Cyclic AMP and AKAP-mediated Targeting of Protein Kinase A Regulates Lactate Dehydrogenase Subunit A mRNA Stability*

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Expression of the lactate dehydrogenase A subunit (ldh-A) gene is controlled through transcriptional as well as post-transcriptional mechanisms. Both mechanisms involve activation of protein kinase A (PKA) into its subunits and subsequent phosphorylation and activation of several key regulatory factors. In rat C6 glioma cells, post-transcriptional gene regulation occurs through PKA-mediated stabilization of LDH-A mRNA and subsequent increase of intracellular LDH-A mRNA levels. Previous studies (Tian, D., Huang, D., Short, S., Short, M. L., and Jungmann, R. A. (1998) J. Biol. Chem. 273, 24861-24866) have demonstrated a cAMP-stabilizing region (CSR) located in the LDH-A 3′-untranslated region which, in combination with several phosphorylated CSR-binding proteins (CSR-BP), regulates the PKA-mediated stabilization of LDH-A mRNA. However, the mechanistic details of interaction of CSR with proteins as they pertain to mRNA stabilization by PKA are so far largely unknown. In this study we tested the hypothesis that ribosomal protein extracts (RSE) from glioma cells contain PKA regulatory (RII) and catalytic (C) subunits that, in combination with a protein kinase A anchoring protein (AKAP 95) and CSR-BPs participate in forming CSR-protein complexes that are responsible for mRNA stability regulation. To demonstrate the importance of CSR-protein complex formation, the PKA subunits and AKAP 95 were removed from the RSE by immunoprecipitation, and the antigen-deleted RSE were subjected to CSR binding analysis using gel mobility shift and UV cross-linking. It was shown that AKAP 95 as well as RII formed a direct linkage with CSR during CSR-protein complex formation. In contrast, the catalytic subunit formed part of the CSR-protein complex but did not bind to CSR directly in a covalent linkage. To determine whether formation of CSR complexes that included C, RII, and AKAP 95 constituted a functional event and was necessary for mRNA stabilization, cell-free decay reactions were carried out with RSE extracts, and the kinetics of decay of LDH-A mRNA was determined. Depletion of PKA subunits and AKAP 95 from RSE extracts by immunoprecipitation resulted in a marked loss of mRNA stabilization activity indicating that the presence of the PKA regulatory and catalytic subunits as well as AKAP 95 in the CSR-protein complexes was absolutely necessary to achieve LDH-A mRNA stabilization.

Lactate dehydrogenase (EC 1.1.1.27) is a tetrameric protein consisting of four subunits of two different types designated the A and B subunits. Genetic evidence shows that they originate from two separate genes located on different chromosomes and are regulated by different molecular mechanisms. The lactate dehydrogenase A subunit (LDH-A) gene is a delayed early serum responsive gene whose expression can be modulated at the transcriptional level by a number of effector agents (1–4) and the oncogene c-myc (5), which have no regulatory effects on LDH-B gene expression. The intracellular level of LDH-A mRNA, however, does not entirely reflect the effector agent-induced increased rate of gene transcription (1), implying that its steady-state level is concomitantly regulated by the ability of cells to modulate LDH-A mRNA stability. Indeed, the relatively short half-life LDH-A mRNA (t1/2 ≈ 55 min) increases markedly in response to activators of the PKA signal transduction pathway (1, 4, 6, 7). As a consequence, the cellular isoenzyme pattern is shifted in favor of LDH-5 altering the aerobic/anerobic metabolic status of cells. Under anaerobic conditions, cells may thus cover a potential energy deficit through an increased conversion of pyruvate to lactate by LDH-5.

A number of reports have demonstrated the existence of cis domains primarily in the 3′-UTR of a number of mRNAs as well as trans-acting regulatory factors that act in combination and are critical in mediating post-transcriptional control of gene expression (8). AU-rich regions and in particular multiple repeats of AUUA motifs located within the 3′-UTR of mRNA function as elements determining the stability/instability of mRNA of a number of genes (9, 10). Moreover, several examples of regulated mRNA stability in eukaryotic cells have been reported indicating that cis/trans-acting factors of the mRNA decay system can be subject to regulation through signal transduction pathways. For instance, LDH-A mRNA, whose stability is regulated via cAMP by PKA (1), contains a number of U-rich (48%) motifs clustered in a 3′-UTR region (nucleotides 1286–1549) of ~99 bases (2, 11). Mutational and deletion analysis have shown that at least three of the AU-rich sequence domains within this region are responsible for the relatively rapid rate of LDH-A mRNA turnover (11). Interestingly, whereas cAMP does not alter the destabilizing action of two of the three domains, one region consisting of bases 1472 through 1501 (gAMP-stabilizing region; CSR) is critically required for cAMP-mediated LDH-A mRNA stabilization (11). However, for the functional effect of CSR to manifest itself, binding of four proteins (CSR-binding proteins; CSR-BP) is required whose

