Protein Stoichiometry of a Multiprotein Complex, the Human Spliceosomal U1 Small Nuclear Ribonucleoprotein

ABSOLUTE QUANTIFICATION USING ISOTOPE-CODED TAGS AND MASS SPECTROMETRY*

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The human U1 snRNP (small nuclear ribonucleoprotein), which is a part of the spliceosome, consists of U1 snRNA and ten different proteins: seven Sm proteins B/B', D1, D2, D3, E, F, and G and the three U1-specific proteins U1–70 K, U1-A, U1-C. To determine the stoichiometry of all ten proteins, the complex was denatured, digested completely with an endoproteinase and labeled with an amine-specific tag. Corresponding peptides were synthesized and labeled with the same tag containing heavier isotopes. The digest was then spiked with defined amounts of the synthetic peptides, and the resulting isotopic peptide pairs were analyzed quantitatively by mass spectrometry. The mass spectra provided information about the absolute amount of each component in the starting protein mixture. The use of the isotope-coded, amine-specific reagents propionyl-*N*-oxysuccinimide and nicotinoyl-*N*-oxysuccinimide was evaluated for stoichiometry determination; the nicotinoyl reagent was found to be advantageous because of its greater mass spectrometric sensitivity. Absolute quantities of all ten proteins were measured, showing equal numbers of all ten proteins in the U1 spliceosomal snRNP. These data demonstrate that quantitative mass spectrometry has great potential for the determination of the stoichiometry of multiprotein complexes.

Many cellular functions are regulated by multiprotein complexes. Mass spectrometry-based methods have allowed significant advances in the discovery and identification of the interacting proteins. Furthermore, this technique has also been used to study the (relative) dynamics of protein complexes in different states by the incorporation of stable isotopes (1, 2), a method widely used in quantitative proteomics. Labeling with stable isotopes can be achieved either by chemical modification of side-chain amino groups with a reagent containing heavier isotopes (^{2}H, ^{13}C, ^{15}N) (3, 4) or by incorporation of isotope-coded amino acids from the growth medium (5, 6). To understand the function of individual proteins in a complex, it is necessary to analyze the interacting proteins in a quantitative way, which reveals whether a certain protein is present in defined stoichiometric amounts or forms only a weak substoichiometric interaction. To gain a knowledge of the stoichiometry of a protein complex, however, determination of the absolute amounts (7, 8) of the complex constituents is required. We recently described a method for the determination of the stoichiometry of protein complexes, which is based on proteolytic digestion of the complex, labeling with a fluorescent reagent specific for amino or sulfhydryl groups, and separation by liquid chromatography with fluorescence and mass-spectrometric detection. The strength of the fluorescence signal of the labeled peptides from different proteins is directly proportional to the stoichiometry of these proteins in the complex (9). For large multiprotein complexes, this method may be limited because of the difficulty involved in the separation of very complex peptide mixtures; poor resolution of these may lead to an overlap of the peptide peaks in the fluorescence chromatogram, preventing correct quantification. Here we describe the application of a similar approach that overcomes this limitation.

We applied the new method to the determination of the protein stoichiometry of the spliceosomal multiprotein complex U1 snRNP. The spliceosome is formed by four small nuclear ribonucleoproteins (snRNPs),[1] U1, U2, U5, and U4/U6 (named after their uridine-rich snRNAs) and numerous additional proteins (10). The snRNPs can be divided into two groups: the U-snRNP-specific proteins and the Sm proteins (11). The Sm proteins, named SmB, SmD1, SmD2, SmD3, SmE, SmF, and SmG, are associated with the Sm binding site of the snRNAs, except for U6, which contains homologous "Sm-like" proteins. Some arginine residues of SmB and SmD3 have symmetrical dimethyl modifications (12). In humans, some tissues, as well as HeLa cells, contain a second SmB protein called SmB', which is a splicing variant in which the last two amino acid residues are replaced by a new sequence of eleven residues (13). When bound to the snRNA, the Sm proteins form a very stable core RNP structure. *In vitro*, Sm proteins form specific protein complexes, and crystal structures have been obtained from the two dimeric SmD1/D2 and SmD3/B complexes. These results prompted the proposal of a heptameric circular model for the Sm core RNP (14). Furthermore, co-precipitation experiments with genetically labeled yeast Sm proteins have suggested that one copy of each Sm protein is present in the core RNP (15). U1

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[1] The abbreviations used are: snRNP, small nuclear ribonucleoprotein; TFA, trifluoroacetic acid; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; NMM, *N*-methylmorpholine; Trt, trityl; *t*Bu, *tert*-butyl alcohol; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-*S*-sulfonyl; Boc, *tert*-butoxycarbonyl; HPLC, high pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ByBOP, benzotriazolyloxy-tris-(pyrrolidino)-phosphonium hexafluorophosphate.

snRNP, purified from HeLa cells, is the smallest and best characterized human spliceosomal RNP subunit. Besides the Sm proteins, it contains only three specific proteins: U1–70 K, U1-A, and U1-C. Electron cryomicroscopy of U1 snRNP revealed a three-dimensional structure consisting of a circular core domain quite similar to the proposed heptameric Sm ring structure and individual additional domains for U1–70 K and U1-A proteins with sizes compatible with one copy of each of these proteins (16). The U1-C protein could not be recognized as an individual domain. Although these results suggested the presence of one copy of U1-C, other biochemical experiments suggested that U1-C in the U1 snRNP could be present as a dimer (17).

