Identification, Purification, and Characterization of Subunits of cAMP-dependent Protein Kinase in Human Testis

REVERSE MOBILITIES OF HUMAN RII, AND RII, ON SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS COMPARED WITH RAT AND BOVINE RII*

Bjenn S. Skålhegg††, Brynjar Landmark‡, Kari B. Foss‡‡, Suzanne M. Lohmann***, Vidar Hansson††, Tor Lea#, and Tore Jahnsen‡‡

From the †Institute of Pathology, Rikshospitalet, N-0027 Oslo 1, Norway, the ‡Institute of Immunology and Rheumatology, Rikshospitalet, N-0027 Oslo 1, Norway, the §Institute of Medical Biochemistry, University of Oslo, P.O. Box 1112 Blindern, N-0317 Oslo 3, Norway, and the ***Labor für Klinische Biochemie, Medizinische Universität Klinik, Josef-Schneider Str. 2, 8700 Würzburg, Germany

The Journal of Biological Chemistry
© 1992 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 267, No. 8, Issue of March 15, pp. 5374-5379, 1992
Printed in U.S.A.


Our results show that human testis contains mRNAs for five out of the seven known subunits of cAMP-dependent protein kinase. We observed strong expression of mRNAs for RII, (1.5 and 3.2 kilobases (kb)), RII, (2.2, 2.4, and 7.0 kb), and RII, (3.3 kb). We also demonstrated mRNAs for two of the three catalytic subunits, C, (2.7 kb) and C, (1.7 kb). Purification of R subunits by DEAE-cellulose and cAMP affinity chromatography revealed three distinct forms with apparent molecular masses of 49, 51, and 53 kDa, respectively. Characterization of these R subunits by their 8-azido-cAMP photoaffinity labeling and immunoreactivity, as well as by a phosphorylation-dependent mobility shift on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), indicated subunit sizes of RII, (53 kDa) > RII, dephosphoform (51 kDa) > RII, (49 kDa). This conclusion was verified by the analysis of RII subunits produced by in vitro transcription/translation of full-length cDNAs for both human RII, and RII, in wheat germ lysates. The in vitro translated products were the same size as the purified human testis subunits, and only the smallest RII subunit (RIIL) revealed a distinct mobility shift on SDS-PAGE after phosphorylation/dephosphorylation. This study supports the conclusion that the mobilities of human RII subunits (RIIL, RIIL) on SDS-PAGE are reversed in contrast with those of other species such as rat and bovine. This has to be taken into consideration when examining R subunits in other human tissues by methods such as photoaffinity labeling which cannot distinguish subtype identities.

Regulatory effects of cAMP in mammalian cells are mediated via activation of cAMP-dependent protein kinase (1). In the testis, cAMP/cAMP-dependent protein kinase mediate the effects of the gonadotropins on testicular somatic cells and also influence sperm motility (2-6). The cAMP-dependent protein kinase holoenzyme consists of a regulatory (R) subunit dimer and two catalytic (C) subunits which are dissociated by the binding of four molecules of cAMP, two to each R subunit. Binding of cAMP to R releases the active C subunit responsible for subsequent phosphorylation of key substrates (7, 8). Type I and type II cAMP-dependent protein kinase have been described and are distinguished by the salt concentration at which they elute from DEAE-cellulose columns (9, 10).

Molecular cloning has demonstrated isoforms of both R and C subunits. At present four different forms of R subunits (RIIL, RIIL, RIIL, RIIL) (11-21) and three different C subunits (C, C, C) (22-24) have been identified at the mRNA/gene level. RIIL, RIIL, and C, represent the ubiquitous mRNA forms found in most tissues, whereas the mRNA for RIIL seems to be cell- and tissue-specific and is hormonally regulated in ovarian granulosa cells and testicular Sertoli and Leydig cells (25, 26). RIIL mRNA has so far been detected in mouse brain and testis (13), rat brain and testis (27), and in several human tissues, with the highest expression in the brain (28). The highest levels of C, mRNA have been observed in rat and mouse brain and in the human prostate, intestine, brain, and

1 The abbreviations used are: RIIL, RIIL, and RIIL isoforms of the regulatory subunits of cAMP-dependent protein kinase; C, C, and C isoforms of the catalytic subunit of cAMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; cAK and cAKII, cAMP-dependent protein kinase type I and II; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

Received for publication, April 30, 1991, and in revised form, December 2, 1991.
testis (23, 25), whereas the message for C, has only been detected in human testis (24). In the species so far examined R subunit sizes follow the order RIIa, 54–56 kDa (14) > RIIb, 51–52 kDa (17–19, 29) > RIα, 49 kDa (11). Both RIIα and RIIβ are autophosphorylated (30), but only RIIα shows an auto-phosphorylation-dependent mobility shift as on SDS-PAGE (30). The RIα protein has been detected in mouse brain with an apparent molecular mass of 55 kDa (13), and C, has an apparent molecular mass of 40 kDa (31), whereas Cβ and Cγ have so far not been demonstrated at the protein level.

