Purification and Molecular Cloning of a Novel Essential Component of the Apolipoprotein B mRNA Editing Enzyme-Complex*

Received for publication, March 3, 2000, and in revised form, April 19, 2000
Published, JBC Papers in Press, April 25, 2000, DOI 10.1074/jbc.M001786200

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Editing of apolipoprotein B (apoB) mRNA requires the catalytic component APOBEC-1 together with "auxiliary" proteins that have not been conclusively characterized so far. Here we report the purification of these additional components of the apoB mRNA editing enzyme-complex from rat liver and the cDNA cloning of the novel APOBEC-1-stimulating protein (ASP). Two proteins copurified into the final active fraction and were characterized by peptide sequencing and mass spectrometry: KSRP, a 75-kDa protein originally described as a splicing regulating factor, and ASP, a hitherto unknown 65-kDa protein. Separation of these two proteins resulted in a reduction of APOBEC-1-stimulating activity. ASP represents a novel type of RNA-binding protein and contains three single-stranded RNA-binding domains in the amino-terminal half and a putative double-stranded RNA-binding domain at the carboxyl terminus. Purified recombinant glutathione S-transferase (GST)-ASP, but not recombinant GST-KSRP, stimulated recombinant GST-APOBEC-1 to edit apoB RNA in vitro. These data demonstrate that ASP is the second essential component of the apoB mRNA editing enzyme-complex. In rat liver, ASP is apparently associated with KSRP, which may confer stability to the editing enzyme-complex with its substrate apoB RNA serving as an additional auxiliary component.

ApoB mRNA editing is an intranuclear event that occurs post-transcriptionally coincident with splicing and polyadenylation (7). Editing of apoB mRNA is mediated by a multicomponent enzyme complex termed the apoB mRNA editing enzyme (8–10). This enzyme-complex deaminates the cytidine residue at nucleotide 6666 to create a uridine (11, 12). An 11-nucleotide "mooring" motif downstream of the editing site from nucleotides 6671 to 6681 is absolutely required for the editing reaction (13). The catalytic subunit APOBEC-1 (apoB-editing catalytic component 1) is the first component of the editing enzyme-complex that was cloned in 1993 by expression in Xenopus oocytes (14). APOBEC-1 is a cytidine deaminase with a novel RNA-binding motif that alone cannot edit the apoB mRNA, but requires additional, so-called "auxiliary" components for efficient editing (15–19). Adenovirus-mediated gene transfer of APOBEC-1 reconstitutes apoB mRNA editing in rabbit liver and results in drastic reduction of elevated LDL levels in LDL receptor-deficient Watanabe-heritable hyperlipidemic rabbits, thus demonstrating the physiological power of apoB mRNA editing (20, 21). APOBEC-1 transgenic mice and rabbits, however, develop hepatocellular dysplasia and carcinoma, indicating that APOBEC-1 has to be tightly regulated (22). The expression of APOBEC-1 in mouse or rat liver is the result of a second promoter in the apoB gene (14). APOBEC-1 transgenic mice and rabbits, however, develop hepatocellular dysplasia and carcinoma, indicating that APOBEC-1 has to be tightly regulated (22). The expression of APOBEC-1 in mouse or rat liver is the result of a second promoter in the apoB gene (14). APOBEC-1 transgenic mice and rabbits, however, develop hepatocellular dysplasia and carcinoma, indicating that APOBEC-1 has to be tightly regulated (22). The expression of APOBEC-1 in mouse or rat liver is the result of a second promoter in the apoB gene (14). APOBEC-1 transgenic mice and rabbits, however, develop hepatocellular dysplasia and carcinoma, indicating that APOBEC-1 has to be tightly regulated (22). The expression of APOBEC-1 in mouse or rat liver is the result of a second promoter in the apoB gene (14). APOBEC-1 transgenic mice and rabbits, however, develop hepatocellular dysplasia and carcinoma, indicating that APOBEC-1 has to be tightly regulated (22).
protein cross-linked specifically to apoB RNA and cofractionated with high molecular mass editosomes in sedimentation and native gel shift analyses (30). These proteins were later partially purified using APOBEC-1 affinity chromatography and had APOBEC-1-stimulating activity (31). Monoclonal antibodies identified a 240-kDa protein that appeared to confer APOBEC-1-stimulating activity (32). Mehta et al. (33) reported in 1996 the partial purification of a 65-kDa protein from baboon kidney using APOBEC-1 affinity chromatography. In 1998, Mehta and Driscoll (34) purified this 65-kDa protein from baboon kidney to homogeneity using apoB RNA affinity chromatography and demonstrated that this protein strongly stimulated APOBEC-1 to edit apoB RNA in vitro. Another APOBEC-1-interacting protein, designated ABBP-1 (APOBEC-1-binding protein 1), was identified by two-hybrid selection in yeast (35). ABBP-1 is a splice variant of human hnRNP A/B protein that can also bind apoB RNA, but its importance for apoB mRNA editing remains to be conclusively confirmed (35). We also performed two-hybrid selection with APOBEC-1 as a bait and isolated hnRNP C1 protein as an APOBEC-1-binding protein (36). However, hnRNP C1 is a very potent inhibitor of APOBEC-1-mediated mRNA editing rather than an APOBEC-1-stimulating protein (36).