For protein stoichiometry determination, up to three reference peptides were synthesized for each U1 snRNP protein and were quantified and labeled with a [^{13}C]-coded tag, whereas the peptides derived from U1 snRNP proteolysis were labeled with a [^{12}C]-coded tag. Mass spectrometric analysis of mixtures of both kinds of labeled peptides provided information about the absolute quantities of the constituents of the U1 spliceosomal snRNP.

EXPERIMENTAL PROCEDURES

Materials—Acetonitrile (gradient grade), urea (analytical grade), dithiothreitol, ammonium chloride, trifluoroacetic acid (TFA, analytical grade), and guanidinium hydrochloride (analytical grade) were obtained from Merck (Darmstadt, Germany). HEPES was obtained from Sigma. Water from an ultrapure water system (Millipore, Bedford, MA) was used for preparation of all aqueous solutions. Trypsin and endoproteinases Lys-C, Glu-C, and Asp-N were from Roche Applied Science (Mannheim, Germany). Tri-isopropylsilane and Me$_2$SO were obtained from Fluka Sigma-Aldrich GmbH (Seelze, Germany), Fmoc amino acids from MultiSynTech GmbH (Witten, Germany), and NMM from Biosolve BV (Valkenswaard, Netherlands).

Synthesis of Reference Peptides—Peptide synthesis was performed using Fmoc chemistry (ByBop/NMM activation, Trt/tBu/Pbf/Boc side-chain protection), preloaded Wang Resins (200–400 mesh) on a multiple peptide synthesizer (AMS 422; Abimed GmbH) on a 20-μmol scale. The peptides were cleaved (92.5% TFA, 2.5% water, 5% tri-isopropylsilane) for 1.5 h, precipitated, and washed three times with cold methyl *t*-butyl ether. Air-dried crude peptides were purified using reversed-phase HPLC (solvents, water-acetonitrile gradients, 0.1% TFA) (HPLC system, Sycam GmbH), a GROM Sil ODS 2 column (25 cm length × 2 cm diameter), and 5-μm C18 silica beads. The purity of the peptide fractions was assessed by analytical reversed-phase HPLC (solvents, water-acetonitrile gradient) (HPLC system, Beckman System Gold), a GE Healthcare SOURCE 5 RPC ST 4.6/150 column (15 cm length × 4.6 mm diameter), and 5-μm C18 polystyrene/divinylbenzene beads. The sequence identity of the peptides was verified by MALDI-TOF mass spectrometry. Absolute quantification of the reference peptide amounts was performed gravimetrically (accuracy >0.05 mg), carefully minimizing errors caused by peptide hygroscopy or electrostatic effects of the sample cup.

Labeling of the Peptides with [^{13}C]Propionyl-N-oxysuccinimide or [^{13}C]Nicotinoyl-N-oxysuccinimide—0.8–6 mg of the reference peptides were dissolved in 1 ml of 10% acetonitrile or 10% acetonitrile and 10% formic acid in water. The sequences of the synthetic peptides matched those of the peptides expected to be created by endoproteinase cleavage of the U1 snRNP. All peptides that corresponded to cleavage products of a distinct proteinase (endoproteinase Lys-C, Asp-N, Glu-C, or trypsin) were combined; this resulted in four peptide mixtures. These solutions were then diluted with water to a volume of 250 μl, resulting in concentrations between 0.03–0.13 μg/μl each peptide. 5 μl of each of these solutions was combined with 45 μl of HEPES buffer (50 mM, pH 8.5). Thereafter, 4 μl of propionyl-*N*-oxysuccinimide (0.15 M in Me$_2$SO) or nicotinoyl-*N*-oxysuccinimide (0.15 M in Me$_2$SO) was added. The mixture was incubated at 25 °C for 1 h. To stop the reaction, 4 μl of ammonium chloride (1 M) was added, and the solution was incubated for 15 min at room temperature. To hydrolyze reaction side products with hydroxyl groups (18, 19), the pH was raised to 12 with NaOH (1 M) for 10 min. The solution was then neutralized by the addition of the same amount of HCl (1 M). 0.5–2 μl of this solution was added to the digest of the human U1 snRNP.