* This work was supported by National Institutes of Health Grant GM53115. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Received for publication, March 7, 2005, and in revised form, May 4, 2005
Published, JBC Papers in Press, May 5, 2005, DOI 10.1074/jbc.M502514200

1 The abbreviations used are: LDH, lactate dehydrogenase; 3′-UTR, 3′-untranslated region; CSR, cAMP-stabilizing region; CSR-BP, cAMP-stabilizing region-binding protein; (S,S)-cAMPS, adenosine 3′,5′-cyclic monophosphorothioate; PKA, protein kinase A; RII, regulatory subunit II of protein kinase A; C, catalytic subunit of protein kinase A; AKAP, PKA-anchoring protein.
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CSR binding and mRNA stabilizing activities require their phosphorylative modification by PKA (12). The participation of PKA in the stabilizing events suggests that it may be targeted to specific substrates (such as CSR-BPs) through its interaction with cellular “anchoring proteins” (13–15).

The experiments described here were designed to identify the potential role of the PKA subunits in CSR-protein complex formation and in the control of LDH-A mRNA stability following PKA activation. Because the mechanism of PKA activation involves dissociation of the holoenzyme into the regulatory and catalytic subunits whose intracellular loci of action may be selected by PKA anchoring protein(s) (AKAP), we additionally investigated a putative interaction of AKAPs with CSR and its role in mRNA stabilization.

EXPERIMENTAL PROCEDURES

Materials—Nucleic acid-modifying enzymes, acrylamide, and nucleoside triphosphates were from Roche Molecular Biochemicals. Radiochemicals were purchased from PerkinElmer Life Sciences or ICN Radiochemicals. Proteinase K and other reagents were of molecular biology grade and purchased from Sigma. Adenosine 3′,5′-cyclic monophosphorothioate, S2,-isomer (S2-cAMP), was from Calbiochem. Antisera were obtained from Calbiochem or Santa Cruz Biotechnology. AKAP 95 was a rabbit polyclonal IgG prepared from an epitope corresponding to amino acids 542 mapping to an internal region of AKAP 95 of rat origin. Two polyacrylamide-purified synthetic RNA oligonucleotides (wild-type and mutated base sequence 1472–1501) were purchased from Dharmaco Research, Inc. Lafayette, CO. Ht-31 and Ht-31-P were obtained from Promega.

Previously Described Experimental Procedures—Cell culture methodology and preparation of protein extracts for the various RNA-protein binding assays were previously described (11). Similarly, RNA gel mobility shift assays and UV–RNA protein cross-linking assays were described before (11).

Cell Stimulation—Rat C6 glioma cells (American Type Culture Collection CCL 107) were grown to about 90% confluency. Serum was withdrawn for an 18-h period after which fetal calf serum (final concentration, 15%) was added. Four hours after the addition protein extracts were prepared.

Preparation of Purified RI Subunit—RII was purified from glioma cell cytosol by affinity chromatography on 8-(6-aminohexyl)amino-cAMP-Sepharose 4B as described (16, 17).

Synthesis of Labeled LDH-A 3′-UTR Transcripts—In the first labeling method, plasmid pH-5 containing the 510-nucleotide 3′-untranslated region of LDH-A mRNA (corresponding to nucleotides 1103–1610) (2) was constructed as previously described (11). A Nael/Bsml fragment containing the entire 3′-UTR region was used for the transcription of a wild-type 3′-UTR fragment containing the 5′-non coding region of LDH-A mRNA. This fragment was synthesized using Ambion MAXiscript in vitro transcription kit with biotin as label. To synthesize the LDH-A 3′-UTR fragment containing the mutated bases shown below, a correspondingly mutated Nael/Bsml fragment was used for transcription.

In the second method, a wild-type and mutated 74-base 3′-UTR fragment, consisting of LDH-A mRNA bases 1453–1527 (12), were synthesized with [α-32P]UTP using the Ambion MAXiscript transcription kit as described above. The labeled RNA probes were purified by denaturing polyacrylamide gel electrophoresis and used for UV/protein cross-linking analysis. The 74-base fragment contained the following sequence: 5′-CGAAGAUAAUGUUUAGUAUGUGCU-3′ (bases forming the CSR region are underlined), and mutated fragment: 5′-CUACAG-GGAUAUGUUuGACGAUAUGUGCU-3′ (mutated bases in small print). For gel shift analysis the oligonucleotides were synthesized, 5′-32P-end labeled, and tested for RNA binding activity. For affinity chromatography, the 30-base sequences were synthesized and biotinylated at the 5′ end with an 18-atom spacer arm. The biotinylated polynucleotides were coupled to magnetic beads with conjugated streptavidin (M-280 streptavidin-coupled Dynabeads).