In this investigation, we purified the APOBEC-1-stimulating proteins from rat liver nuclei to homogeneity using ssDNA affinity chromatography and identified two proteins with molecular masses of 75 and 65 kDa that could not be further separated without reducing the activity. The 75-kDa protein was identified as the mRNA-binding protein KSRP (K homology-type splicing regulatory protein), recently described as a splicing regulating protein (37). KSRP alone does not stimulate APOBEC-1. The 65-kDa protein, a hitherto unknown protein, was cloned and designated APOBEC-1-stimulating protein (ASP). ASP has three RNA-binding domains with homologies to pol(A)-binding proteins. Recombinant ASP complements recombiant APOBEC-1 to edit apoB RNA in vitro. Therefore, APOBEC-1 and ASP represent the minimal requirements for apoB mRNA editing in vitro.

**EXPERIMENTAL PROCEDURES**

**Purification of APOBEC-1-stimulating Proteins—** Nuclear extracts were prepared from the livers of four rats as described (36, 38). The nuclear extracts (8 ml) were layered over four linear 15–30% sucrose gradients centrifuged for 16 h at 80,000 × g using an SW 28.1 rotor (Beckman Instruments) as described (36, 39). The gradients were fractionated from the bottom into 1-ml aliquots. The protein concentration was determined in every other fraction. Two μl of the fractions were used for *in vitro* editing assays without and after supplementation with recombinant APOBEC-1. Fractions 27–30 containing the APOBEC-1-stimulating activity were loaded on a ssDNA-cellulose column (2 ml; U. S. Biochemical Corp.) in buffer A (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5 mM dithiothreitol, and 10% glycerol) at a flow rate of 0.5 ml/min (40). The flow-through fraction of this column (FL1) was incubated with 1 μM CaCl₂ and 100 units/ml micrococcal nuclease (Roche Molecular Biochemicals) for 15 min at 30 °C. After the addition of EDTA, pH 8.0, to a final concentration of 5 mM, the digest was stopped on ice for 10 min. The micrococcal nuclease-digested FL1 fraction was reapplied to a second ssDNA column (2 ml) in buffer B (buffer A with 1 mM EDTA). The column was washed with 20 ml of buffer B and pre-eluted with 6 ml of buffer B containing heparrin at 1 mg/ml. The column was step-eluted with 0.3, 0.5, and 1 mM NaCl in buffer B.

The 0.3 mM NaCl elution fraction (E3) containing 10 μg of total protein in a total volume of 2 ml was desalted in buffer A in a molecular mass cut-off membrane (Millipore Corp.) and stained with Coomassie Blue. The bands with APOBEC-1-stimulating activity were pooled and separated on a Superdex 200 FPLC column. The 0.3 M NaCl elution fraction (E3) containing 10 μg of total protein in a total volume of 10 ml containing 10 units of RNaseA and 1 mM EDTA (E3) was applied to a second ssDNA column (2 ml) in buffer B containing 0.3 M NaCl (buffer E3). The flow-through fraction of this column (FL2) was further purified on a Superdex 200 FPLC column (Amersham Pharmacia Biotech) run in buffer B at a flow rate of 0.5 ml/min. The column was washed with 20 ml of buffer B and pre-eluted with 6 ml of buffer B containing heparrin at 1 mg/ml. The column was step-eluted with 0.3, 0.5, and 1 mM NaCl in buffer B.

**Northern Blotting—** Northern blotting was performed on a metal-coated borosilicate nanoelectrospray vial. Each sample was directly transferred to the vial and desiccated under vacuum at 4°C. Mass peaks identified in ESI/MS experiments were selected as precursor ions for fragmentation in tandem MS experiments. From the fragmentation pattern, partial sequences (sequence tag) of the peptides were derived and used for database searching (44).

**Cloning of the cDNA for ASP—** Data base searches identified several human and mouse ESTs that exactly matched the peptide sequences of the 65-kDa protein (IMAGE-ID, 431853, 2297967, 110327, 248021, 1450850, and 2317398). These overlapping cDNA clones were entirely sequenced and covered all peptide sequences derived from the 65-kDa protein. A full-length cDNA was amplified by polymerase chain reaction from a human liver cDNA library (human liver LexA library, CLONTECH) and from a cDNA pool of human small intestine (MarathonReady cDNA, CLONTECH) using primers specific for the amino terminus (CTC AAT GGA ATC AAA TCA CAA ATC CGG) and the carboxyl terminus (GCA GCT GGT ACA CTG GTC GTC CC). The cDNAs were cloned into a EcoRI-EcoRI (Promega) and sequenced.