In rat testis, CAMP-dependent protein kinase subunits for RIα, RIIα, RIIβ, Cβ, and Cγ have been identified both at the mRNA and protein level, whereas the RIβ and Cß subunits have only been described at the mRNA level.

In our laboratory, human cDNAs for RIα, RIIα, RIIβ, Cß, Cγ, and Cγ have been cloned (12, 16, 21, 24). In the present study we have used Northern blot analysis to examine mRNAs for the various CAMP-dependent protein kinase subunits in human testis. We have further purified and characterized the R subunits of CAMP-dependent protein kinase from human testis and demonstrate that the largest RII subunit is RIβα (63 kDa) followed by the dephosphorylated form of RIβ (51 kDa) and RIβ (49 kDa). We have verified this by showing that RIβ and RIβ produced by in vitro transcription/translation behave identically to the native RII subunits purified from human testis. We also demonstrated that the mobility shift produced by phosphorylation/dephosphorylation is a more reliable characteristic for identification of RII, than is apparent size determination by SDS-PAGE.

MATERIALS AND METHODS AND RESULTS

In the present study we have purified and characterized the regulatory subunits of CAMP-dependent protein kinase from human testis. We have further shown that mRNAs for five out of seven known R and C subunits are expressed in the testis of elderly men. Strong signals for RIα, RIIα, RIßα, Cβ, and for the newly cloned Cγ, were seen. We observed small molecular weight messages for RIα and RIIα, which have been shown to be germ cell-specific in rats (25). In the rat, RIα mRNA has also been primarily found in germ cells (25). However, the fact that this subunit was nearly undetectable in our human RNA preparations may indicate that the spermatogenesis in elderly men is reduced to such an extent that the germ cell specifically containing this mRNA is lacking. Despite the fact that Cγ mRNA has been demonstrated in human testis of a 14-year-old boy (25), we could not find significant levels of this message in our RNA preparations.

Purification of R subunits by DEAE-cellulose and CAMP affinity chromatography revealed three distinct bands with apparent molecular masses of 49, 51, and 53 kDa, respectively. The three protein bands were shown to be CAMP-binding proteins by photoaffinity labeling using 8-azido-[γ-32P]cAMP. The smallest protein (49 kDa) was identical in size and immunoreactivity to rat RIα, and was considered to represent the human testis RIα subunit. Further support for this was the fact that this subunit could not be autophosphorylated in the presence of γ-[32P]ATP and catalytic subunit (results not shown).

The smallest  protein (49 kDa) was identical in size and phosphorylation-dependent mobility shift  as on SDS-PAGE. Although we have not been able to detect any 55-kDa proteins either by photoaffinity labeling or Western analysis before or after R subunit phosphorylation, these results indicate that the RIα protein is not present in the testis of elderly men.

In rats and in other species showing the phosphorylation-dependent mobility shift on SDS-PAGE, the larger protein band is 55 kDa. In several species a clear cut phosphorylation-dependent mobility shift has been observed for RIα but not for RIβ. Whereas rat and bovine RIα has a greater molecular weight than RIβ, the purified human testis RIα subunit which showed a mobility shift in response to autophosphorylation was the smaller (51 kDa) subunit. Thus, both immunoreactivity as well as phosphorylation-dependent change in apparent size by SDS-PAGE clearly indicated that the smaller (51 kDa) human testis RIβ subunit was RIα, whereas the larger (53 kDa) subunit was RIβ. Further studies and in light of the fact that the skeletal muscle RIβ antibody used (40) in their studies specifically recognizes the RIβ subunit, it is possible that changes in protein levels attributed to RIβ were instead changes in RIβ. An alternative possibility is that the isomeric RIβ subunits of CAMP-dependent protein kinase in human display tissue- as well as cell-specific differences in size. However, results from our laboratory indicate that normal human peripheral blood T lymphocytes express an RIβ subunit of 51 kDa as well.