Gel Mobility Shift Assays—Cell extracts (5–10 μg of protein) were incubated in RNA binding buffer (10 mM Hepes, pH 7.4, 40 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EDTA, 5% glycerol, 1 μg/mL of heparin, and 0.5 μg/mL of yeast tRNA) with 32P-labeled RNA probe (bases 1472–1501) (50,000 cpm) on ice for 1 h in a total volume of 25 μL. Electrophoresis was carried out on 6% non-denaturing polyacrylamide gels 1472–1501 (molar ratio of acrylamide/bisacrylamide, 90:1) in 45 mM Tris, 45 mM borate acid, and 1.2 mM EDTA buffer, pH 7.4, for 4 h at 4 °C. Gels were dried and exposed to Hyperfilm-MP (Amersham Biosciences) film for autoradiography or to a PhosphorImager plate (Fujifilm imaging plate, type BAS-IIU) for quantitation.

UV RNA/Protein Cross-Linking—RNA/protein binding reactions were carried out in 96-well tissue culture dishes. The binding conditions were identical as described for the gel mobility shift with the exception that the randomly 32P-labeled wild-type and mutated 74-nucleotide fragments (50,000 cpm) were used as probes. Samples were placed on ice and exposed to 600 mJ of ultraviolet light (5-cm distance for 15 min using a 254-nm ultraviolet lamp, model R-52G, UVG, Inc.). After irradiation, RNase A (0.2 μg/mL) and RNase T1 (40 units/μL) were added, and samples were incubated at 37 °C for 15 min. The irradiated samples were adjusted to 62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol and heated to 90°C for 2 min. An aliquot was subjected to electrophoresis on 8% SDS-polyacrylamide gel along with pre-stained protein molecular size markers (rainbow marker; U.S. Biochemical Corp.). The gels were fixed for 2 h in 50% methanol, followed by 2 h in 10% acetic acid, and 1 h in 5% methanol, 7% acetic acid after which the gels were dried. Gels were exposed to film for autoradiography or to an imaging plate for quantitation as described above.

Western Immunoblot Analysis—RSW extracts (20–30 μg of protein) were separated by SDS-PAGE and then transferred to polyvinylidine difluoride membranes. Blots were processed using the appropriate antisera as described in the text following the manufacturer’s protocol.

Assay of in Vitro mRNA Decay—mRNA decay reactions were performed in a total volume of 20 μL at 37 °C using 5.0 μg/μL of R5W protein in 10 mM Tris acetate, pH 7.8, 2 mM magnesium acetate, 1 mM potassium acetate, 2 mM dithiothreitol, and 0.2 mM dithiothreitol. Aliquots (25 μL) contained 1 μM [32P]cAMP (50 Ci/mmol), 90 μg of R5W protein or regulatory subunit RIIs in the absence and presence of 20 μM competitor nonradioactive cAMP. The mixture was placed on ice for 60 min in the dark. The incubation was followed by a 10-min irradiation (254 nm) at a distance of 8.5 cm with a UV lamp (UV Products R-52) rated at 1200 μW/cm2 at a distance of 15 cm. Samples were cooled on ice and unreacted 8-azido-[32P]cAMP was removed by centrifugal filtration on ultra free-MC 0.45-μm Amicon filter units (Millipore).

Assay for RII Binding Activity by the Overlay Procedure—The overlay binding assay was performed as described (16, 17). Western blots were probed with 32P-labeled RII using 5 × 105 cpm of 32P radioactivity/μL and RII-binding proteins were visualized by autoradiography.

Affinity Purification of CSR-binding Proteins—5′-Biotinylated 30-base wild-type and mutated CSR with an 18-atom spacer arm were linked to magnetic M-280 streptavidin-coupled Dynabeads according to the instructions given in the Dynal Technical Handbook (Dynal Biotech). To remove most of the nonspecifically binding proteins, RSW extracts were first incubated with beads carrying mutated CSR in Hepes buffer (150 mM KCl, 20 mM Hepes, pH 7.9, 0.05% Nonidet P-40, 0.2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) on a rotary shaker for 1 h at 20 °C. Beads were collected by centrifugation. The supernatant fraction was subsequently similarly incubated with beads carrying the wild-type CSR. To reduce nonspecific binding, 0.5 μg/0.25 μL of poly(A) were added to the incubation mixture. After incubation beads were washed three times with incubation buffer. CSR-binding proteins were subsequently dissociated from the bead-linked CSR with high-salt buffer (incubation buffer containing 0.4 M potassium acetate).