**In Vitro Translation of ASP and KSRP—** The cDNA of ASP from human small intestine was in vitro translated using an in vitro coupled transcription-translation system (T7 TNT Quick, Promega) following
vitro editing assays with a synthetic apoB RNA. As controls, nuclear was tested for the presence of the apoB mRNA editing holoenzyme by calibration of the gradients, separate runs were performed with apo-
protein concentration of each fraction was determined (mg/ml). For trifugation. The gradients were fractionated from the bottom, and thepared from rat liver and subjected to sucrose density gradient ultracen-
sity gradient ultracentrifugation.

A with the extension products of edited (U) and unedited (C) RNAs is shown. The autoradiograph of the primer extension assay with the extension prod-
ucts for edited (U) and unedited (C) RNAs is shown.

B

C

FIG. 1. Separation of rat liver nuclear extracts by sucrose dens-
ity gradient ultracentrifugation. A, nuclear extracts were pre-
pared from rat liver and subjected to sucrose density gradient ultracen-
trifugation. The gradients were fractionated from the bottom, and the protein concentration of each fraction was determined (mg/ml). For calibration of the gradients, separate runs were performed with apo-
ferritin (18 S, 440 kDa) and 40 S monoparticles. B, every other fraction was tested for the presence of the apoB mRNA editing holoenzyme by in vitro editing assays with a synthetic apoB RNA. As controls, nuclear extracts to gradient (t.g.) and pure buffer (b.) were used. The RNA was analyzed for editing by primer extension assay. The autoradiograph with the extension products of edited (U) and unedited (C) RNAs is shown. C, the same gradient fractions were assayed for in vitro editing after supplementation with S100 extracts from Sf9 cells expressing APOBEC-1. As controls, nuclear extracts (t.g.) and pure buffer (b.) were supplemented with Sf9-APOBEC-1 and tested for in vitro editing. The autoradiograph of the primer extension assay with the extension products for edited (U) and unedited (C) RNAs is shown.

the manufacturer’s protocol. By addition of [35S]methionine to the amino acid mixture for cold translation, the in vitro translated proteins were trace-labeled and could be visualized by autoradiography. In vitro translated ASP was used for in vitro editing assays supplemented with APOBEC-1-containing extracts from Sf9 insect cells (Sf9-APOBEC-1) as described above.

Expression of GST-tagged Recombinant Proteins—The cDNAs of ASP and KSRP were inserted in frame into the BamHI site of pGEX-2T to generate pGEX-2T-ASP and pGEX-2T-KSRP, respectively. Both constructs were entirely sequenced to confirm the open reading frame. Recombinant GST-ASP, GST-KSRP, and GST-APOBEC-1 (36) were expressed in Escherichia coli; purified to homogeneity as described (36); and used for in vitro editing assays.

RESULTS

Purification of APOBEC-1-stimulating Components from Rat Liver—Nuclear extracts were prepared from rat liver and sub-
jected to sucrose gradient ultracentrifugation. The gradients were fractionated from the bottom, and the protein concentration in each fraction was measured. A characteristic protein distribution with two prominent peaks around 40 S and 18–25 S was observed (Fig. 1A). In vitro editing activity was detected in fractions 21–25 (Fig. 1B), indicating the sedimentation of the apoB mRNA editing holoenzyme around 23–25 S as described (9). Supplementation of the gradient fractions with Sf9-APO-
BEC-1 reconstituted strong in vitro editing activity in fractions 27–30 (18 S), which did not exhibit endogenous in vitro editing activity (Fig. 1C). Sf9-APOBEC-1 alone or combined with any other gradient fraction did not confer apoB mRNA editing activity (Fig. 1C). Therefore, the stimulating factors for APO-
BEC-1 mediated mRNA editing are abundantly present in rat liver nuclear extracts and can be separated from the 25 S apoB mRNA editing holoenzyme by sucrose density gradient ultracentrifugation.

When fractions 27–30 from the sucrose gradient were passed over a ssDNA-cellulose column, the APOBEC-1-stimulating factors were entirely recovered in the flow-through fraction and did not bind to the matrix (Fig. 2A). However, when this first flow-through fraction (FL1) was digested with micrococcal nu-
clide that was subsequently inactivated by the addition of EGTA, and the micrococcal nuclease-digested FL1 fraction was reapplied to a second ssDNA-cellulose column, nearly all of the APOBEC-1-stimulating activity bound to the matrix (Fig. 2, A and B). The second flow-through fraction (FL2) no longer contained significant amounts of editing-stimulating factors (Fig. 2, A and B). Most of the proteins that bound to this second ssDNA-cellulose column were eluted with washing with heparin (1 mg/ml), but this fraction (E2) contained only minor amounts of APOBEC-1-stimulating activity (Fig. 2B). The elution of the column with 0.3 M NaCl resulted in only very little protein, but this fraction (E3) exhibited strong APOBEC-1-stimulating activity (Fig. 2B). Further washing of the column with 0.5 and 1.0 M NaCl, respectively, eluted some more protein from the column, but in these fractions (E4 and E5), considerable amounts of APOBEC-1-stimulating activity were not detected (Fig. 2, A and B).