Denaturation and Digestion of the Human U1 snRNP—2.5–4 μl of
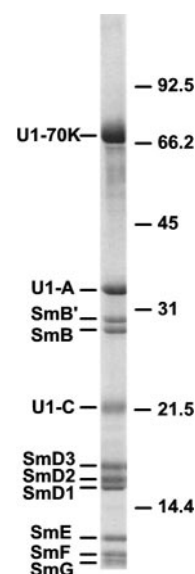

FIG. 1. **Coomassie-stained one-dimensional SDS gel of the U1 snRNP.**

the U1 snRNP (0.5 mg/ml (20), dissolved in 20 mM Tris-HCl, pH 7.9, 1.5 mM MgCl$_2$, 150 mM KCl, 0.5 mM dithioerythritol, 0.5 mM phenylmethylsulfonyl fluoride), purified essentially according to the methods of Bach *et al.* (21) and containing its complete set of proteins (see Fig. 1 for a Coomassie-stained electrophoresis gel), were mixed with 20 μl of 6 M guanidinium hydrochloride. 1 μl of dithiothreitol (1 mg/ml) was added, and the solution was incubated at 60 °C for 45 min. The solution was then diluted with 180 μl of 25 mM HEPES buffer (pH 8.5), after which 40 μl of endoproteinase Lys-C (0.05 μg/ml), trypsin, endoproteinase Glu-C, or endoproteinase Asp-N were added, and the digests were incubated at 37 °C.

Labeling of the Digest with [^{12}C]Propionyl-N-oxysuccinimide or [^{12}C]Nicotinoyl-N-oxysuccinimide—4 μl of propionyl-*N*-oxysuccinimide or nicotinoyl-*N*-oxysuccinimide (0.15 M in Me$_2$SO) was added to 75 μl of the endoproteinase digest (see above), and the solution was incubated at 25 °C for 1 h. Subsequently, 4 μl of ammonium chloride (1 M) was added, and the mixture was incubated for a further 15 min. The pH was raised to 12 with NaOH (1 M) for 10 min, and the solution was then neutralized with the same amount of HCl (1 M).

Capillary Liquid Chromatography and MALDI Target Preparation—Separation of the peptides was achieved with a capillary liquid chromatography system (Ultimate, LC Packings) and a reversed-phase column (LC Packings Pepmap reversed-phase C18 column; 15 cm length × 75 μm inner diameter). 25 μl of the peptide solution was injected and the peptides were trapped on a short reversed-phase column. For the separation of the peptides, the solvents A (0.05% TFA) and B (80% acetonitrile, 0.04% TFA) were used with a linear gradient from 10 to 100% B in 30 min with subsequent 20-min isocratic intervals at 100% B. The eluting peptides were spotted robotically onto a 192-well MALDI sample plate and mixed with a matrix solution (5 mg/ml of α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA) during the spotting process. The sample spots were dried at room temperature.

Tandem Time-of-flight Mass Spectrometry—Mass spectrometric analysis was performed on a 4700 Proteomics Analyzer from Applied Biosystems (Framingham, MA) equipped with a neodymium-YAG (yttrium-aluminum-garnet) laser that produces pulsed power at 355 nm at pulse rates of 200 Hz. Mass analysis was performed using the positive reflector mode with a deflection cutoff range of m/z 700. 5000 laser shots were accumulated to produce a single spectrum (Fig. 1). Subsequently, high energy MALDI-TOF/TOF collision-induced dissociation spectra were recorded on selected ions from the same sample spot. The collision energy was 2 keV. Air was used as the collision gas.

RESULTS

Peptide Selection—Fig. 2 shows a schematic summary of the method we used for the determination of the stoichiometry of the U1 snRNP. The complex was denatured and digested completely with an endoproteinase. The proteolytic peptides were then labeled with an amine-specific ^{12}C isotope-coded tag (18,
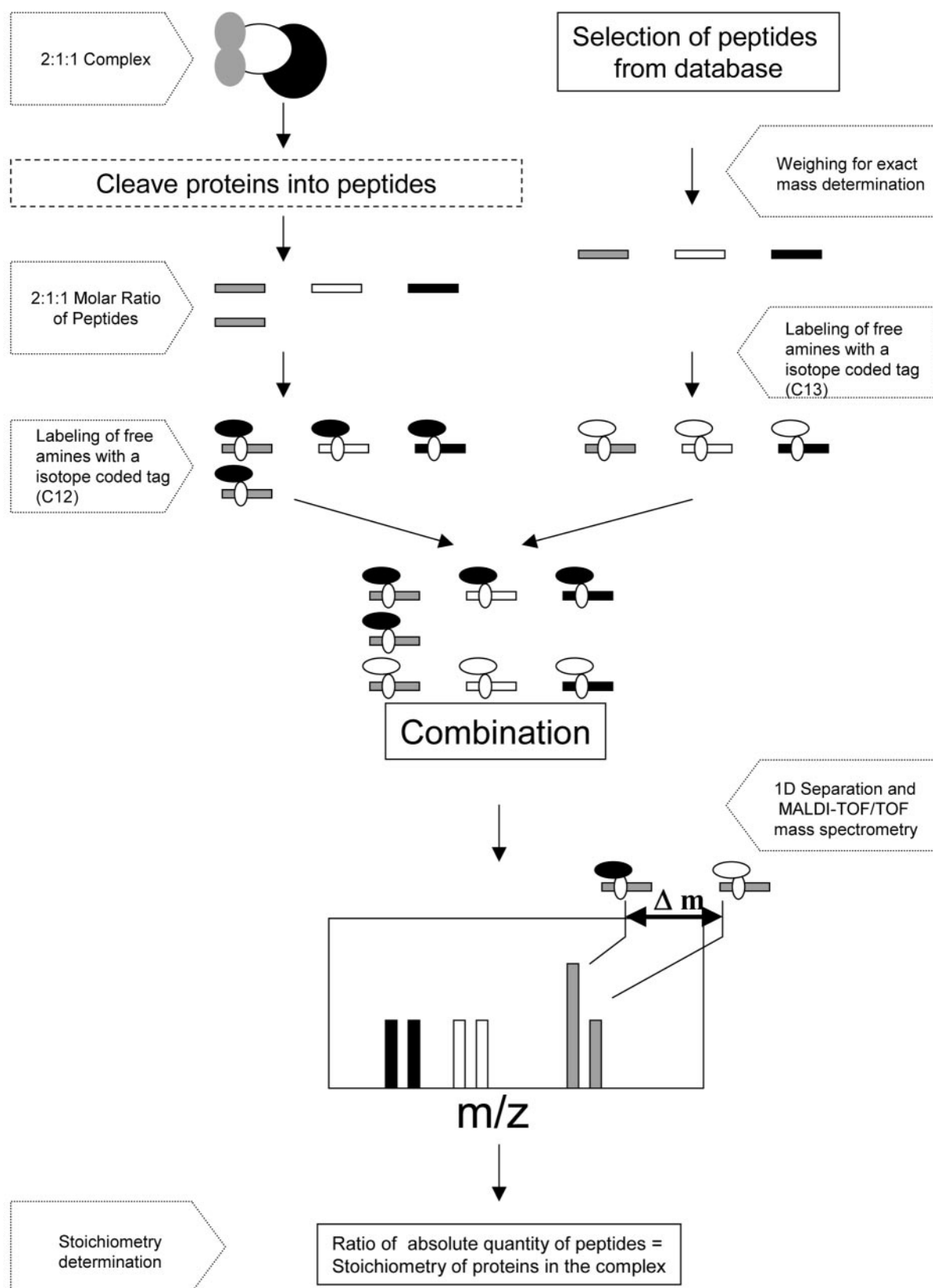
FIG. 2. **Analytical strategy illustrated for a 2:1:1 complex.**

