cAMP-Dependent Protein Kinases I and II: Divergent Turnover of Subunits†

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Received February 21, 1986; Revised Manuscript Received May 23, 1986

ABSTRACT: cAMP-dependent protein kinase subunits were isolated from livers of rats that had been subjected to biosynthetic labeling with radioactive leucine. By application of ligand and antibody affinity techniques pure regulatory (R I; R II) and catalytic (C) subunits could be obtained in high yields, which allowed measurement of the apparent degradation rate constants and half-lives following a double isotope labeling protocol. In this way marked differences of apparent half-lives of regulatory subunits R I ($t_{1/2}$ = 31 h) and R II ($t_{1/2}$ = 125 h) were observed. To avoid the negative influence of reutilization inherent in the decay experiments, specific radioactivities were determined after a short isotope pulse. This parameter, which under steady-state conditions reflects the fractional turnover rate of the subunits, was found to be different for all three protein kinase subunits. Relative to total liver protein, the ratios R I:R II:C corresponded to 3.9:0.6:2. Our data indicate that in each type of protein kinase isoenzymes regulatory and catalytic subunits turn over with similar rates. The type I isoenzyme, however, is renewed much faster than protein kinase II. Furthermore, our findings are consistent with the thesis that free subunits as generated by activation are more susceptible to degradation than the holoenzymes, leading under steady-state conditions to compensatory resynthesis. Since renewal of R I exceeded that of R II also in two other tissues, the elevated turnover of protein kinase I as an indicator of preferential activation appears to be a general phenomenon. The different turnover of the two isoenzymes, then, may relate to different cellular functions like modulation of enzyme activity vs. modulation of gene activity.

The physiological effects of cAMP in mammalian tissues are thought to be mediated by the action of cAMP-dependent protein kinases [for a review, see Nimmo and Cohen (1977)]. These enzymes are composed of regulatory (R^1) and catalytic (C) subunits. Determination of the quaternary structure (Rosen et al., 1975) and of the stoichiometry of cAMP binding led to the formulation of the activation reaction as follows (cf. Corbin et al., 1978; Weber et al., 1979; Weber & Hilz, 1979; Builder et al., 1980):

$$R_2C_2 + 4cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

In most, if not all, tissues, cAMP-dependent protein kinases occur in the form of two isoenzymes, I and II (Hofmann et al., 1975; Corbin et al., 1975). The fact that the two holoenzymes contain different regulatory but apparently identical catalytic subunits represents a unique situation: Dissociation by the allosteric effector cAMP releases from both isoenzymes an active protein kinase with the same substrate specificities (cf. Nimmo & Cohen, 1977). Yet, the simultaneous presence of both enzyme forms in most mammalian cells strongly suggests divergent functions. These functions should pertain to basic processes of the cellular metabolism as both isoenzymes are found in proliferating and nonproliferating and in differentiated and less differentiated cells (Nimmo & Cohen, 1977). Apart from a possible "fixation" of C by the regulatory subunits to certain cellular compartments (Nigg et al., 1985a), the significance of the two isoenzymes could relate to the different sensitivities toward activation by cAMP (Schwoch, 1978; Byus et al., 1977), thus allowing selective modulation by phosphorylation of defined functions, like enzyme activities vs. gene activities. Recent data on a reversible translocation of C to the nucleus from Golgi complex-associated holoenzyme II under conditions of maximal stimulation (Nigg et al., 1985b) as well as other data on a nuclear function of C (Maurer, 1981; Murdoch et al., 1982; Lamers et al., 1982; Boney et al., 1983; Jungmann et al., 1983; Lewis et al., 1983) lend support to such an interpretation.

Signal substances like neurotransmitters when released from storage forms are usually rapidly degraded by catabolic enzymes present in the milieu of action. There are several findings suggesting that this may also be true for polypeptide messengers like the free catalytic subunit of cAMP-dependent protein kinase. C activity was found to decrease in Leydig cells following stimulation by hCG (Schumacher et al., 1979). Also, a proteinase has been described that degrades the free catalytic subunit while leaving it untouched when present in the holoenzyme form (Alhanaty & Shaltiel, 1979). Furthermore, free forms of the regulatory subunits also appear to be more susceptible to limited proteolysis than R protein bound to C (Sugden & Corbin, 1976; Potter & Taylor, 1979; Steinberg & Agard, 1981; Brochetto-Braga et al., 1982; Butley et al., 1985). If this applies generally to in vivo conditions, then it is to be expected that degradation of protein kinase subunits will be higher the longer activation lasts. Under steady-state conditions, as found in the liver of normally fed rats undergoing continuous hormonal activation/deactivation cycles, then that type of protein kinase which is activated by low cAMP concentration should be subject to a higher turnover than the other isoenzyme, elevated rates of degradation being compensated for by increased rates of resynthesis.

Here, we report on a divergent turnover of protein kinase isoenzyme subunits in rat liver as well as in two other organs. These findings suggest that protein kinase I is the isoenzyme

† This work was supported by the Deutsche Forschungsgemeinschaft.