In addition to these findings, previous results from Øyen et al. (16) demonstrate that human RIα diverge markedly in the N-terminal end (amino acids 45–87) compared with species such as rat, bovine, mouse, and porcine, whereas the RIβ sequence is more conserved. Taken together, these data strongly suggest that the human RIα may display a different apparent molecular weight compared with RIβ in other species and may be smaller than RIβ.

In a final attempt to prove this conclusion, we performed in vitro transcription/translation of full-length cDNAs for RIα, RIβ. We also determined on SDS-PAGE the extent to which the in vitro translated proteins demonstrated a mobility shift upon phosphorylation/dephosphorylation. In

2 K. Taskén, S. M. Lohmann, B. Landmark, O. Øyen, B. Skåløegg, and T. Jahnsen, manuscript in preparation.
3 Portions of this paper (including "Materials and Methods," "Results," Figs. 1–5, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

these studies we clearly found that the in vitro transcribed/translated subunits possess properties identical to the purified human testis RII subunits, both with respect to size and mobility shift upon phosphorylation/dephosphorylation. RII, (dephosphorylated) had an apparent molecular mass of 51 kDa, whereas RII, repeatedly was determined to 63 kDa. After in vitro phosphorylation, the RII, subunits increased its molecular mass to about 54 kDa, whereas the in vitro translated RII, showed no apparent change in size. In support of previous data from several species, our results demonstrate that the autophosphorylation-dependent mobility shift is a characteristic feature of the RII, subunit and a useful criterion for distinguishing RII, from RII, it further suggests that the mobility shift due to autophosphorylation is a more characteristic property of the RII, subunit than is its apparent size on SDS gels. Our results indicate that some of the previous studies assuming that the 51-kDa RII subunit found in human cells represents the RII, subunit need to be reevaluated.

Acknowledgment — The antiserum against rat liver RL was a gift from Dr. Stein O. Døskeland, Institute of Anatomy, University of Bergen, N-5009 Bergen, Norway.

REFERENCES


5376 cAMP-dependent Protein Kinase in Human Testis
CAMP-dependent Protein Kinase in Human Testis

Preparation of membrane extracts

Membrane extracts were prepared from testes obtained from 10 to 12 weeks of gestation, and from testes removed from patients in the postnatal period. The testes were homogenized in 0.8 M potassium phosphate buffer, pH 7.4, containing 0.1 M sodium chloride and 1.0 mM EDTA. The crude homogenates were centrifuged for 10 minutes at 100,000 g. The supernatants were then collected and dialyzed against 0.05 M Tris-Cl (pH 7.4) containing 0.1 M sodium chloride and 1.0 mM EDTA. The dialyzed supernatants were centrifuged at 100,000 g for 1 hour. The resulting supernatants were used for further experiments.

Preparation of radiolabeled substrates

For the preparation of radiolabeled substrates, the following procedures were used: 1. Tissue extracts were incubated with [32P]glycogen (50,000 cpm/pg) in incubation buffer containing 0.8 M potassium phosphate buffer, pH 7.4, 0.1 M sodium chloride, 1.0 mM EDTA, and 1.0 mM magnesium chloride. After incubation for 10 minutes, the reaction was terminated by adding SDS sample buffer, and the samples were analyzed by SDS-PAGE electrophoresis.

Detection of CAMP-dependent protein kinase activity

CAMP-dependent protein kinase activity was measured by the incorporation of [32P]ADP into histone H1 in the presence of 8-bromo-cAMP and 32P-ADP. The reaction mixture contained 50 mM Tris-Cl (pH 7.4), 0.1 M sodium chloride, 0.5 mM magnesium chloride, 0.1 mM calcium chloride, 2 mM DTT, and 0.01% Tween 80. The reaction was initiated by the addition of histone H1 (50 ng) and terminated by the addition of SDS sample buffer. The samples were subjected to SDS-PAGE electrophoresis, and the gels were dried and exposed to film for autoradiography.

Results

The specific activity of CAMP-dependent protein kinase in human testis was found to be 0.7 units/mg protein, as determined by the incorporation of [32P]ADP into histone H1 in the presence of 8-bromo-cAMP and 32P-ADP. The activity was stimulated by the addition of 8-bromo-cAMP, with a half-maximal stimulation observed at 1 mM 8-bromo-cAMP.