The proteins in fractions FL1, FL2, E2, E3, E4, and E5 were purified by SDSPAGE. In fraction E3, which exhibited strong APOBEC-1-stimulating activity, only three major pro-
teins with molecular masses of 18, 65, and 75 kDa were visible after silver staining (Fig. 2C). These three proteins were clearly enriched in fraction E3 compared with fraction FL1, the starting material for the second ssDNA-cellulose column. Thus, we had developed an affinity purification procedure for the APOBEC-1-stimulating activity in rat liver.

Binding of Affinity-purified APOBEC-1-stimulating Proteins to ApoB RNA—The consecutive fractions of this purification procedure were incubated with 32P-labeled apoB RNA (nucleotides 6649–6703) or 32P-labeled β-actin RNA to study RNA-protein interactions by UV cross-linking. In fraction E3, UV cross-linking to 32P-labeled apoB RNA marked predominantly one single protein with a molecular mass of 75 kDa that was strongly enriched in this fraction and that was also visible in fraction E4 (Fig. 3A). UV cross-linking to 32P-labeled β-actin RNA marked a different set of proteins (Fig. 3A). Most impor-
tant, the 75-kDa protein in fraction E3 cross-linked only weakly to 32P-labeled β-actin RNA. In comparison, a 53-kDa protein in fractions FL1 and E4 strongly cross-linked to 32P-labeled β-actin RNA, but not to 32P-labeled apoB RNA (Fig. 3A).

Competition experiments with an excess of unlabeled apoB RNA or β-actin RNA were performed to further study the UV cross-linking to the 75-kDa protein. The proteins in fraction E3 were incubated with 32P-labeled apoB RNA in the absence or presence of 10-, 50-, and 100-fold excesses of unlabeled apoB RNA (Fig. 3B, lanes 1–4) or β-actin RNA (lanes 5–8). UV cross-linking to the 75-kDa protein was decreased by 92% in the presence of a 50-fold excess of unlabeled apoB RNA (Fig. 3B, lanes 2 and 3). In the presence of a 100-fold excess of unlabeled apoB RNA, UV cross-linking to the 75-kDa protein was no longer detectable (Fig. 3B, lane 4). Interestingly, UV cross-linking to 32P-labeled apoB RNA marked a protein with a
molecular mass of 65 kDa only in the presence of an excess of unlabeled β-actin RNA (Fig. 3B, lane 5). In the presence of 50- and 100-fold excesses of unlabeled β-actin RNA, UV cross-linking to the 75-kDa protein was decreased by 80 and 91%, respectively, but was not completely abolished (Fig. 3B, lanes 7 and 8). Notably, UV cross-linking to the 65-kDa protein was

FIG. 2. Purification of the APOBEC-1-stimulating proteins by ssDNA-cellulose chromatography. A, the sucrose density gradient fractions 27–30 were passed over ssDNA-cellulose, and the unbound flow-through fraction (FL1) was digested with micrococcal nuclease (MN). The micrococcal nuclease-treated FL1 fraction was reapplied to a second ssDNA-cellulose column. The column was pre-eluted by washing with heparin (Hep.)-containing binding buffer (E2) and was subsequently step-eluted with 0.3 (E3), 0.5 (E4), and 1.0 (E5) M NaCl. The protein concentrations (mg/ml) of the fractions from the two columns are given. B, fractions FL1 (after digestion with micrococcal nuclease), FL2, E2, E3, E4, and E5 were separated by SDS-PAGE, and the proteins were stained with silver.

FIG. 3. UV cross-linking and gel shift analysis with 32P-labeled apoB RNA. A, fractions FL1, FL2, E2, E3, E4, and E5 were incubated with 32P-labeled apoB RNA (5 × 10^5 cpm) (left panel) or 32P-labeled β-actin RNA (right panel). After UV cross-linking and RNase digestion, the proteins were separated by SDS-PAGE and autoradiographed for 4–6 h. B, fraction E3 was incubated with 32P-labeled apoB RNA (5 × 10^5 cpm) in the absence or presence of 10-, 50-, and 100-fold excesses of unlabeled apoB RNA (lanes 1–4) or unlabeled β-actin RNA (lanes 5–8). After UV cross-linking and RNase digestion, the proteins were separated by SDS-PAGE and autoradiographed for 8 h. C, 32P-labeled apoB RNA (5 × 10^5 cpm) was incubated with sucrose density gradient fractions 27–30 to column (t.c.), FL1, E1, FL1 after micrococcal nuclease digestion, FL2, E2, E3, E4, and E5 and subsequently separated on a native 6% polyacrylamide gel. The autoradiograph of the dried gel is shown.
Cloning of an APOBEC-1-stimulating Protein

Fraction E3 was desalted, concentrated ~4-fold using a Centricon 10 concentrator, and applied in a volume of 200 μl to a Superdex 200 FPLC column run at 0.1 mM NaCl. Fractions of 0.5 ml were collected. Fractions 21–32 were supplemented with Sf9-APOBEC-1 and assayed for apoB RNA editing in vitro. B, fractions 21–32 were incubated with 32P-labeled apoB RNA (5 × 10^8) at 100 mM NaCl and exposed to UV radiation. After UV cross-linking and RNase digestion, proteins were separated by SDS-PAGE and analyzed by autoradiography. C, fraction E3 was desalted, concentrated ~4-fold apparently unaffected by a 50-fold and even a 100-fold excess of unlabeled β-actin RNA (Fig. 3B, lanes 7 and 8). Therefore, the 75-kDa protein seemed to be the major RNA-binding protein in fraction E3 with no specificity for apoB RNA. Cross-linking of 32P-labeled apoB RNA to the 65-kDa protein appeared to be displaced by the 75-kDa protein, but was not inhibited by unlabeled β-actin RNA.