[1] Abbreviations: R, regulatory subunit; C, catalytic subunit; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-5661$01.50/0

preferentially activated in vivo.

Materials and Methods

Buffers. Buffers included (A) 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3; (B) 30 mM Tris–acetate, 4 mM EDTA, 20 mM benzamidine, 200 units/mL Trasylol (Bayer), 10% glycerol, pH 7.4; (C) 20 mM Tris–acetate, 4 mM EDTA, 6 mM mercaptoethanol, 0.5 mM methylisobutylxanthine, 200 units/mL Trasylol, 10% glycerol, pH 7.4; (D) 25 mM Tris·HCl, 192 mM glycine, 20% (v/v) methanol; (E) 10 mM Tris–acetate, 4 mM EDTA, 135 mM NaCl, 5 mM benzamidine, 6 mM mercaptoethanol, 200 units/mL Trasylol, 10% glycerol, pH 7.4; and (F) 5 mM Tris·HCl, 145 mM NaCl, 0.05% sodium azide, pH 7.4.

cAMP Affinity Gel. 8-(2-Hydroxyethyl)thio-cAMP was synthesized and coupled to epoxy-activated Sepharose (Pharmacia) as described previously (Weber et al., 1979).

Regulatory Subunit R I and R II Standards. Protein kinase regulatory subunits type I and type II were isolated from rabbit muscle and bovine heart, respectively, as described by Weber et al., 1979.

Anti-R I, Anti-R II, and Anti-C Antibodies. Antibodies to the regulatory subunit type I were raised in guinea pigs and goats as described (Weber et al., 1981). Antibodies to the regulatory subunit type II and to the catalytic subunit of cAMP-dependent protein kinase were obtained from rabbits (Weber et al., 1981; Schwoch et al., 1980).

Immobilized Anti-R I and Anti-R II Antibodies. Anti-R I immunoglobulin was separated from goat immune serum by affinity chromatography on a column of R I covalently coupled to Sepharose 4B [activated by CNBr (Axen & Ernback, 1971)]; 6 M urea was used for the separation of immune complexes. Anti-R II immunoglobulin was concentrated from rabbit immune serum by ammonium sulfate precipitation (60% saturation). Immunoglobulins were dialyzed against buffer A and coupled to Sepharose 4B by the standard cyanogen bromide procedure using a 10% (w/v) CNBr solution for the activation of an equal volume (7 mL) of Sepharose 4B and 50 mg/5 mL protein for the coupling reaction (2 h, 25 °C).

Labeling of Proteins and Preparation of Liver Extracts. Female rats (Wistar, 200–250-g body weight) were injected ip with about 0.3 mCi of L-(4,5-^{3}H)leucine (Amersham/Buchler; 60–70 Ci/mmol) in 0.5 mL of saline. In pulse labeling experiments the animals were sacrificed after 90 min by decapitation under ether narcosis. Livers were rapidly removed and homogenized with 30 mL of buffer B in a glass/teflon homogenizer. A sample of 400 μL of phenylmethanesulfonyl fluoride (100 mM in 2-propanol) was added immediately before homogenization. After centrifugation (15 min, 11000*g*), the sediment was washed once with 10 mL of buffer B, and the combined supernatants were subjected to ultracentrifugation (45 min; 150000*g*). The cytosol fraction obtained was concentrated by ammonium sulfate precipitation (60% saturation), and the precipitate was dissolved in 7 mL of buffer C.

When protein kinase subunits were to be purified by chromatography on antibody columns, livers were homogenized with the inclusion of 2 mL of charcoal suspension (5% in 2% bovine serum albumin) in order to minimize dissociation of holoenzymes by endogenous cAMP. Also, the ammonium sulfate step was omitted.

Isolation and Identification of Regulatory Subunits. cAMP affinty chromatography was applied to obtain R subunits directly from the ammonium sulfate fraction of liver extracts: 2 mL of 8-(2-hydroxyethyl)thio-cAMP agarose was rotated with the extract of one liver for 2 h at 4 °C. R protein adsorbed to the gel was then successively washed with buffer C, 2 M NaCl, and 1 mM AMP and was finally desorbed with 20 mL of 7 M urea (all in buffer C). In some experiments, combined immune/cAMP affinity chromatography was used to obtain pure R I and R II in separate fractions: Protein kinase holoenzymes were adsorbed to anti-R columns (see "Isolation and Identification of Catalytic Subunits"); after the release of C subunits by cAMP, the R proteins remained bound to the antibody gels and could be eluted with 25 mL of 6 M urea; the eluates were dialyzed against buffer C and subjected to affinity chromatography as outlined above. To concentrate the fractions of purified R proteins for gel electrophoresis (Laemmli), dialysis against 0.05% SDS and lyophilization were applied.