19). Reference peptides were selected from the data base, synthesized, and labeled with a ^{13}C isotope-coded tag. Mass spectrometric comparison of the intensities of the ^{12}C- and ^{13}C-labeled peptide pairs allows the absolute quantification of the peptides. The ratio of the absolute quantities of peptides re-

sulting from different proteins corresponds to the stoichiometry of the proteins in the complex. In this study, we selected two peptides/protein from the protein sequences in the NCBI data base for the absolute quantification of all ten U1 proteins. The sequences of the peptides are shown in Tables I and II. We used

TABLE I
Quantification of human U1 snRNP labeled with [¹²C]propionyl-N-oxysuccinimide

This table summarizes the results of two independent experiments for the absolute quantification of the human U1 snRNP denatured, digested with endoproteinase Lys-C, Glu-C or trypsin, and labeled with [¹²C]propionyl-*N*-oxysuccinimide. Absolute quantification was achieved by the addition of synthetic peptides labeled with [¹³C]propionyl-*N*-oxysuccinimide. Two peptides were used for the quantification of most proteins. Quantification was compromised for some peptides of the Sm proteins because of methylation of arginine residues (SmB, SmD3).

Protein	Peptide	Experiment 1		Experiment 2		Enzyme
		pmol	Mean value	pmol	Mean value	
U1–70K	LRREFEVYGPIK (104–118)	1.46	1.47	1.43	1.44	Lys-C
	TLFVARVNYDTTESK (119–130)	1.48		1.44		Lys-C
U1-C	TTAAFQQGK (53–61)	Not detected				Lys-C
	DYYQK (36–40)	Not detected				Lys-C
U1-A	GTFVERDRK (99–107)	1.53	1.53	1.25	1.25	Lys-C
	SQETPATK (115–122)	Not detected				Lys-C
SmB	KRVLGLVLLRGE (64–75)	Not detected				Glu-C
	VLGLVLLR (66–73)	Not detected				Trypsin
	VPLAGAAGGPGIGR (98–108)	Not detected				Trypsin
SmD1	LVRFLMK (3–9)	1.57	1.57	1.38	1.38	Lys-C
SmD2	DRYISK (93–98)	1.46	1.46	1.44	1.44	Lys-C
	NNTQVLINCRNNK (38–50)	12.34		12.79		Lys-C
SmD3	NQGSGAGRGK (90–99)	< 0.0783		< 0.0688		Lys-C
	VAQLEQVYIR (55–64)	1.49	1.49	1.50	1.50	Trypsin
SmE	QLGRIMLK (73–80)	1.50	1.50	1.67	1.67	Lys-C
	VMVQPINLIFR (13–23)	Not detected				Trypsin
SmF	YIDGALSGHLGE (9–17)	Not detected				Glu-C
	CNNVLYIR (65–73)	Not detected				Trypsin
SmG	AHPPELK (4–10)	1.60	1.60	1.30	1.30	Lys-C
	HVQGILR (26–32)	Not detected				Trypsin