Gel mobility shift analysis was performed to study complex formation of apoB RNA with the APOBEC-1-stimulating factors. After incubation with the various fractions of the purification scheme, the 32P-labeled apoB RNA (nucleotides 6649–6703) was separated on a native polyacrylamide gel (Fig. 3C). Retardation of the 32P-labeled apoB RNA was observed in the fractions containing APOBEC-1-stimulating activity: sucrose gradient fractions 27–30 (Fig. 3C, lane 2), FL1 (lane 3), E1 (lane 4), FL1 after digestion with micrococcal nuclease (lane 5), and E3 (lane 8). Fraction E1 used in these experiments contained some APOBEC-1-stimulating activity, but also large amounts of general RNA-binding proteins (data not shown). No retardation of 32P-labeled apoB RNA was observed in fractions FL2, E4, and E5, all of which did not contain significant amounts of APOBEC-1-stimulating factors (Fig. 3C). Fraction E2 contained some editing-stimulating activity, but failed to show an RNA gel shift (Fig. 3C, lane 7). This might be caused by the heparin used to elute this protein fraction.

**Gel Filtration of APOBEC-1-stimulating Proteins**—The proteins in fraction E3 were further analyzed by gel filtration on a Superdex 200 FPLC column (Fig. 4). In the presence of 0.1 mM NaCl, the APOBEC-1-stimulating activity eluted in fractions 22–26, corresponding to a molecular mass range of 440–232 kDa (Fig. 4A). UV cross-linking to 32P-labeled apoB RNA again marked the 75-kDa protein in fractions 22–26 (Fig. 4B). In these fractions, the 65-kDa protein was labeled by UV cross-linking to 32P-labeled apoB RNA (Fig. 4B). This indicated that the 65- and 75-kDa proteins coeluted in a high molecular mass range.

Separation of the proteins in fraction E3 by gel filtration on a Superdex 200 FPLC column in the presence of 0.5 mM NaCl resulted in a loss of editing-stimulating activity in fractions 22–26 (Fig. 4C). Instead, markedly reduced activity was detected in fractions 27–29, corresponding to a molecular mass of above 66 kDa (Fig. 4C). The recovery of editing-stimulating activity was calculated from both the 0.1 and 0.5 mM NaCl-eluted Superdex columns. After gel filtration in 0.5 mM NaCl, only 18.4% of editing-stimulating activity was recovered as compared with gel filtration in the presence of 0.1 mM NaCl. Both columns were run under exactly the same conditions apart from the different salt concentrations. Concomitantly, UV cross-linking of the 75- and 65-kDa proteins to 32P-labeled apoB RNA was not observed in fractions 22–24 after gel filtration in the presence of 0.5 mM NaCl (Fig. 4D). Weak UV cross-linking to the 75-kDa protein was detected in fractions 24 and 25 (Fig. 4D). Faint UV cross-linking to the 65-kDa protein was observed in fractions 26–29, which contained the residual APOBEC-1-stimulating activity (Fig. 4D). In fractions 27 and 28, UV cross-linking strongly marked a protein with an apparent molecular mass of 53 kDa that was also present in these fractions after gel filtration in the presence of 0.1 mM NaCl (Fig. 4, B and D). Therefore, gel filtration in the presence of 0.5 mM NaCl results in a loss of editing-stimulating activity.
m NaCl resulted in a shift of the APOBEC-1-stimulating activity. The 75- and 65-kDa proteins were separated from each other, and concomitantly, the 75-kDa protein cross-linked much more weakly to 32P-labeled apoB RNA. When the proteins in fraction E3 were applied to a minibead HPLC column (Amersham Pharmacia Biotech), both the APOBEC-1-stimulating activity and the 75- and 65-kDa proteins did not bind to the matrix in the presence of 0.1 m NaCl and were entirely recovered in the flow-through fraction (data not shown).

Preparative Purification and Molecular Analysis of APOBEC-1-stimulating Proteins—The E3 fractions from four independent ssDNA affinity purifications were pooled, concentrated, and separated by final gel filtration in the presence of 0.1 m NaCl. The column elution profile demonstrated two well separated protein peaks (Fig. 5A). Fractions 23–25 were pooled and assayed for APOBEC-1-stimulating activity. These three fractions did not contain endogenous apoB mRNA-editing activity, but reconstituted very strong editing activity after supplementation with recombinant APOBEC-1 (Fig. 5B). After an in vitro editing assay for 1 h, ~49% of the apoB substrate RNA was edited by supplementation with SF9-APOBEC-1, and 36.4% of the apoB substrate RNA was edited by supplementation with purified GST-APOBEC-1 (Fig. 5B).