Discussion

The results presented in this study indicate that CAMP-dependent protein kinase activity is present in human testis and is stimulated by 8-bromo-cAMP. These findings support the hypothesis that CAMP-dependent protein kinase plays a role in the regulation of testicular function, possibly through modulation of protein phosphorylation and thereby influencing the expression of specific genes.

Acknowledgments

This work was supported by grants from the National Institutes of Health (GM-30122 and GM-30229). We thank Dr. John M. Meyer for providing the histone H1 used as substrate, and Dr. Susan L. Hepler for helpful discussions.
Identification of mRNAs for different subunits in human testis.

To identify the mRNA expression of R and C subunits of cAK in human testis we probed Northern filters with cDNA's for all known subunits of cAK, RIα, RIIα, RIIβ, Cα, Cγ and Cδ. As shown in figure 1, mRNAs for the subunits RIα, RIIα, RIIβ, Cα and Cγ were readily recognized by their corresponding cDNA probes, indicating that 5 out of 7 known genes for cAK subunits are expressed in the human testis. In some experiments very low levels of the subunits RIIβ (0.6 kb) and Cγ (0.8 kb) were also detected (data not shown). RIα expressed two distinct mRNAs of 1.5 and 3.2 kb, the latter being the ubiquitous form found in somatic cells, whereas the 1.5 kb mRNA is primarily expressed in germ-cells (13). In the case of RIγ, three distinct mRNAs were observed (2.2, 2.4, 7.0 kb), the largest mRNA of which represents the form found in most tissues, whereas the 2.2 and 2.4 kb mRNAs are germ cell specific (16,25). The RIIα and Cγ genes both expressed one mRNA of 3.3 and 3.7 kb, respectively. Cα cDNA detected two distinct mRNAs of 1.7 and 2.7 kb. Only the smaller mRNA is considered to be the specific Cα message, whereas the 2.7 kb mRNA represents cross hybridization with Cγ mRNA.

Identification of proteins for cAK R subunits in human testis.

To identify human testis cAK R subunits at the protein level, cell extracts were examined for cAMP-binding proteins, specifically incorporating the cAMP analog 8-azido-3H-cAMP. Four major proteins with apparent molecular weights ranging from 37 to 54 kDa were specifically labeled by the analog (Figure 2). The 37 kDa protein binding 8-azido-cAMP represents a degradation product of the RII subunits (38). To further classify these proteins as human R subunits we compared them with cAK R subunits in rat testis cell extracts and purified rat brain RIα (form 1) and RIIα (forms 1 and 2) forms, for their comigration with anestra raised against rat liver RIα, rat liver RIIα and rat brain RIα. This identified the immunoreactive R subunits in human testis with molecular weights between 49 and 54 kDa (figure 3).

Figure 4: Purification of human testis cAK R subunits. Fifty g of human testis was homogenized and the cell extract fractionated by DEAE-cellulose chromatography and ion-exchange step-wise with 100 mM and 400 mM NaCl. The proteins eluted were separately purified by cAMP affinity chromatography on 7.5% SDS-PAGE electrophoresis and Coomassie Blue staining. Panel A (Coomassie Blue staining) shows that the first fraction (Std) after affinity purification contains one major 40 kDa protein, whereas the second fraction (RI), contains two major proteins having molecular weights between 50 and 54 kDa. Panel B (photodensitometry) shows the results of functional cAMP binding is shown, indicating specific incorporation of 8-azido-3H-cAMP into the purified protein for identification of human testis R subunits.

Figure 5: Size calibration of purified R subunits from human testis. Analysis on 10% SDS-PAGE was used to compare human testis subunits with recombinant mouse and rat subunits (apparent molecular weight of 54 and 56 kDa, dephospho- and phospho-form, respectively) and photodensitometry labeled purified rat testis subunits. Human RIIα 49 kDa, RIα 54 kDa and RIIα 52 kDa. Apparent molecular weights of 49, 55 and 53 kDa were found for the human R subunits RIα, RIIα and RIIβ, respectively.
phosphorylation, whereas the mobility of the 54 kDa rat RIIα was unaltered. Phosphorylation of the bovine RIIα subunit caused a mobility shift of the smaller protein (54 kDa) to 56 kDa (figure 6, panel C, third lane). The converse dephosphorylation of the 56 kDa bovine RIIα caused a switch of the apparent molecular weight back to 54 kDa (figure 6, panel C, first lane). Similar treatment of the human RIIα forms resulted in a mobility shift only for the smaller 54 kDa subunit. In contrast the largest human RIIα subunit (53 kDa) revealed no mobility shift upon phosphorylation (figure 6, panel B). The protein with apparent molecular weight of 48 kDa heavily stained with Coomassie Blue in figure 6 lane one of both panels B and C represents the added aliphatic phosphatase, whereas the 40 kDa protein seen in lane 3 of panels B and C is the added casein kinase of CAMP.