the following selection criteria for the reference peptides. (i) No peptides starting with proline or glutamic acid were chosen, considering the possibility of incomplete cleavage by some endoproteinases (*e.g.* trypsin) before these amino acids. (ii) Amino acids that can be oxidized or transformed were avoided, such as methionine and tryptophan (which are easily oxidized) and N-terminal glutamine (which can be transformed into pyroglutamic acid). All the peptides chosen fulfill these requirements, except for the methionine-containing peptides 3–9 of SmD1 and peptides 73–80 and 13–23 of SmE. Peptides 10–20 and 66–86 of SmD1 and 81–92 of SmE, which would fit the criteria, could not be synthesized in sufficient purity. All reference peptides correspond to peptides that are expected to be produced from the spliceosomal proteins by endoproteinase digestion. As shown in Tables I and II, most peptides are created by endoproteinase Lys-C digestion. Alternatives were trypsin, endoproteinase Glu-C, and Asp-N.

Peptide Derivatization—There are two possibilities for the incorporation of stable isotopes into peptides: peptide synthesis using isotope-coded amino acids (such as [¹⁵N]-, [¹⁸O]-leucine) (8) or derivatization of their side chains with isotope-coded reagents (22). Munchbach *et al.* (23) describes the advantages of amine-specific isotope-coded reagents, such as d0/d4 nicotinoyl-*N*-oxysuccinimide carrying a fixed positive charge and increasing the mass spectrometric response for labeled peptides significantly, compared with the acetylated peptide as well as with their unlabeled counterpart. The increase in mass spectrometric response is most pronounced for small peptides (up to 10 amino acids), which can also be synthesized more reliably. The use of nicotinoylation may therefore result in an increase in sensitivity for the absolute quantification and stoichiometry determination, even for protein complexes that can only be isolated in low quantities.

Determination of Optimal Conditions for Denaturation and Digestion—To determine the correct stoichiometry with this method, complete denaturation and digestion needs to be achieved for the U1 snRNP. Initially, urea was used for the denaturation of the complex. Urea is tolerated by several endoproteinases up to a concentration of 4 M, which allowed the digestion of the denatured protein complexes without prior dilution or dialysis (9). However, even with a concentration of 8

M urea, complete denaturation of the U1 snRNP was not achieved (data not shown). Therefore, 5 M guanidinium hydrochloride (GHCl) was tested. Because most endoproteinases already show a significant loss of activity at a concentration of 1 M, the denatured complex was diluted to 0.5 M GHCl with HEPES buffer. Subsequently, the enzyme was added and incubated overnight. After labeling with a ¹²C amine-specific tag, the digest was spiked with the synthetic peptides, which were derivatized with a ¹³C amine-specific tag. 0.36 μg of the complex was used in each experiment; assuming a hypothetical 1:1 stoichiometry of all complex constituents, this would correspond to ~1.7 pmol of the complex. Approximately the same molar amounts of the reference peptides were added, which should therefore theoretically result in a similar intensity of the ¹²C- and ¹³C-labeled peptides, supposing the complex to have been pure and denaturation and digestion to have been complete. Figs. 3 and 4 show the MALDI-TOF mass spectra of the peptides acquired after liquid chromatographic separation, which demonstrated a similar intensity of the isotopic peptide pairs.

Stoichiometry Determination Using Propionyl-N-oxysuccinimide—Table I summarizes the results of two independent experiments for the absolute quantification of the U1 proteins using propionyl-N-oxysuccinimide, using ~0.36 μg of the complex/experiment. Half of all the peptides, however, could not be detected because of their low mass spectrometric response. Peptides from the proteins U1–70 K, U1-A, SmD1, SmD2, SmD3, SmE, and SmG showed absolute amounts in the range of 1.25–1.67 pmol, suggesting 1:1 stoichiometries of these proteins in the complex. The observed deviations of approximately ±16% may be due to variations in sample handling, such as pipetting of peptide samples and dilutions. Peptides 38–50 of SmD2 showed a very high amount (12.4 pmol), which may have been due to solubility problems of the reference peptide. Concentrations below 0.069 pmol were found for peptides 90–99 of SmD3, which was attributed to arginine dimethylation of Arg-97 (12).

Stoichiometry Determination Using Nicotinoyl-N-oxysuccinimide—To quantify all the proteins in the complex, the experiments were repeated with the same absolute amounts of the U1 snRNP as described above, replacing propionyl-*N*-oxysuc-

TABLE II
Quantification of human U1 snRNP labeled with [¹²C]nicotinoyl-N-oxysuccinimide

This table summarizes the results of two independent experiments for the absolute quantification of the human U1 snRNP denatured, digested with endoproteinase Lys-C, Glu-C, trypsin, or Asp-N, and labeled with [¹²C]nicotinoyl-*N*-oxysuccinimide. Absolute quantification was achieved by the addition of synthetic peptides labeled with [¹³C]nicotinoyl-*N*-oxysuccinimide. Two peptides were used for the quantification of most proteins. Quantification was compromised for some peptides of the Sm proteins because of methylation of arginine residues (SmB, SmD3).