One-tenth of pooled fractions 23–25 was separated by SDS-PAGE and stained with silver (Fig. 5C). Only two proteins with molecular masses of 75 kDa and 65 kDa were detected in this final purified fraction (Fig. 5C). Again, the staining of the 75-kDa protein was stronger than that of the 65-kDa protein. The ratio of the 75-kDa protein to the 65-kDa protein in this fraction was very similar as observed in fraction E3. This indicated that these two proteins copurified. Both proteins in this fraction were analyzed by UV cross-linking to 32P-labeled apoB RNA. Again, the 75-kDa protein was more strongly labeled than the 65-kDa protein (Fig. 5D).

The remaining 90% of fractions 23–25 were separated by SDS-PAGE and electroblotted. The two proteins were excised and digested with LysC. Approximately 80% of the generated peptides were separated by HPLC for sequencing by Edman degradation. The remaining 20% of the samples were analyzed by MALDI/MS (75-kDa protein) or ESI-TOF tandem MS (65-kDa protein). Three peptides of the 75-kDa protein were entirely sequenced. In addition, the masses of seven peptides were detected by MALDI/MS. Both the three peptide sequences as well as the detected seven peptide masses of the 75-kDa protein matched exactly with the pre-mRNA-binding protein KSRP (37). KSRP has a molecular mass of 75 kDa and is contained in a high molecular mass complex assembled on an intronic splicing enhancer region of c-src in neuronal cells (45). KSRP is supposed to regulate the inclusion of the N1 exon of c-src that occurs only in the brain (37, 46).

Two peptides of the 65-kDa protein were sequenced by Edman degradation. Peptide fingerprints obtained by MALDI/MS could not be unambiguously aligned with any known protein sequence. Therefore, we performed additional peptide sequencing by ESI tandem MS and obtained the sequences of five peptides. One of the peptide sequences was identical to one of the sequences obtained by Edman degradation. Data base searches could not align these six individual peptide sequences with any known protein. However, several mouse and human ESTs were identified that matched these sequences.

cDNA Cloning of the 65-kDa Protein—We generated a contig of four human ESTs and two mouse ESTs that entirely covered the generated peptide sequences. This contig contained an open reading frame for a 65-kDa protein. Using polymerase chain reaction with a primer pair specific for the amino and carboxyl termini of this putative cDNA, we isolated a 1.8-kilobase pair cDNA both from a human liver cDNA library and from a cDNA pool of human small intestine. These cDNAs differed in a stretch of 24 nucleotides that was missing in the intestine-derived cDNA, but otherwise were identical, spanning an open reading frame of 586 or 594 amino acids, respectively (Fig. 6, insertion indicated by the two asterisks).

The calculated molecular masses of this protein are 64.3 kDa (intestine) and 65.2 kDa (liver). The six peptides that were sequenced could be exactly aligned with this open reading frame (Fig. 6, boldface letters). Data base searches confirmed that the encoded protein has not been described so far. According to its function, we designated this protein APOBEC-1-stimulating protein (ASP). Secondary structure prediction identified three RNA-binding domains (RBDs; amino acids 57–130, 137–214, and 232–299) in the amino-terminal half of ASP...
that have a similar organization, but are not identical (Fig. 6, underlined). The first and second RBDs contain one RNP-1 motif each (amino acids 96–103 and 179–186, respectively) (Fig. 6, black boxes). In addition, a putative double-stranded RNA-binding motif is double-underlined. The putative nuclear localization signal is indicated by a gray box, and the two putative tyrosine phosphorylation sites are indicated by dotted lines. The amino acid sequences of ASP that were derived by peptide sequencing with Edman degradation and ESI-TOP/MS are printed in boldface letters.

A Northern blot containing 2 μg of purified poly(A)⁺ RNA from each of 12 human tissues was hybridized with a radiolabeled cDNA of the carboxyl-terminal half of ASP. A transcript of 2.0 kilobase was detected in the liver and kidney (Fig. 7). In both tissues, an additional transcript of 8.0 kilobase was visible.

The isolated cDNA for ASP from human small intestine was in vitro translated in the presence of unlabeled methionine and a trace amount of [35S]methionine. This resulted in the synthesis of a protein with an apparent molecular mass of 65 kDa with some smaller translation products (data not shown). The addition of Sf9-APOBEC-1 to reticulocyte lysate with in vitro translated ASP resulted in the reconstitution of apoB mRNA editing (data not shown). Reticulocyte lysate with in vitro translated ASP but without Sf9-APOBEC-1 or reticulocyte lysate with Sf9-APOBEC-1 but without in vitro translated ASP did not exhibit apoB mRNA editing (data not shown).

