide invaluable insights for target recognition, biomarker discovery, and drug development. Unique to the BONCAT approach are the selective labeling and enrichment of the subproteome of newly synthesized proteins, thereby raising the chances of identifying new proteins made against a bewildering background of preexisting proteins. Protein labeling using BONCAT is analogous to metabolic labeling with radioactive amino acids such as [³⁵S]cysteine or [³⁵S]methionine and allows the immediate validation of candidate proteins using AHA- or d₁₀-Leu-based modifications as identification constraints, thereby omitting obligatory and often time-consuming secondary validation steps. In conjunction with short incubation times, metabolic labeling with AHA will be particularly useful for postmitotic cell culture systems. Although stable isotope labeling by amino acids in cell culture-based approaches has proven to be very powerful for dividing cell systems for which a complete incorporation of a given “light” or “heavy” form of an essential amino acid is desirable, this is not an easy feat in postmitotic cells (8, 50). In this regard, it is worth noting that labeling of primary hippocampal neurons with AHA did not appreciably affect their survival, morphology, or protein synthesis. The BONCAT technique, combined with subcellular fractionation or microdissection of tissue (51), could provide a means for the development of a comprehensive picture of temporal and spatial aspects of cellular proteomes.

Methods

Reagents. Information on reagents and antibodies used in this study can be found in *Supporting Text*.

Cell Culture. HEK293 cells were cultivated in DMEM plus 10% FCS and penicillin/streptomycin (all from Invitrogen). Dissociated hippocampal neuron cultures were prepared from newborn rat pups (P0), as published previously (38). Cell viability assays, measurement of ubiquitination levels, radioactive metabolic labeling, transient transfection, and viral infection are described in *Supporting Text*.

[3 + 2] Cycloaddition Chemistry and Purification of Tagged Proteins. AHA, biotin-PEO-propargylamide, and the triazole ligand were prepared as described (52–54). The cleavable biotin-FLAG-alkyne tag was synthesized by the GenScript Corporation (Piscataway, NJ). d₁₀-Leu was purchased from Sigma. A time window of 2 h was chosen for the AHA incubation step to allow for new protein

synthesis to take place without any further manipulation of the cells such as stimulation or starvation being necessary. Cell pellets, typically 1.95–2.1 mg of total protein per single purification and MS analysis, as well as subcellular fractions were lysed in 1% SDS in PBS/PI-E (tagging buffer) with genomic DNA sheared with a syringe and a needle and boiled for 10 min at 96–100°C. Lysates were diluted to 0.2% SDS/0.2% Triton X-100 in PBS, pH 7.4/PI-E before addition of 200 mM triazole ligand/50 mM alkyne tag/100 mg/ml copper (I) bromide. The reaction was allowed to proceed for 6 h at room temperature, and excess reagents were removed by gel filtration through PD-10 columns (Amersham Pharmacia Bioscience). Optimal concentrations (2.86–4.0 mM) of AHA were determined by monitoring the biotin signal strength on the Western blot level with no change in the level of ubiquitination of proteins (data not shown). Protein extracts were adjusted to 1% Nonidet P-40 (Sigma)/0.1% SDS in PBS, pH 7.4/PI-E, and tagged proteins were purified on Immobilized Neutravidin affinity resin (Pierce). After extensive washing in incubation buffer, followed by washes in 1% Nonidet P-40 in PBS as well as 50 mM ammonium bicarbonate buffer, resin suspensions were incubated for 10 min at 70°C and adjusted to 2 M urea as 25–33% slurries. Trypsination was done as described in ref. 55. In a typical experiment, 400 μl of peptide solution was loaded for MS analysis.

MS and Data Analysis. Analysis of peptide mixtures by MudPIT was done essentially as described in Graumann *et al.* (55), using a HP-1100 quaternary HPLC pump (Agilent, Palo Alto, CA) and a LCQ-DecaXP electrospray ion trap MS (ThermoElectron, Palo Alto, CA). Proteolytically digested samples were separated on a triphasic microcapillary column basically as described in McDonald and Yates (56). First, samples were loaded onto a biphasic column (2.5 cm of Aqua C18 reverse-phase material (Phenomenex, Ventura, CA) placed upstream of 3.5 cm of SCX resin (Partisphere SCX, Whatman) packed into a 100-μm internal diameter-fused silica column (PolyMicro Technology, Phoenix) fitted into an Inline MicroFilter Assembly unit with a 0.5-μm PEEK Frit (all from Upchurch Scientific, Oak Harbor, WA). After sample loading, a column head packed with 6.5-cm Aqua C18 resin was attached at the other end of the MicroFilter Assembly unit, creating a triphasic RP-SCX-RP column. Sample separation was achieved with a six-step chromatography program, and the column eluate was analyzed as described in Graumann *et al.* (55). Centroided fragmentation spectra acquired by XCALIBUR 1.3 software (Thermo Electron) were evaluated for spectrum quality and charge state by using 2TO3 (57) and searched against the translated ORFs of the human IPI database ipi.HUMAN.v3.06.fasta (www.ebi.ac.uk/IPI/IPIhelp.html) by SEQUEST (58), Version 27, Rev. 9, using unified input and output files (59). Details of relevant SEQUEST parameters, DTASELECT, and CONTRAST (56, 60) settings, as well as assessment of false-positive rates, can be found in *Supporting Text*.

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