| Protein	Peptide	Experiment 1		Experiment 2		Enzyme
		pmol	Mean value	pmol	Mean value	
U1–70K	LRREFEVYGPIK (104–118)	1.83	1.67	1.52	1.53	Lys-C
	TLFVARVNYDTTESK (119–130)	1.51		1.54		Lys-C
U1-C	TTAAFQQGK (53–61)	1.38	1.59	1.25	1.32	Lys-C
	DYYQK (36–40)	1.79		1.38		Lys-C
U1-A	GTFVERDRK (99–107)	1.53	1.41	1.20	1.40	Lys-C
	SQETPATK (115–122)	1.30		1.59		Lys-C
SmB	KRVLGLVLLRGE (64–75)	1.65	1.48	1.38	1.37	Glu-C
	VLGLVLLR (66–73)	1.35		1.36		Trypsin
	VPLAGAAGGPGIGR (95–108)	< 0.0065		< 0.0054		Trypsin
SmD1	LVRFLMK (3–9)	1.57	1.57	1.38	1.38	Lys-C
SmD2	DRYISK (93–98)	1.88	1.86	1.51	1.65	Lys-C
	NNTQVLINCRNNK (38–50)	11.51		13.50		Lys-C
	DSVIVVLRNPLIAGK (104–118)	1.84		1.79		Asp-N
SmD3	NQGSGAGRGK (90–99)	< 0.0087	1.40	< 0.098	1.60	Lys-C
	VAQLEQVYIR (55–64)	1.40		1.60		Trypsin
SmE	QLGRIMLK (73–80)	1.73	1.55	1.53	1.51	Lys-C
	VMVQPINLIFR (13–23)	1.35		1.48		Trypsin
SmF	YIDGALSGHLGE (9–17)	0.050	1.71	0.035	1.23	Glu-C
	CNNVLYIR (65–73)	1.71		1.23		Trypsin
SmG	AHPPELK (4–10)	1.45/1.31	1.50	1.55/1.67	1.51	Lys-C/Trypsin
	HVQGILR (26–32)	1.54		1.46		Trypsin
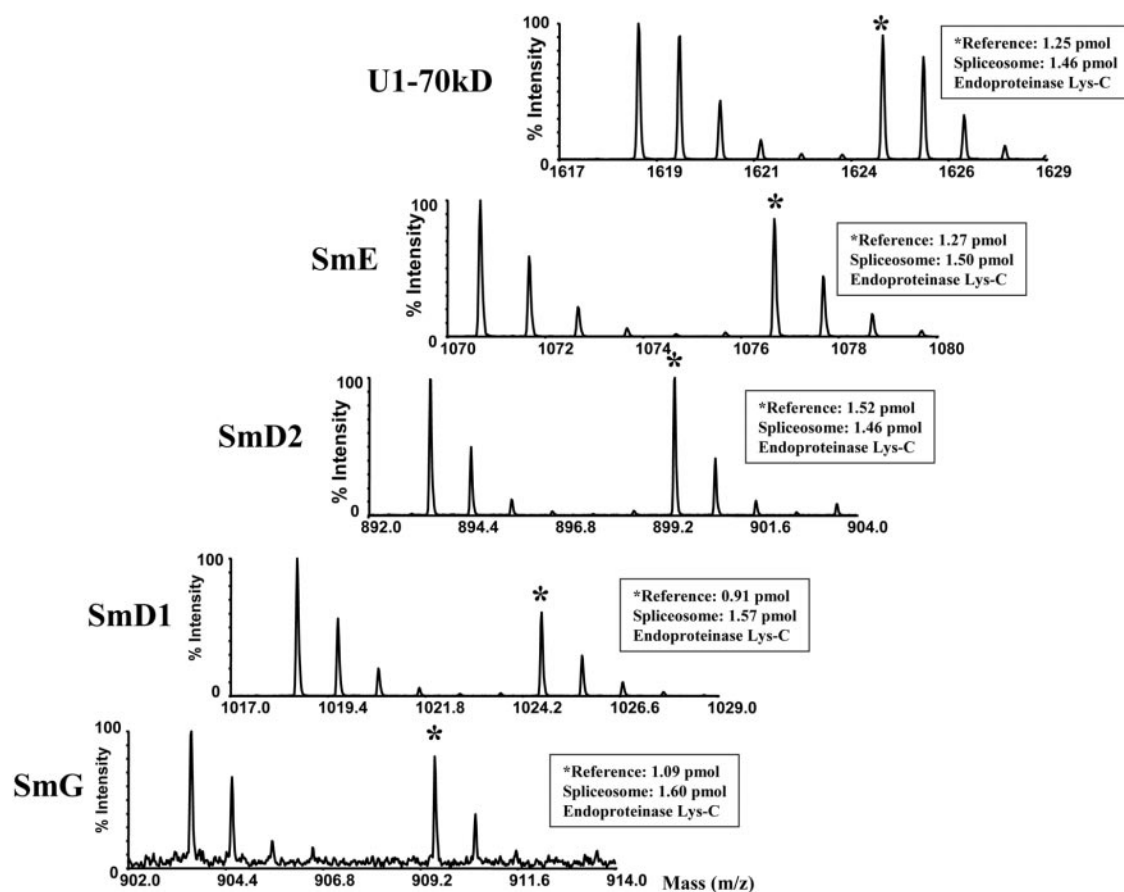
FIG. 3. **MALDI-TOF mass spectra used for the absolute quantification of spliceosomal proteins U1–70 K, SmE, D2, D1, and G of the U1 snRNP.** The U1 spliceosomal snRNP was denatured and then digested completely with endoproteinase Lys-C as described under "Experimental Procedures." The resulting peptides were labeled with [¹²C]propionyl-*N*-oxysuccinimide. Known amounts of synthetic peptides, labeled with the corresponding ¹³C isotope-coded amine-specific tag, were added (*Reference). MALDI-TOF mass spectrometry was used to absolutely quantify the proteins in the U1 spliceosomal snRNP.