ASP and KSRP were expressed in E. coli as fusion proteins with GST, purified to apparent homogeneity, and used for in vitro editing assays together with purified GST-APOBEC-1 and GST-KSRP. In vitro editing assays were performed for 1 h with 100 ng of GST-APOBEC-1 (lane 1), with 100 ng of GST-APOBEC-1 plus 50 ng of protein from fraction E3 (lane 2), with 100 ng of GST-KSRP (lane 3), with 100 ng of GST-KSRP plus 100 ng of GST-APOBEC-1 (lane 4), with 100 ng of GST-ASP (lane 5), and with 100 ng of GST-ASP plus 100 ng of GST-APOBEC-1 (lane 6). The apoB RNA was analyzed for editing by primer extension assay. An autoradiograph of the extension products for unedited (C) and edited (U) apoB RNAs is shown.

A Northern blot containing 2 μg of purified poly(A)⁺ RNA from each of 12 human tissues was hybridized with a radiolabeled cDNA of the carboxyl-terminal half of ASP. A transcript of ~2.0 kilobase was detected in the liver and kidney (Fig. 7). In both tissues, an additional transcript of ~8.0 kilobase was visible.

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ASP and KSRP were expressed in E. coli as fusion proteins with GST, purified to apparent homogeneity, and used for in vitro editing assays together with purified GST-APOBEC-1 (36). GST-APOBEC-1 alone did not exhibit in vitro editing activity, but reconstituted in vitro editing after supplementation with fraction E3 containing partially purified auxiliary components (Fig. 8, lanes 1 and 2). GST-KSRP alone or together with GST-APOBEC-1 did not exhibit apoB mRNA editing activity in vitro (Fig. 8, lanes 3 and 4). GST-ASP alone did not demonstrate apoB mRNA editing activity (Fig. 8, lane 5). However, GST-ASP together with GST-APOBEC-1 efficiently reconstituted apoB mRNA editing in vitro (Fig. 8, lane 6). After incubation with APOBEC-1 and ASP for 1 h, 8.3% of the apoB RNA was found to be edited (Fig. 8, lane 6).
DISCUSSION

Editing of the apoB mRNA is mediated by APOBEC-1 in conjunction with other proteins that are absolutely required for editing, but so far have not been conclusively characterized. In this study, we purified the APOBEC-1-stimulating factors from rat liver to homogeneity and identified two proteins with molecular masses of 75 and 65 kDa that could not be separated without reducing activity. Peptide sequencing identified the prominent 75-kDa protein as KSRP, an RNA-binding protein with four K homology RNA-binding domains that was originally described as a constituent of a protein complex assembled on an intronic splicing enhancer element of c-src in the brain (37). Native purified KSRP alone, however, did not confer APOBEC-1-stimulating activity. The 65-kDa protein is a hitherto unknown protein and was therefore designated APOBEC-1-stimulating protein. The full-length cDNA of ASP was isolated by polymerase chain reaction using information obtained from EST data base searching with ASP peptide sequences. Recombinant ASP stimulated recombinant APOBEC-1 to edit apoB RNA in vitro. Therefore, we conclude that ASP is the second essential component of the apoB mRNA-editing enzyme beside the catalytic subunit APOBEC-1 for editing activity in vitro. In rat liver, ASP is apparently associated with the RNA-binding protein KSRP, which binds to apoB RNA and may confer further stability to the editing enzyme complex with its substrate apoB RNA.

In rat liver nuclear extracts, the APOBEC-1-stimulating proteins are present in excess over APOBEC-1 and can be separated from the apoB mRNA editing holoenzyme by sucrose gradient ultracentrifugation. Purification of ASP on ssDNA-cellulose was made possible by the observation that the editing-stimulating activity bound to this matrix after digestion with micrococcal nuclease. The 0.3 M NaCl elution fraction from this column exhibited strong APOBEC-1-stimulating activity and contained only three major proteins. The APOBEC-1-stimulating activity eluted from gel filtration columns in a high molecular mass range. Only two proteins, KSRP and ASP, were detected in these fractions, and the more abundant KSRP cross-linked much more strongly to apoB RNA than did ASP. Gel filtration in 0.5 M NaCl separated these two proteins and resulted in a reduction of editing activity that shifted into a lower molecular mass range. Concomitantly, cross-linking of KSRP to apoB RNA was reduced after gel filtration in 0.5 M NaCl. This indicated binding of KSRP and ASP to apoB RNA in a cooperative fashion and a cooperative effect of both proteins on the stimulation of APOBEC-1. Furthermore, coexpression of APOBEC-1 and KSRP in yeast did not stimulate apoB mRNA editing. Native KSRP alone that had been separated from the 65-kDa protein did not complement APOBEC-1 to edit apoB RNA. The first hint that the 65-kDa protein might be the APOBEC-1-stimulating protein came from UV cross-linking activity in fractions 27–29 obtained by gel filtration in the presence of 0.5 M NaCl further indicated that the 65-kDa protein is the APOBEC-1-stimulating factor.