RESULTS

Identification of the Catalytic and Regulatory RII Subunit of PKA as Components of the CSR-Protein Binding Complex—We have recently identified a 30-nucleotide base CSR (nucleotides 1472 through 1501) within the 3′-UTR of LDH-A mRNA, which

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in combination with four as yet uncharacterized 96-, 67-, 52-, and 50-kDa CSR-BP is required to achieve LDH-A stabilization in response to PKA activation (11, 12). As part of this process, CSR together with CSR-BP forms multiple ribonucleoprotein complexes whose formation requires a reversible phosphorylation cycle involving PKA and as yet unknown protein phosphatases. The precise mechanism of participation of PKA in complex formation is unknown. One can speculate that based on a size comparison the regulatory subunit RII might be identical with either one or the other of the 52- or 50-kDa CSR-BPs. Thus, it is conceivable that the catalytic and RII subunits of PKA may also participate, in concert with other proteins, in the formation of functional CSR-multiple protein complexes that modulate mRNA stability.

First, to ascertain the presence of RII in glioma cell RSW extracts, we probed the extracts by photoaffinity labeling with 8-azido-[32]P)cAMP. The resulting data confirm the presence of regulatory subunit RII by photoaffinity labeling (Fig. 1, lanes 1 and 2). The specificity of RII photoaffinity labeling was demonstrated by the competition of radioactive label with nonradioactive cAMP (lane 3) and lack of [32]P labeling after depletion of RII from RSW by immunoprecipitation (lane 4). Immunoprecipitated RII extracted from protein A-agarose could successfully be photoaffinity-labeled demonstrating the efficiency of immunoprecipitation and presence of RII in the RSW extract (lane 5).

To investigate the presence of PKA subunits in CSR-multiple protein complexes and to identify a potential direct binding of the subunits RII and C to CSR, we carried out electrophoretic band-shift analysis and UV cross-linking assays as described under “Experimental Procedures.” Lane 1, extract from unstimulated cells; lane 2, extract from (S)_cAMP-stimulated cells; lane 3, extract from (S)_cAMP-stimulated cells with added 1 mM nonradioactive cAMP as competitor; lane 4, extract from (S)_cAMP-stimulated cells after immunodepletion of RII with anti-PKA RII antibody and subsequent photoaffinity labeling; lane 5, SDES-eluete obtained from protein A-agarose after immunoprecipitation of RII from extracts of (S)_cAMP-stimulated cells.

whereas the above studies allowed us to identify the presence of RSW proteins that exhibit binding affinity for CSR, it is necessary to ascertain the specificity of CSR-protein complex formation. In detailed previous studies (not shown here but see Ref. 12) that identified four CSR-BPs in RSW, we allowed complexes to form in the presence of a [32]P-labeled CSR probe and different unlabeled nonspecific competitor LDH mRNA fragments. Under these conditions, [32]P-CSR-protein complex formation was competitively eliminated by increasing molar concentrations of unlabeled CSR but not by nonspecific unlabeled RNA fragments indicating the specificity of complex formation of CSR to four RSW proteins.

To test whether the catalytic subunit was required for complex formation, we incubated the extracts from (S)_cAMP-treated cells with anti-PKA catalytic C antibody. Following removal of the immunoprecipitate with protein A-agarose, the subunit-depleted cell extract was examined for CSR-protein complex formation by gel mobility shift. Under these conditions, formation of the three CSR-protein complexes was markedly reduced (Fig. 2A, lane 4). Incubation of [32]P)CSR with a preincubated combination of highly purified C and RII subunits in the absence of RSW and (S)_cAMP-treated cells did not produce shifted band(s) (lane 5) indicating the lack of direct binding affinity of catalytically inactive PKA holoenzyme for the CSR. The need for active PKA subunit was convincingly confirmed by the fact that addition of C to the C-depleted RSW partially restored at least two of the faster moving CSR-protein complexes (Fig. 2A, lane 7). The slower moving complex (arrow 1) was not restored suggesting the need of an as yet unknown auxiliary component for complex formation to occur.