cinimide with nicotinoyl-*N*-oxysuccinimide (Table II). In this case, all the peptides were detected; in particular, small peptides showed a significant increase in detectability as compared

with propionyl-*N*-oxysuccinimide. One peptide of SmG (4–10, sequence: (K) AHPPELK), which is created by complete digestion with endoproteinase Lys-C or trypsin, was quantified in
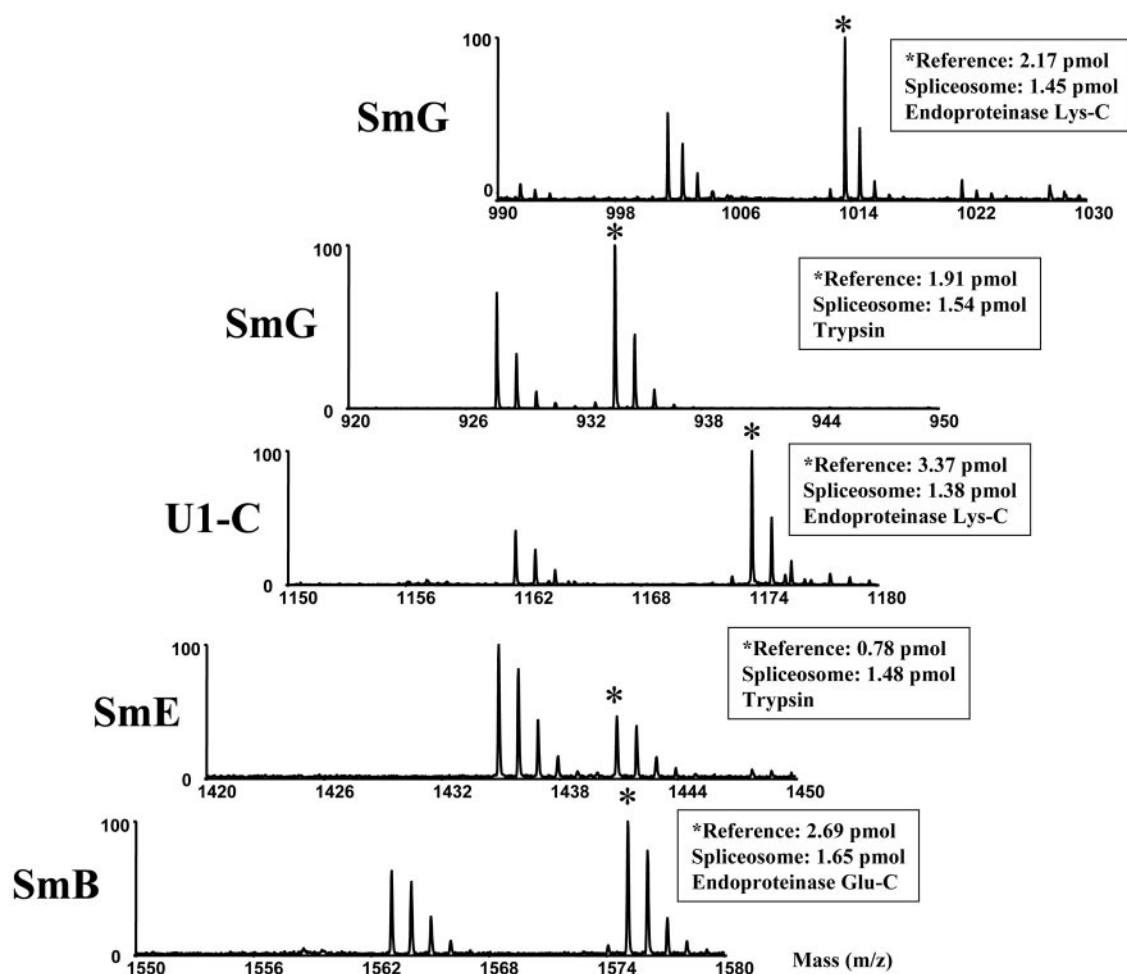
FIG. 4. **MALDI-TOF mass spectra used for the absolute quantification of spliceosomal proteins SmG, U1-C, SmE, and SmB of the U1 snRNP.** The U1 spliceosomal snRNP was denatured and then digested completely with endoproteinase Lys-C (*U1-C, SmG*), trypsin (*SmG, SmE*) and endoproteinase Glu-C (*SmB*) as described under "Experimental Procedures." The resulting peptides were labeled with [¹²C]nicotinoyl-*N*-oxysuccinimide. Known amounts of synthetic peptides, labeled with the corresponding ¹³C isotope-coded tag were added (*Reference). MALDI-TOF mass spectrometry was used to absolutely quantify the proteins in the U1 spliceosomal snRNP.