For immunoblotting, the Coomassie Blue stained gel was equilibrated with water and then incubated for 30 min at 25 °C with 0.1% SDS in buffer D prior to the electrotransfer to nitrocellulose paper (Schleicher & Schüll BA 85). After 4 h at 3 V/cm, the total Coomassie Blue stain and part of the protein were transferred to the paper. The blots were incubated for 2 h each with 1:50 dilutions of first (guinea pig anti-R I or rabbit anti-R II) and second antibody [peroxidase-labeled rabbit anti-guinea pig or goat anti-rabbit IgG (Miles)]. Buffer F was used for thorough washings subsequent to incubations and, with 3% bovine serum albumin added, for the dilution of antisera. Blots were developed by using 3,3′,5,5′-tetramethylbenzidine (Miles) and H_2O_2 in the peroxidase reaction. In spite of the Coomassie Blue stain still present on the blots, immunoreactive bands could be detected by the superimposed brown peroxidase reaction product. To demonstrate the two colors in black/white print, however, a selective filtering was achieved by the use of a Coomassie Blue filter (25-mL culture flask filled with a dilution of the stain); thereby, mainly the immunoreactive bands, and not the Coomassie Blue stain, were detected on photographs (cf. Figure 1).

Isolation and Identification of Catalytic Subunits. This new one-step purification procedure is based on the ability of our anti-R antibodies to adsorb protein kinase holoenzymes without causing dissociation. Specific release of C is then brought about by cAMP. About 30 mL of extract, prepared by homogenizing one rat liver in the presence of charcoal as described above, was passed at 3 mL/h through a column containing anti-R I agarose, and the effluent was connected to an anti-R II column (1.5 × 3 cm each). After the extensive washing of the columns with 150 mL of buffer E, C subunits were eluted with 25 mL of 30 μM cAMP in buffer E. The concentration of this fraction by lyophilization and the analysis by SDS gel electrophoresis were performed as described for the R subunits. Identification of the 40-kDa band as catalytic subunit was confirmed by immunoblotting with an antiserum raised against the bovine heart C subunit (Schwoch et al., 1980). The blotting procedure was performed as described for the identification of R proteins.

Determination of Protein. Quantification of R and C proteins was carried out by SDS gel electrophoresis (8% acrylamide) and densitometry of Coomassie Blue stained protein bands. Areas of densitometer peaks were calculated and compared with those of standards of R I and R II (from rabbit muscle and bovine heart, respectively (Weber et al., 1979) or bovine serum albumin run on the same gel. Since the staining efficiency proved to be similar for the individual proteins, all values in this study were based on albumin as standard. Protein determinations in cell extracts were performed according to Lowry.

Determination of (^3H)Leucine Incorporation. (a) Total soluble protein: 50-μL samples of cytosol were precipitated and washed with 15 mL of 5% trichloroacetic acid, heated for 15 min at 95 °C in 1 mL of 5% trichloroacetic acid, and washed with 1 mL of ethanol; residues were dissolved in 100 μL of H_2O/1 mL of Soluene 350 (Packard Instruments) and counted after addition of 10 mL of Dimilume (Packard Instruments). Protein pellets from aliquots were used for determination of protein. (b) Purified subunits: Subunit preparations representing the yield from one rat liver were separated on SDS flat gels (3 mm); destained gels were rinsed in water to remove acetic acid; the stained bands of R and C proteins were cut out, and the pieces were minced and incubated in a glass vial with 2 mL of Soluene 350 at 50 °C for 2 h under vigorous shaking; 20 mL of Dimilume was then added, and samples were counted repeatedly. In a typical experiment 500–4000 dpm were found per sample. The efficiency of this method was controlled by ashing excised bands in a Packard sample oxidizer; a comparison of both procedures using identical R I and R II samples yielded the same values within the limits of the methods.

Degradation Rate Constants for R I and R II. Three rats were injected each with 0.12 mCi of L-(U-^{14}C)leucine (Amersham/Buchler; 340 mCi/mmol) at 0 h followed by injections of 0.24 mCi of (^{3}H)leucine (see above) at defined times (0–95 h) thereafter. Animals were killed 1 h following the ^{3}H-pulse, and R proteins were purified and analyzed as described above. The radioactivity incorporated was measured on a scintillation counter with optimized window setting for the differential ^{3}H/^{14}C determination. Samples were counted repeatedly and corrected for overspill, and ^{3}H:^{14}C ratios were calculated to allow the estimation of degradation rate constants according to Arias et al. (1969) and Bock et al. (1971). The inclusion of the ^{3}H:^{14}C ratio at time zero when both isotopes were injected simultaneously served to correct for unequal precursor radioactivity (Bock et al., 1971).

Enzyme Assays. Protein kinase activity and cAMP binding were determined as described previously (Wittmaack et al., 1983; Weber et al., 1979).

Results

(1) Isolation and Quantitation of Regulatory Subunits. Determination of turnover of protein subunits requires the isolation of homogeneous polypeptides in order to be able to quantify the extent of specific labeling in decay studies or in labeling up experiments. In rat liver, the regulatory subunits of cAMP-dependent protein kinases comprise less than 0.2% of total soluble protein. In order to separate homogeneous R polypeptides in amounts sufficient for a reliable determination of the isotope incorporated, we applied affinity chromatography on 8-(2-hydroxyethyl)thio-cAMP–agarose (Weber et al., 1979) and gel electrophoresis. Due to the high affinity of R proteins to the immobilized cAMP analogue, columns could be directly loaded with crude liver extracts. After extensive washings, the regulatory subunits were eluted with urea and separated on SDS gels. Two major bands comigrating with reference rabbit muscle R I and bovine heart R II, respectively, were found. When specific anti-R I and anti-R II antisera were used, these 49- and 54-kDa bands were identified by immunoblotting as protein kinase regulatory subunits I and II (Figure 1).