Furthermore, in several gel shift experiments we tested the specificity of the observed CSR-protein complex formations and immunodepletion experiments. To that effect we incubated [32]P)CSR with 7 units of C subunit in the absence of RSW (lane 6) and with preimmune serum alone (lane 8). In neither experiment could CSR-protein complexes be identified. In contrast,
incubation of [\textsuperscript{32}P]CSR with preimmune serum in the presence of RSW from unstimulated or (S\textsubscript{p})-cAMPS-treated cells allowed identification of the CSR-protein complexes as shown in lanes 2 and 3. These experiments clearly show the specificity of the immunodepletion reactions and identify a selectivity of CSR-protein complex formation.

A similar pattern of CSR-protein complex formation was obtained when the binding characteristics of the regulatory subunit RI\textalpha were examined by gel shift analysis. The typical band shift patterns seen in Fig. 2A were also observed with RSW from untreated and (S\textsubscript{p})-cAMPS-treated cells (Fig. 2B, lanes 2 and 3). After treatment of RSW extract from (S\textsubscript{p})-cAMPS-stimulated cells with anti-RI\textalpha, antisera and protein A-agarose complex formation was markedly reduced (lane 4) indicating that RII was required for complex formation. Addition of 0.5 \mu g of purified RI\textalpha to the RI\textalpha-depleted RSW restored two of the faster migrating complexes (lane 5, arrows 2 and 3) but not the slowest moving one (arrow 1) similar to the restored banding pattern in Fig. 2A (lane 7) suggesting that this complex contains a component that is required for its formation with RII and C but is removed by immunoprecipitation and cannot be restored by addition of either subunit to the immunodepleted RSW. Incubation of RII with CSR in the absence of RSW did not result in complex formation (lane 6) showing that the regulatory subunit RII itself exhibits no direct binding affinity for CSR as analyzed by gel shift. It is, nevertheless, part of and required for CSR-protein complex formation conceivably mediated through interaction with protein(s) that bind directly to CSR. As we discuss below, a PKA anchoring protein required for complexing RII (and C) to CSR might be removed from the extract by immunoprecipitation together with the subunits.

Our failure to identify by gel shift a direct binding of the subunits to CSR raises the question whether the use of different methodology, e.g., UV cross-linking, which results in a covalent linkage might be successfully applied to demonstrate direct linkage of the subunits to CSR. Covalently linked RNA-protein complexes can be formed by irradiation of cell extracts with UV light (19). Using competition assays, we previously demonstrated that the technique can be applied with great specificity to the study of CSR/protein interaction (12). To determine whether either the RII and/or C subunit could be linked to the CSR base sequence, we performed a series of experiments similar to the ones previously described (12) applying UV irradiation of [\textsuperscript{32}P]-labeled CSR in the presence of RSW protein from untreated and (S\textsubscript{p})-cAMPS-treated glioma cells and to test the functional effects of subunit deletion. Accordingly, uniformly [\textsuperscript{32}P]-labeled CSR was incubated with RSW, irradiated, separated by denaturing PAGE, and subjected to autoradiography. Additionally, RSW extracts were used from which RII and C had been deleted by immunoprecipitation as described in the legend to Fig. 2. Representative experiments analyzing the UV-catalyzed binding characteristics of the catalytic subunit are shown in Fig. 3A. Three proteins of apparent molecular masses of 96, 52, and 50 kDa were detected in RSW from control (lane 1) and (S\textsubscript{p})-cAMPS-treated glioma cells (lane 2) as previously shown (12). Treatment of the RSW extract from (S\textsubscript{p})-cAMPS-treated glioma cells with anti-C\textalpha antiserum and protein A-agarose did not qualitatively affect the [\textsuperscript{32}P]-CSR/protein cross-linking patterns (Fig. 3A, lanes 1–3) but decreased the density of the 50-kDa band suggesting that only the 50-kDa band shown in lane 3 represented the covalently linked C subunit. We were unable to demonstrate UV-mediated linkage of the C subunit to [\textsuperscript{32}P]CSR in the absence of RSW extract (lane 4). Thus, we conclude that the catalytic subunit can ligate directly to the base sequence of CSR when present in the CSR-protein complex.

We next tested the ability of the regulatory subunit RI\textalpha to form a direct covalent CSR linkage. UV cross-linking of [\textsuperscript{32}P]CSR with RSW proteins resulted in the banding patterns shown in Fig. 3B. The pattern of CSR-protein complexes (see Fig. 3B, lanes 1 and 2) identify three autoradiographic bands of 96, 52, and 50 kDa as previously reported (12). Incubation of the RSW from control and (S\textsubscript{p})-cAMPS-stimulated extracts with anti-RI\textalpha antiserum resulted in the deletion of the 52-kDa band (Fig. 3B, lanes 3 and 4) indicating a direct binding of RII to CSR. Addition of RII to the RI\textalpha-depleted extract before UV linking resulted in the restoration of the original banding pattern (lane 5). UV cross-linking yielded negative results when mutated CSR was used as probe (lane 6). We conclude from these results that the previously identified 52-kDa CSR-BP (12) is identical with the regulatory subunit RII.