both digests. Quantification of this peptide in trypsin and endoproteinase Lys-C digests resulted in similar quantities (Table II), which suggests that the digestion was complete. In similarity to the experiments with propionyl-*N*-oxysuccinimide, mean absolute quantities of 1.40–1.86 pmol (Experiment 1) and 1.23–1.65 pmol (Experiment 2) were measured for most peptides. For peptides 95–108 of SmB and 90–99 of SmD3, quantities below 0.01 pmol were determined. This was ascribed to dimethylation of arginine 97 of SmD3 and arginine 108 of SmB (12). For peptides 9–17 of SmF, a quantity of 0.035–0.05 pmol was found. This peptide contains aspartic acid, which is also cleaved by endoproteinase Glu-C to a lesser extent. For peptides 38–50 of SmD2, very high quantities (11.5–13.5 pmol) were found. These data also demonstrated that one peptide/protein may be insufficient for the reliable absolute quantification of a protein. A third peptide-(104–118) from SmD2 was therefore quantified, resulting in 1.79–1.84 pmol, which is comparable with the quantity of peptides 93–98 (1.65–1.88 pmol).

DISCUSSION

Recently, we described a method for the determination of the stoichiometry of protein complexes. Absolute quantification of the complex constituents was achieved by comparing the areas of fluorescently labeled peptides resulting from different proteins in the fluorescence chromatogram. This method was used for the determination of the stoichiometry of the complex of

hFc1 and sFcγRIII (9). The method allows relatively fast stoichiometry determination of complexes consisting of a moderate number of proteins. Although cysteine-specific rather than amine-specific labeling reduced significantly the complexity of the fluorescently labeled peptide mixture resulting from hFc1 and sFcγRIII, the number of peptides resulting from very large multiprotein complexes may still be too high to allow a good separation. Therefore, we tested the strategy depicted in Fig. 2 for stoichiometry determination of multiprotein complexes. Because reference peptides have to be synthesized for this approach, this method is more time-consuming than the fluorescence method; however, it overcomes several limitations for the analysis of large multiprotein complexes. First of all, it is absolutely compatible with multidimensional separations. Losses of peptides during the transfer from the first to the second dimension have no influence on the absolute quantification, because the ¹³C-labeled synthetic reference peptides show chromatographic behavior identical to that of their ¹²C-labeled counterparts. Furthermore, even poorly separated peptides in the chromatogram can be quantified, because the quantification is based on the comparison of the mass spectrometric intensities belonging to the isotopic peptide pairs. A similar approach was suggested for absolute quantification in proteomics, using isotope-coded affinity tag-labeled idiotypic synthetic reference peptides as internal standards (22).

However, several circumstances should be considered when

using this approach for absolute protein quantification. Complete denaturation and digestion of the complex is a prerequisite for absolute quantification and stoichiometry determination. Furthermore, because unmodified synthetic peptides are used for quantification, unexpected post-translational modifications of the proteolytic peptides can falsify the result in quantification of the protein. The use of absolute quantification on a proteome-wide scale may therefore still be problematic. Multiprotein complexes, however, consist of a moderate number of proteins, which are usually better characterized. Another issue is that of miscleavage by endoproteinases, such as chymotryptic activity in trypsin or endoproteinase Glu-C cleavage at aspartic acid.

Therefore, we used in this study two or more peptides/protein for the absolute quantification of most proteins. We found similar absolute quantities for all ten proteins, suggesting equal stoichiometries of all ten proteins in the U1 snRNP, including U1-C. For quantification of the SmB protein, only peptides derived from sequences common to both SmB and SmB′ were used. Consequently, there is only one type of SmB protein in the Sm core, and SmB can be replaced by SmB′ or vice versa. Because Coomassie-stained gels of electrophoretically separated snRNP proteins always show the two proteins SmB and SmB′ as two bands of equal intensity (see Fig. 1), the two proteins are present in approximately similar amounts. The 1:1 stoichiometry of the Sm proteins is in good agreement with a model proposing that the Sm proteins form a heptamer ring with a single copy of each Sm protein in the snRNPs (14, 15). A 1:1 stoichiometry is also in best agreement with the three-dimensional structure of U1 snRNP derived from single particle electron cryomicroscopy (16). However, the stoichiometric analysis does not exclude the possibility that two kinds of U1 snRNP are present in equal amounts: one with two copies of the protein U1-C protein and the other with none. Nevertheless, this possibility can be excluded, because the ion exchange chromatography used here to purify U1 snRNP separated U1 snRNPs lacking protein U1-C from those containing it (Ref. 21

and data not shown). The method described here should therefore be well suited for the determination of the stoichiometric composition of a variety of snRNPs and (spliceosomal) multiprotein complexes.

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