This type of purification protocol not only provided sufficient amounts of R proteins (about 50 μg of each R subunit per rat liver) for the determination of radioactivity. It also allowed to quantitate the amounts of R subunits in the individual gels, thereby yielding specific radioactivities of R isoproteins obtained from a single rat liver. Protein determination was carried out by scanning the Coomassie Blue stain of the gel bands and comparing their intensities with a series of standard proteins.

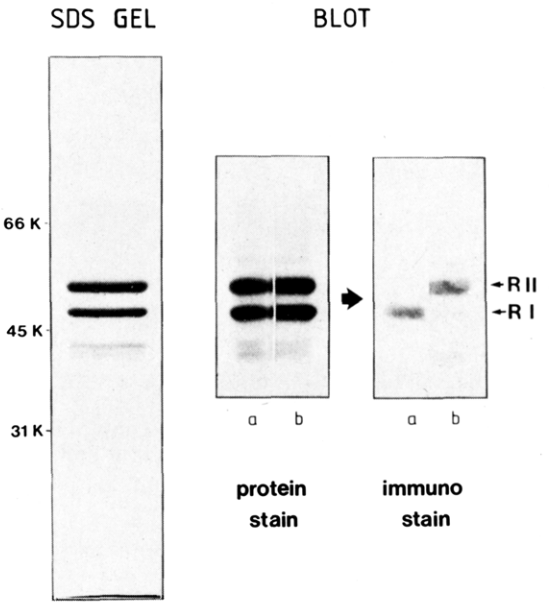

FIGURE 1: Immunological identification of purified R I and R II after separation by SDS gel electrophoresis. The extract of one rat liver was applied to affinity chromatography on 8-(2-hydroxyethyl)thio-cAMP–Sepharose (2 mL); an aliquot (5%) of the fraction eluted with urea was analyzed on a 8.5% polyacrylamide gel in the presence of SDS (left). The Coomassie Blue stained bands of the isolated proteins were blotted onto a nitrocellulose paper. The blot was cut longitudinally into halves, one section (a) treated with guinea pig anti-R I/anti-guinea pig IgG peroxidase, the other (b) with rabbit anti-R II/anti-rabbit IgG peroxidase. After development, the two halves of the blot were aligned again and photographed without (protein stain) and with (immuno stain) a "Coomassie Blue filter", thus preferentially marking the superimposed brown color of the peroxidase-catalyzed reaction product (for further details, see Materials and Methods).

(2) Turnover of Regulatory Subunits R I and R II. The term "turnover" is commonly used to denote the overall renewal process of a cellular component, as a result of its synthesis and degradation. Under steady-state conditions, both processes should be in equilibrium, thereby maintaining a constant level of the component in question. On the basis of this premise the turnover rate can be determined by measuring either the rate of synthesis or the rate of degradation. Since absolute rates (in terms of mass per time) are difficult to assess, isotope labeling experiments are usually applied that yield parameters describing the rate of synthesis or the rate of degradation relative to the existing pool of the respective protein (fractional turnover rate). Application of the double-isotope labeling procedure (Arias et al., 1969; Bock et al., 1971) enabled us to determine the apparent rate constants of degradation and the apparent half-lives of rat liver regulatory subunits R I and R II. Normally fed rats were injected initially with (^{14}C)leucine; at defined times thereafter the animals received additional injections of (^{3}H)leucine and were then sacrificed 1 h later. Assuming first-order kinetics for decay, apparent rate constants of degradation could be calculated from the ^{3}H:^{14}C ratios according to the equation (Arias et al., 1969; Bock et al., 1971)

$$k_d(h^{-1}) = [2.303/(t_1 - t_0)][\log (^3H/^{14}C)_{t_1}/(^3H/^{14}C)_{t_0}]$$

Two experiments with two periods of decay each (24 and 48 h) were performed. Average rate constants of degradation were calculated to be $2.64 \pm 0.78 \times 10^{-2}$ h^{-1} and $0.60 \pm 0.22 \times 10^{-2}$ h^{-1} for R I and R II, respectively, indicating a sig-