**AKAP 95 Participates in CSR-Protein Complex Formation.—**To obtain specific functionality of PKA subunits at their sites of action, the subunits must be compartmentalized in close proximity to their targets. It has been shown that this can be achieved through interaction of PKA substrates with a class of proteins termed “protein kinase A-anchoring proteins” (13–15). Thus, it is conceivable that attachment of type II PKA to CSR occurs through mediation of an as yet unknown AKAP(s). To determine whether PKA anchoring is involved in regulating mRNA stability and whether one or more of the previously (12) identified four CSR-BPs might be an anchoring protein, we proceeded to identify protein(s) with AKAP activity in glioma cell RSW. To do this, we first partially purified previously identified CSR-BPs (12) and then tested their potential to form ligands with [\textsuperscript{32}P]-labeled RII. Using affinity chromatography with magnetic Dynabead-streptavidin in combination with biotinylated CSR, we recovered four CSR-BPs of 96, 67, 52, and...
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FIG. 4. Detection of AKAP 95 in RSW fractions from rat C6 glioma cells. A, affinity purification of CSR-binding proteins was carried out as described under "Experimental Procedures." The RSW extract was passed through a column linked to bases 1471 through 1507 of LDH-A mRNA. Fractions were assayed for CSR-binding proteins by UV cross-linking as described in legend of Fig. 3. Lane 1, extract from unstimulated cells; lane 2, extract from (S_p)-cAMPS-stimulated cells eluted with 0.4 mM potassium acetate; lane 3, eluted with 0.5 mM potassium acetate; lane 4, extract from (S_p)-cAMPS-stimulated cells eluted with 0.5 mM potassium acetate and incubated with anti-AKAP 95 antiserum. The immunoprecipitate was removed with protein A-agarose. B, protein eluted with 0.4 mM potassium acetate was dialyzed, separated by 10% denaturing PAGE, and transferred to polyvinylidene difluoride membrane. Blots were subjected to analysis of RII binding by overlay ligand blot of RSW from control and (S_p)-cAMPS-stimulated cells; lanes 2 and 3, lanes 4 and 5, extract from (S_p)-cAMPS-stimulated cells with Ht31-P (1 μM). C, protein eluted with 0.4 mM potassium acetate was dialyzed, separated by 10% denaturing PAGE, and transferred to polyvinylidene difluoride membrane. Blots were subjected to Western blot analysis using specific antibody to AKAP 95. Lane 1, extract from (S_p)-cAMPS-stimulated cells; lane 2, extract from unstimulated cells.

50 kDa size (Fig. 4A, lanes 2 and 3). Using a commercial antiserum preparation against the anchoring protein AKAP 95, we first tested the possibility whether the 96-kDa protein is identical with a previously identified AKAP 95 of nuclear origin (20, 21). Incubation of the 0.5 mM potassium column eluate with anti-AKAP 95 antiserum and subsequent treatment with protein A-agarose resulted in a protein that migrated on standard SDS-PAGE of the properties of an AKAP class protein. Using a commercial antiserum to AKAP 95, the blot demonstrated a band of 95−96 kDa (see Fig. 4C) identical to the band identified by ligand blotting suggesting that both are identical. An anchoring protein of similar molecular size (AKAP 95) has previously been identified that possesses affinity for the nuclear matrix (13, 14, 20).

To determine whether the 96-kDa protein binds to RII in a manner specific to AKAPs, the RII overlays were performed in the presence of the AKAP inhibitor peptide Ht31 or the control peptide Ht31-P, which lacks inhibitory activity. Peptide Ht31 acted as an inhibitor of AKAP-RII binding (Fig. 4B, lane 3), whereas Ht31-P, the control peptide, was unable to prevent binding of RII to the 96-kDa protein in an AKAP-specific manner (lanes 4 and 5) (22–24).