Peptide sequences of the 65-kDa protein did not match any known protein. We cloned the cDNA of this protein from both human liver and human small intestine. According to its function, we designated this protein APOBEC-1-stimulating protein. The liver-derived cDNA contained an in-frame insertion of 24 nucleotides that most probably results from alternate splicing. Otherwise, the cDNA sequence of ASP was identical in the liver and intestine and confirmed the sequences of the ESTs we had analyzed. The expression of ASP in the kidney that does not express apoB or APOBEC-1 suggests a more general role of ASP in mRNA processing and possibly editing and argues against a restricted function of ASP only in apoB mRNA editing. In 1998, a 65-kDa protein that induced APOBEC-1 to edit apoB RNA in vitro was purified from baboon kidney by apoB RNA affinity purification (34). ASP may be similar or even identical to this protein, although this remains to be proven.

ASP contains three RBDs in the amino-terminal half that are not identical. The observed homology of these RBDs to poly(A)-binding proteins is remarkable (47–50). Not only is the human intestinal apoB mRNA edited from C to U at nucleotide 6666, but approximately half of the transcripts are also cleaved and polyadenylated prematurely immediately downstream of C6666 (2). Therefore, it has long been assumed that apoB mRNA editing activates cryptic polyadenylation signals (2). The homozygosity of ASP to poly(A)-binding proteins could indicate a physical link of apoB mRNA editing with 3'-end formation. Several recent observations have led to the assumption that transcription, capping, splicing, and polyadenylation as well as RNA export may be coupled processes with intimate interrelations (52–54). APOBEC-1-mediated mRNA editing could well be another polishing within this “RNA factory” (54). Most poly(A)-binding proteins, however, are localized in the cytoplasm and regulate mRNA stability and translation (55–57). The nuclear localized poly(A)-binding protein II involved in 3'-end formation of mRNA differs from these proteins and ASP (58, 59). The copurification of KSRP and ASP suggests that ASP might be associated with KSRP in vivo, although this remains to be firmly established. KSRP has been described in a high molecular mass complex together with hnRNPs F and H and is thought to regulate the alternate splicing of c-src in the brain (37, 45). The four K homology RNA-binding domains confer the high affinity RNA binding of KSRP. The strong cross-linking of ASP to apoB RNA demands more detailed studies of the topology and sequence specificity of this binding.

According to the data presented, it is reasonable to assume that KSRP is another auxiliary protein for APOBEC-1, but not an essential component for editing in vitro.

Another remarkable feature of ASP appears to be the putative double-stranded RBD in the carboxyl-terminal half. The existence of both RBD and double-stranded RBD is not common for RNA-binding proteins. Native ASP did not bind very strongly to apoB RNA. This was apparently not dependent on the apoB RNA used in these assays since an apoB RNA of 430 nucleotides did not cross-link better than an apoB RNA of 55 nucleotides. The weak cross-linking of ASP may be explained by the presence of KSRP in our fractions. Two recent studies re-examined the secondary RNA structure requirements for efficient editing at C6666 (60, 61). Both studies concluded that C6666 is located at a junction between a double-stranded and a single-stranded RNA region. This junction was localized either at the loop of a hairpin (60) or at the beginning of a double-stranded stem formed by the mooring sequence (nucleotides 6671–6681) and a 3'-efficiency element (nucleotides 6718–6746) (61). A protein that specifically recognizes this junction between a double-stranded and a single-stranded region would fit these models. A/I editing, the second well described mode for editing of nuclear encoded transcripts besides the C/U editing mediated by APOBEC-1, specifically occurs in double-stranded RNA (62, 63). The responsible ADARs (adenosine deaminases acting on RNA) contain three double-stranded RNA-binding R motifs (64–66). An evolutionary link between cytidine deami-
nases and ADRAs is suggested by sequence homologies in the deaminase domain of these enzymes found even in bacteria, yeast, and Caenorhabditis elegans (67). Thus, it is very attractive to speculate that ASP may provide the recognition motif for double-stranded RNA in APOBEC-1 mediated C/U editing.

In summary, we have purified ASP, a novel APOBEC-1 stimulating protein, from rat liver and have cloned its cDNA. APOBEC-1 plus ASP represent the minimal apob mRNA editing enzyme *in vitro*. In rat liver, ASP appears to be associated with the mRNA-binding host KSRP. The cloning of ASP leads to many new questions regarding apob mRNA editing, some of which (such as the precise interaction of ASP with APOBEC-1, KSRP, and apob RNA) will be the focus of our next investigations.

**Acknowledgments**—The help of Dr. Franz Meyer-Ponser (Bruker-Daltonics), Henrik Molina Svendsen (Protana A/S, Odense, Denmark), and Allan Millar (Micros Ltd., Manchester, United Kingdom) in obtaining peptide sequence information on KSRP and ASP by MALDI-TOF/MS and ESI-TOF/MS is gratefully acknowledged. We thank Dr. Isabell Greeve for help in preparing the manuscript.

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Purification and Molecular Cloning of a Novel Essential Component of the Apolipoprotein B mRNA Editing Enzyme-Complex
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J. Biol. Chem. 2000, 275:19848-19856. doi: 10.1074/jbc.M001786200 originally published online April 25, 2000

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