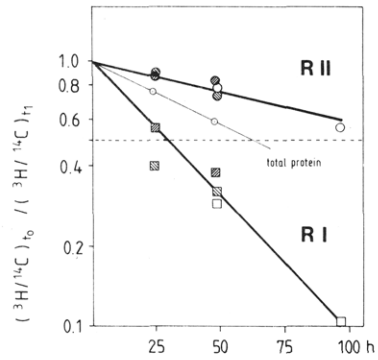

FIGURE 2: Decay of regulatory subunits R I and R II and of total soluble protein. In three separate experiments three animals each were subjected to the double-isotope labeling procedure. The protocol for sequential injections of (^{14}C)leucine and (^{3}H)leucine is described in Materials and Methods. The animals were killed 1 h after (^{3}H)leucine application, and R subunits were isolated. ^{3}H:^{14}C ratios were determined by repeated scintillation countings in the R I and R II protein bands excised from SDS gels. For the analysis of the total protein aliquot fractions of liver, supernatants were precipitated and washed with trichloroacetic acid to determine the radioactivity associated with the pellets. $(^{3}H/^{14}C)_{t_0}/(^{3}H/^{14}C)_{t_1}$ values were plotted on a logarithmic scale versus time. In one experiment (□) only the ^{14}C radioactivity relative to the amount of protein was plotted. Linear regression was applied for the estimation of half-lives.

Table I: Specific Radioactivities of Regulatory Subunits and Total Soluble Protein after a Single Pulse with (^{3}H)Leucine

	(^{3}H)leucine incorpn (dpm/μg of protein)			
expt	total protein	R I	R II	R I:R II
I	18.5	73.2	11.4	6.4
II	17.1	62.5	10.0	6.3 $\bar{x}$ = 6.1
III	28.4	117.4	19.7	5.6

nificantly ($p < 0.02$) faster breakdown of the R subunit type I. Plotting the values from three experiments vs. time (Figure 2) yielded the decay curves of both R subunits and of the total soluble liver protein. From the graph, an apparent half-life for R I = 31 h and for R II = 125 h could be derived. Both parameters indicate that R I turns over 4 times faster than R II. Figure 2 also demonstrates that the breakdown of R I is markedly faster and that of R II significantly slower than that of average liver protein.

Decay experiments as applied here are subject to interference by reutilization of the labeled amino acid, causing a possible overestimation of half-lives. This overestimation would be higher when the turnover of the polypeptide in question is faster (Koch, 1962). Therefore, the actual difference in turnover rates of R I and R II may still be greater than indicated from the decay experiments described above. In order to approach true turnover rates more closely, we have determined the renewal of the existing subunit pools by evaluating specific radioactivities after a short (^{3}H)leucine pulse. This determination of the fractional turnover rate (Rowe & Wyngaarden, 1966) practically eliminates the error introduced by the reutilization of the labeled precursor because of an only marginal influence of degradation in this type of pulse experiment. The procedure that involves determination of protein and radioactivity from the same SDS gel band has the advantage of being fast, and independent of yields. A direct comparison of the protein kinase subunits in terms of (^{3}H)-leucine incorporation is legitimate because according to the available sequence data (Titani et al., 1984) these proteins contain almost identical amounts of leucine. Table I compares the specific radioactivities of R I, R II, and total soluble protein after a 90-min pulse of (^{3}H)leucine. The specific radioactivities

Table II: Specific Radioactivity of Regulatory Subunits R I and R II from Rat Brain and Rat Kidney[a]

	(^{3}H)leucine incorpn (dpm/μg of protein)			
tissue	total protein	R I	R II	R I:R II
brain	5	42	2	21
kidney	11	62	12	5

[a] Organs from three animals were pooled 90 min after injection with (^{3}H)leucine and analyzed by the same procedure as described in the legend of Table I.

demonstrate indeed that the turnover rate of R I exceeds that of R II by a significantly higher factor than that obtained by the decay experiments (R I:R II ratio = 6.1, compared to 4).

The marked difference in R I and R II turnover led us to verify the protein isolation procedure and to exclude the presence of possible contaminants in the R protein bands. We included immunoaffinity chromatography on columns of immobilized anti-R I and anti-R II antibodies as an additional purification step prior to cAMP-affinity chromatography. Thus, separate fractions of highly purified R I and R II subunits were obtained. The specific radioactivities of these differently purified subunits were practically identical with those listed in Table I.

About 90% of total cAMP binding activity of rat liver is found in the cytosol that was used for these studies. To examine whether the result of diverging biosynthetic labeling holds also for the minor portion of R isoproteins associated with the particle fraction, the insoluble material of homogenized rat liver after pulse-labeling was extracted with 1% Triton X-100. Specific radioactivities of R I (34 dpm/μg) and R II (9 dpm/μg) purified from that extract exhibited a ratio similar to that of the cytosolic R isoproteins.

We have also determined for both regulatory subunits the relative rates of synthesis expressed as percentage of isotope incorporated relative to the radioactivity of the total soluble protein. On the basis of the actual yields of isolated R proteins and their amounts in liver,[2] incorporation after a 90-min pulse of (^{3}H)leucine into either pool of R isoprotein could be calculated to be $5.1 \pm 0.5 \times 10^{-2}$% and $0.8 \pm 0.2 \times 10^{-2}$% for R I and R II, respectively ($n = 3$). On the average, the rate determined for R I exceeded that of R II by a factor of 6.5. It is not surprising that this difference closely resembles that found for the fractional turnover rates because the amounts of R I and R II in rat liver are nearly equal (Wittmaack et al., 1983; Weber et al., 1981). In this tissue, therefore, not only the existing pool of R I is renewed much faster than the R II pool. The absolute amounts of R I synthesized per time unit are also much higher.