Additionally, we investigated whether AKAP is part of the RNA-protein complex(s) formed when RSW is incubated with CSR. Gel shift analysis was carried out with RSW from which AKAP had been removed by immunoprecipitation with a specific antibody to AKAP 95. The results are shown in Fig. 5. Lanes 1 and 2 represent the typical band shift patterns (two and three bands, respectively) of RSW extracts prepared from unstimulated or (S_p)-cAMPS-stimulated cells as seen above in Figs. 2 and 3. The band shifts in lanes 3 and 4 were obtained from the extracts of unstimulated and stimulated cells, respectively, after incubation with anti-AKAP 95 antiserum. The upper and lower complexes (seen in lane 2) were completely eliminated and the density of the middle band was markedly reduced.

The Patterns of Decay and Regulation of LDH-A mRNA Stability in Cell-free Systems Correlate with the Patterns Observed in Intact Cells—To determine whether the process of complex formation of CSR with PKA subunits and AKAP 95 is functionally involved in the mechanism of LDH-A mRNA stabilization, we used a cell-free system to study the kinetics of decay of LDH-A mRNA fragments that contained the CSR regulatory domain. Cell-free decay systems, provided they mimic the regulation observed in intact cells, are suitable to study the function of trans-regulatory factors. To assess whether the cell-free system is functioning properly in a physiologic proper fashion, it is helpful to determine the decay rates of control mRNAs that are known to have distinct half-lives in intact cells. Messenger RNAs that are unstable in intact cells should also exhibit instability in cell-free systems. Similarly, stable mRNAs should exhibit stability in cell-free systems as well as in intact cells. Furthermore, and most importantly, regulatory events observed in intact cells should be mimicked in cell-free systems if they are to be useful for mechanistic studies.

Several laboratories have successfully developed and used cell-free decay systems (25–28). Accordingly, we have prepared RSW fractions from unstimulated and (S_p)-cAMPS-treated C6 glioma cells and used them as decay systems and source of PKA subunits and AKAP 95. β-Globin mRNA as well as wild-type LDH-A mRNA and LDH-A mRNA with a deleted CSR were incubated in RSW extracts from unstimulated and stimulated C6 glioma cells for increasing time intervals after which the rate of RNA decay was analyzed after Northern blotting. As expected, wild-type LDH-A mRNA decayed rapidly with a half-life of t_1/2 = 16 min in RSW from unstimulated glioma cells, whereas the stability of wild-type mRNA decaying in RSW from (S_p)-cAMPS-stimulated cells decreased ~4.5 times to a half-life of to 70 min (Fig. 6A). In contrast, β-globin mRNA was remarkably stable in the cell-free decay system (Fig. 6A).
similar to its known stability in intact cells (29). To expand and confirm these data, we carried out a stability analysis of LDH-A mRNA from which the 22-base CSR (fragment 1478–1499), a critical determinant for mRNA stability and regulation (11), had been deleted. The decay blots shown in Fig. 6A demonstrate that LDH-A mRNA from which the CSR had been deleted is remarkably more stable than wild-type mRNA, and importantly, its stability is not regulated by (S\text{p})-cAMPS.

PKA Subunits and AKAP95 Are Involved in the Regulation of LDH-A mRNA Stability—Considered together, the above findings point to a pivotal regulatory role for PKA and AKAP in the mechanism of LDH-A mRNA stabilization and suggest that PKA subunits together with AKAP can be part of an RNA-binding protein complex that controls mRNA stability. Hence, we sought additional evidence that subunits are involved in the regulation of LDH-A mRNA stability. To determine whether the PKA subunits and AKAP 95 are involved in mRNA stabilization, we incubated anti-C, anti-RII, and anti-AKAP 95 antibody with the RSW extracts from control and (S\text{p})-cAMPS-treated glioma cells. A biotin-labeled LDH-A mRNA fragment (bases 1103–1610) was added to the subunit-depleted RSW extracts and decay reactions were carried out. As shown in Table I and illustrated in Fig. 6, the half-life of mRNA incubated with extracts from (S\text{p})-cAMPS-treated cells exhibited much greater stability than mRNA incubated with extracts from control cells. However, immunodepletion of both subunits as well as AKAP 95 from the RSW extracts resulted in each case in a marked reversal of the stabilizing effect indicating an absolute requirement for AKAP 95 and the C and RII subunits in the mRNA decay mechanism.