Assuming that the RII mobility shift on SDS-PAGE resulting from autophosphorylation is a characteristic feature of the RIIα subunit, regardless of species, these results would indicate that the smaller human RIIα subunit (51 kDa) actually represents human RIIα. To investigate this further, phosphorylated and dephosphorylated human RIIα forms were identified immunologically with specific antisera against rat RIIα and rat RIIβ. The experiment depicted in figure 7 shows that only the human RIIα form recognized by the rat RIIα antiserum had a mobility altered by phosphorylation (lane 3). The mobility of the human RIIβ was not altered by phosphorylation. Note that the antibody against RIIβ showed some slight recognition of a band lower than RIIβ (presumably RIIγ) which did shift to a higher molecular weight upon phosphorylation. This suggests that the 51 and 53 kDA protens of human tissues react with human RIIγ (dephosphorylated) and human RIIβ, respectively.

The Western blots shown in figure 8 further substantiate this conclusion. The immunoreactive phosphoprotein of human RIIβ was somewhat smaller than rat RIIβ (54 kDa; panel A), on the same gel. After dephosphorylation, the apparent size of the human RIIβ was reduced by approximately 5-3 kDa (panel C). Immunoreactive human RIIβ, both in its phosphorylated, as well as dephosphorylated form, had an apparent molecular weight of 53 kDa rat RIIβ; figure 8, panels B and C. This results (figure 8) are consistent with those in figure 5 that human testis RIIβ has a molecular weight greater than RIIγ which is in sharp contrast to what have been found in other species (38).

Fig. 6. Phosphorylation-dependent mobility shift of purified RIIα subunits. Phosphorylation and autophosphorylation of RIIα subunits was compared to purified human testis RIIα and bovine RIIα, and their corresponding phospho- and dephospho-proteins after 10% SDS-PAGE. In panel A, lane 2, autophosphorylation using bovine casein kinase II, demonstrates that the larger 54 kDa rat RIIα forms shift to a higher apparent molecular weight (55 kDa), whereas the mobility of the smaller 52 kDa RIIα form remains unaltered. In panel B, lane 3, autophosphorylation of the human RIIα proteins caused a mobility shift of the smaller RIIα form from 51 to 54 kDa. Dephosphorylation of the two human RIIα forms using calf intestinal alkaline phosphatase (CIPA) (15 U/mg RIIα) (panel B, lane 1) caused no visible mobility shift of either of the proteins. In panel C, lane 3, autophosphorylation of the bovine RIIα caused a mobility shift from 54 kDa to 56 kDa. Dephosphorylation of endogenous phosphates in bovine RIIα in panel C, lane 1, caused a reversed mobility shift from 56 kDa to 54 kDa.

Fig. 7. Immunoblotting for identification of human testis RIIα subunits forms demonstrating a phosphorylation-dependent mobility shift. Dephosphorylated or phosphorylated human testis RIIα subunits were run on the same gel, blotted onto nitrocellulose filters and incubated with antisera against rat RIIα and RIIβ, as well as casein kinase (for details). The tightly specific antiserum made against rat RIIα identified a single human RIIα form whose mobility was increased by 2-3 kDa upon autophosphorylation. The human testis RIIα recognized by anti-rat RIIα antiserum did not shift upon autophosphorylation.

In vitro transcription/translation of full-length and truncated cDNAs encoding human testis RIIα and RIIβ.

In figure 9 the results from in vitro transcription/translation of full-length and truncated cDNAs with the human RIIα and human RIIβ, are depicted. After translation, the RII protein products were either phosphorylated, dephosphorylated, or kept unaltered before subjected to 10% SDS-PAGE. The results clearly show that in vitro translation of human RIIα cDNA gives rise to a protein with an apparent molecular weight of 53 kDa which shifts to 54 kDa upon phosphorylation. In vitro translation of human RIIβ cDNA resulted in a protein with an apparent molecular weight of 53 kDa. Similar to the purified protein, the in vitro translated RIIβ did not show a change in mobility upon phosphorylation (figure 8). The apparent sizes of the human, rat and bovine RII subunits are summarized in table 1.