(*3*) *Turnover Rates of R Isoproteins in Other Organs.* To find out whether the divergence of R isoprotein turnover is a liver-specific phenomenon, two other tissues, rat brain and kidney, were also analyzed. Brain was selected because it is very active in cAMP-dependent phosphorylation reactions, while the rate of general protein synthesis is significantly lower than that in rat liver (only 24% of the ^{3}H present in brain tissue after 90 min was in the protein fraction vs. 70% in liver). Furthermore, neural R II is a tissue-specific form of the molecule (Erlichman et al., 1980), and it represents the predominant type of R protein in this tissue (R I/R II = 30/70) as found by immunotitration (Weber et al., 1981). The data

[2] Amounts of 7.1 ● 1.6 μg of R I and 7.2 ● 3.4 μg of R II per g of rat liver were isolated ($n = 3$). This corresponds to an average yield of 80% as calculated from total cAMP binding sites and from the R I:R II ratio of 55:45 in this tissue (Wittmaack et al., 1983; Weber et al., 1981).

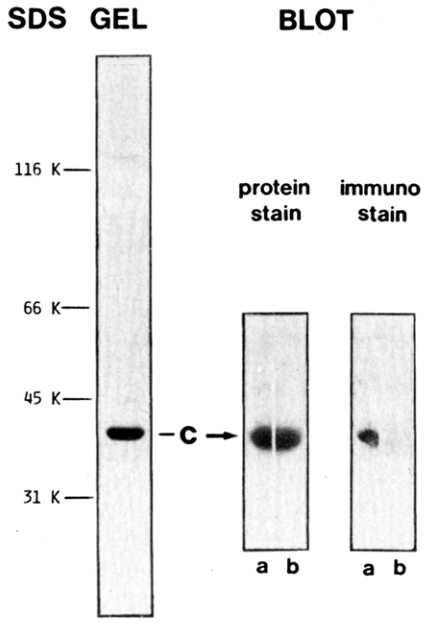

FIGURE 3: SDS gel electrophoretic analysis and immunoblotting of purified protein kinase catalytic subunits. Catalytic subunits C were specifically released by cAMP from protein kinase holoenzymes adsorbed to anti-R columns that had been loaded with rat liver extracts (see Materials and Methods). SDS gel electrophoresis and immunoblotting was applied to identify C protein in the cAMP eluate: A Coomassie Blue stained gel was used for the electrotransfer of proteins to nitrocellulose paper. The blot was cut longitudinally into halves, one section (a) treated with rabbit anti-C, the other (b) with normal rabbit serum. After the development of the blots with peroxidase-labeled anti-rabbit IgG, the blots were photographed by using the selective filtering procedure as described in the legend of Figure 1.

presented in Table II demonstrate that in spite of these differences, R I was again labeled to a much higher degree than total soluble brain protein, while R II had incorporated markedly less ^3H.

In rat kidney, protein synthesis was faster than in brain (54% of ^3H label in the protein fraction). Here, too, R I exhibited a higher fractional turnover rate than that of average protein, and still higher than that of R II. The data suggest that the high turnover of the regulatory subunit R I compared to that of R II is a general phenomenon. This appears to hold also for tissues containing specific isoforms of R II.

(*4*) *Apparent Turnover of the Catalytic Subunit C.* In view of the divergent turnover of protein kinase regulatory subunits R I and R II, it was important to extend these studies to the catalytic subunit C that forms holoenzymes with either type of R protein. The isolation of C protein from one rat liver in sufficient quantity and purity was achieved by an "unusual" application of anti-R I and anti-R II: These antibodies form immune complexes not only with their respective R subunits but also with the corresponding R2·C2 holoenzymes (Weber et al., 1981). Columns of immobilized anti-R I and anti-R II were therefore used in tandem arrangement to adsorb protein kinase holoenzymes from liver extracts. By subsequent incubation of the loaded immuno gels with cAMP, catalytic subunits were selectively released from immunoadsorbed R subunits and identified by SDS gel electrophoresis and immunoblotting (Figure 3). The amounts of C polypeptide (13–20 μg) purified from a single rat liver by this method were sufficient for a reliable determination of fractional turnover rates. Since the yield of C subunits achieved with this one-step purification procedure depends on the amount of protein kinase holoenzyme present in the tissue extracts, livers were homogenized in the presence of charcoal. By this treatment, free cAMP as generated by the "killing stress" is instantly adsorbed, and protein kinase subunits are allowed to reassociate. In this process a redistribution of C subunits between PK I and PK II will occur; likewise, any physiological activation/deactivation cycle affecting both isoenzymes should result, at least in a partial "mixing" of C subunits. For this reason, we did not attempt to discriminate C polypeptides as released from anti-R I and anti-R II columns, respectively. The C protein preparation analyzed in this study, therefore, is considered to represent total catalytic subunits.