**DISCUSSION**

We have continued our investigation into the mechanisms that are responsible for the regulation of LDH-A mRNA stability by the PKA signal transduction pathway. In our past work we have identified a cis-regulatory site in the 3'-UTR of LDH-A acting as a CSR (11) and which, in combination with several CSR-BPs (12), is responsible for the stabilization of LDH-A mRNA. The CSR-BPs of 96, 67, 52, and 50 kDa link directly to the CSR base sequence with a high degree of structure specificity (12). Importantly, CSR binding activity of the four proteins was up-regulated after their PKA-mediated phosphorylation, whereas their dephosphorylation caused a loss of CSR binding activity suggesting a functional cause and effect relationship between CSR-BP complex formation and mRNA stabilization. The necessity for a phosphorylative modification of the CSR-BPs to acquire functionality also implies a key role for the PKA subunits and AKAP 95 in CSR complex formation. In view of these overall findings, the question arose concerning the functional relationship between CSR-BPs action on the one hand and the role of AKAP 95 and PKA subunits in mRNA stabilization on the other hand. AKAP 95, RII, the 96- and 52-kDa CSR-BPs bind CSR directly with high specificity in a
sequence-dependent manner, possess similar immunological properties, and require phosphorylative modification for their mRNA binding and stabilizing activities to manifest themselves. Thus, the high probability of identity of RII and AKAP 95 with the 52- and 96-kDa, respectively, CSR-BP suggests that together with the C subunit a concomitant association of these proteins with an RNA loop structure-positioned CSR (12) will lead to a CSR-protein complex that structurally and functionally allows modification of mRNA stability. This hypothesis is supported by the fact that CSR with a mutated base sequence is unable to undergo complex formation (12). We also demonstrated for the first time a direct linkage of the RII subunit and AKAP 95 with the CSR base sequence. Disruption of anchoring of AKAP 95 to CSR as well as preventing the RII and C subunits from complex formation with CSR will lead to a decrease of LDH-A mRNA stability, lower intracellular LDH-A mRNA levels and, hence, reduced expression of LDH-A subunit containing isoenzymes.

A key finding is the identification of AKAP 95 in CSR-BP complexes and its dependence upon PKA-mediated phosphorylation to achieve direct linkage to CSR. A multitude of AKAPs have been identified over recent years. They represent a functionally related class of proteins that specifically act to anchor and localize PKA to specific subcellular structures (13, 14). In the brain both RII isoforms are localized through binding with specific anchoring proteins. AKAP 95 contains both RII and DNA-binding domains and binds DNA in a sequence-independent manner primarily in nuclear matrix fractions derived from rat pituitary GH4 and NIH-3T3 cells. Our present data clearly show the target-specific action and the ability of glioma cell AKAP 95 to act as an RNA-anchoring protein in its phosphorylated state. It has been shown that most AKAPs are targets for PKA suggesting that their biological activity may be modulated through phosphorylative modification.

Ranganathan and co-workers (22) recently reported that translational regulation of lipoprotein lipase in adipocytes by the β-adrenergic pathway involved formation of an RNA binding complex that included the catalytic subunit of PKA and the PKA anchoring protein 121/149 but not the regulatory subunit RII (22). Whereas the AKAP 121/149 was identified as an RNA-binding protein, we were unable to find any structure similarities of the lipoprotein lipase 3'-UTR with other regulatory RNA domains that respond to cAMP and PKA stimulation.

One restriction in the interpretation of our experimental data is the sparseness of information about the molecular nature of CSR-protein complex formation that would allow us to predict more precisely the structural and functional consequences of their interaction. There is a great variety of different cis-acting mRNA recognition sequences and corresponding trans-acting proteins, but a computer-aided search for a consensus CSR sequence has so far failed. In those instances in which mRNA stability is regulated by PKA (1, 6, 30–35), the binding activity of several proteins to AU-rich regions is up-regulated by cAMP (31, 35–36). However, no uniform mechanism of cAMP action and common feature at the mRNA level has emerged other than the potential necessity for a phosphorylative modification of trans-proteins to achieve RNA binding.

TABLE I

| Subunit analyzed | Treatment | Half-life (t1/2) |
|-----------------|-----------|----------------|
| Catalytic subunit | Control | 16 ± 2.1 |
| (S-p)-cAMPS | 55 ± 4.2 |
| (S-p)-cAMPS C-depleted | 18 ± 1.9 |
| (S-p)-cAMPS C-depleted + Sigma catalytic subunit | 48 ± 4.3 |
| Regulatory subunit RII | Control | 15 ± 1.9 |
| (S-p)-cAMPS | 60 ± 5.1 |
| (S-p)-cAMPS RII-depleted | 20 ± 1.6 |
| (S-p)-cAMPS RII-depleted + purified RII subunit | 51 ± 6.7 |
| AKAP 95 | Control | 19 ± 2.5 |
| (S-p)-cAMPS | 65 ± 6.7 |
| (S-p)-cAMPS AKAP 95-depleted | 15 ± 1.3 |
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J. Biol. Chem. 2005, 280:25170-25177.
doi: 10.1074/jbc.M502514200 originally published online May 5, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M502514200

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