Specific radioactivities of isolated C subunits were measured after a 90-min pulse with (^3H)leucine as described above for the R subunits. To correct for unequal precursor radioactivities administered in three separate experiments, values were divided by the specific radioactivities of the total soluble protein: The result of 2.05 ± 0.1 indicates that the pool of catalytic subunits is renewed twice as fast as the average protein. When the specific radioactivities of all three protein kinase subunits were related to that of total protein, the fractional turnover rates of the subunits could be compared: R I:R II:C = 3.9:0.6:2.0 (values for R I and R II were calculated from Table I). These data show that the fractional turnover rate of the catalytic subunit is intermediate between those of R I and R II.

Although this finding does not rule out independent turnover of C, it rather seems to indicate that total C turnover is coordinated with that of (R I + R II), and it may arise as the result of isoenzyme-specific turnover: If the thesis is correct that free subunits as generated by activation are subject to increased degradation and increased compensatory resynthesis, the overall turnover of C subunits should depend on (i) the activation of PK I and PK II as reflected by R I and R II turnover rates and on (ii) the PK I/PK II ratio that we have determined to be 55:45 in rat liver (Weber et al., 1981). Assuming relative amounts of R I:R II:C = 55:45:100 and introducing the fractional turnover rates of R subunits relative to total protein, the relative turnover rate of total C can be predicted to be (0.55 × 3.9) + (0.45 × 0.6) = 2.4. This calculated value is close to the experimentally derived relative turnover rate for C of 2.1. It therefore appears that, in each isoenzyme, catalytic and regulatory subunits turn over with similar rates but that these rates differ for the two types of protein kinase by a factor of 6.

Discussion

By application of ligand and immune affinity procedures for the isolation of pure subunits of cAMP-dependent protein kinase it was possible to determine for the first time the fractional turnover rates of all three subunits. Our findings have demonstrated that under steady-state conditions R I had a much shorter half-life than average protein while R II was significantly slower. A faster decay of R I compared to that of R II has also been deduced from experiments involving cycloheximide treatment of neuroblastoma cells (Walter et al., 1981). To minimize interference by tracer reutilization, which affects components with fast turnover to a higher degree, we have applied pulse labeling of the tissue proteins to deduce from the specific radioactivities of R (and C) subunits the fractional turnover rates of their existing pools. It could be shown that the R I pool was renewed 6 times faster than the R II pool and that also the absolute amount of R I synthesized per time unit was 6.5 times higher than that of R II. Increased turnover rates of R I compared to that of R II were also seen in kidney as well as in brain containing a tissue-specific R II isoform (Erlichman et al., 1980).

In all normal tissues so far analyzed, equimolar amounts of regulatory and catalytic subunits were found (Corbin et al., 1977; Hofmann et al., 1977). Furthermore, the R:C ratio

remained constant during rat liver development in spite of 4–6-fold changes in absolute amounts (Wittmaack et al., 1983). In view of the different turnover of the R isoproteins, equimolarity of (R I + R II) and C can only be guaranteed if the biosynthesis of C is coordinated with that of both regulatory subunits. In accordance with this postulate, it was found that the catalytic subunit exhibited a renewal rate that was to be expected if C in protein kinase type I turned over at a rate similar to that of R I, while C in the type II isoenzyme approached turnover rates of R II.

The basic molecular mechanism leading to the higher turnover of protein kinase I appears to relate to the greater instability of free subunits compared to that of the holoenzymes. Accelerated degradation of R proteins in S49 lymphoma cells (Steinberg & Agard, 1981) and in other cell types has been observed under conditions that favor dissocation from C (Brochetto-Braga et al., 1982; Butley et al., 1985). Furthermore, degradation of free catalytic subunit C was indicated by experiments with hormone-activated cells (Schumacher et al., 1979; Hemmings, 1986) and in vitro by a specific protease from small intestinal brushborder membranes (Alhanaty & Shaltiel, 1979). Our own work and that of others has shown that both types of R subunits are subject to limited proteolysis (Sugden & Corbin, 1976; Weber & Hilz, 1978, 1979; Corbin et al., 1978). Hydrolysis occurs at defined "hinge regions" (Takio et al., 1980) which are exposed only in the free subunits (cf. Sugden & Corbin, 1976; Potter & Taylor, 1979). One might expect R I to be more sensitive to proteolysis than R II because of a higher accumulation of arginines in the hinge region (Titani et al., 1984). Our in vitro experiments, however, indicated that R II rather than R I is the more susceptible substrate when treated with various proteases (Weber & Hilz, 1979). Since the resulting 35–37-kDa cAMP binding products of R I and R II were unable to recombine with C to form (protected) holoenzymes, cleavage of released subunits at these protease-sensitive sites might provide the signal for further degradation. Although the contribution to the unusually high turnover rate by a hypothetical R I-specific protease cannot be excluded, we feel that the available experimental evidence points to the frequency of protein kinase activation as the deciding factor in proteolytic degradation of the subunits.

To maintain steady-state levels of protein kinase subunits, compensatory resynthesis must be induced in response to each activation/deactivation cycle. The higher turnover of protein kinase I, therefore, indicates that this isoenzyme is responding more readily to moderate elevations of cAMP than protein kinase II. Chromatographic analysis of the protein kinase holoenzymes remaining after hormonal stimulation indicated selective activation of the type I isoenzyme in a wide range of cells and tissues (Schwoch, 1978; Byus et al., 1977; Hunzicker-Dunn, 1981). Conflicting reports from one other group (Livesey et al., 1982; Livesey et al., 1984) may relate to the difficulties inherent in this type of analyses that cannot exclude reassociation and redistribution of subunits during preparation of extracts. Our data do not exclude additional factors modulating protein kinase activation. However, it appears that preferential activation of protein kinase I leading to a preferentially increased turnover of its subunits is the basic phenomenon which holds also for situations after acute and chronic stimulation by hormones (Schwantke et al., in preparation).

Activation of both protein kinase isoenzymes releases catalytic subunits with apparently identical affinities and specificities. Yet, their mere existence in practically all mammalian cells and tissues as well as changes in their absolute and relative amounts during differentiation (cf. Wittmaack et al., 1983; Schwarz & Rubin, 1983), suggests separate functions. The extremely divergent turnover rates of the two isoenzymes observed in three different tissues with widely varying R I:R II ratios further support this interpretation. These findings also indicate that the functional difference of the two isoenzymes must pertain to general cellular processes not specifically related to phenomena like differentiation or cell proliferation. Rather, the difference may be connected with processes like transient vs. sustained response to hormones or modulation of enzyme activities vs. modulation of gene activities. Such differences are suggested by recent findings (Nigg et al., 1985a) in which specific association of protein kinase II with the Golgi complex and with centrosomes was demonstrated. Maximal activation by forskolin-induced cAMP accumulation led to a rapid translocation of C to the nucleus, which was reversible upon removal of forskolin (Nigg et al., 1985b). Together with other reports on the activation of certain genes by cAMP-dependent protein kinases (Maurer, 1981; Murdoch et al., 1982; Lamers et al., 1982; Boney et al., 1983; Jungmann et al., 1983; Lewis et al., 1983), these data may suggest that protein kinase II is dissociated only after a strong stimulus as evoked by high levels of cAMP, thus allowing induction of key enzymes by specific gene activation. Special functions of protein kinase II are also indicated by the existence of tissue-specific isoforms of R II subunits (Erlichman et al., 1980; Robinson-Steiner et al., 1984; Jahnsen et al., 1985). Protein kinase I, as the more sensitive isoenzyme, on the other hand is preferentially activated by relatively small elevations of cAMP and may serve mainly to modulate by phosphorylation target enzymes. These divergencies in function appear to find their expression in different turnover rates.

ACKNOWLEDGMENTS

We thank E. Spikofski for competent assistance and Dr. G. Schwoch for anti-C antibodies.

Registry No. Protein kinase, 9026-43-1.

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Thrombospondin Is a Substrate for Blood Coagulation Factor XIIIa†

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Received January 31, 1986; Revised Manuscript Received April 21, 1986

ABSTRACT: Thrombospondin (TSP) is released from α granules of activated platelets, binds to platelet surfaces, and copolymerizes with fibrin. In the present experiments, we investigated the action of factor XIIIa (plasma transglutaminase) on TSP. Factor XIIIa catalyzed incorporation of [^{14}C]putrescine into soluble TSP and ligation of TSP to itself and to fibrin intermediates. Proteolytic digestion of [^{14}C]putrescine-labeled TSP with trypsin or thrombin yielded a labeled disulfide-bonded core of 90 or 120–130 kilodalton (kDa) subunits, labeled fragments of less than 10 kDa, and an unlabeled 30-kDa heparin-binding fragment, indicating the presence of multiple factor XIIIa reactive glutaminyl residues located in several domains of the molecule. TSP became ligated in fibrin clots formed from amidinated fibrinogen, i.e., fibrin that could not contribute lysyl residues to factor IIIa catalyzed cross-links. The disulfide-bonded core of TSP formed upon thrombin digestion copolymerized with fibrin as efficiently as intact TSP. However, a lower proportion of the disulfide-bonded core became ligated. These results indicate that TSP, both in clots and in solution, contributes glutaminyl and lysyl residues to factor XIIIa catalyzed ligation. Cross-linking may be important in stabilizing interactions among TSP, fibrinogen, or fibrin and other molecules in hemostatic plugs.

Thrombospodin (TSP) is a major protein secreted from the α granules of activated platelets (Hagen, 1975). It is present in plasma at a concentration of only 20–100 ng/mL, but its concentration in serum is about 20 μg/mL due to platelet release (Saglio & Slayter, 1982; Dawes et al., 1983; Mosher et al., 1983). TSP is also secreted by a variety of cells in culture (McPherson et al., 1981; Sage et al., 1981; Mosher et al., 1982; Raugi et al., 1982; Jaffe et al., 1983).

TSP may be involved in several aspects of blood coagulation.

† These studies were supported in part by National Institutes of Health Research Grant HL 29586 (D.F.M.) and National Institutes of Health Postdoctoral Fellowship Grant HL 06868 (M.D.B.).
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0006-2960/86/0425-5667$01.50/0