Cyclic Adenosine 3′:5′-Monophosphate-Dependent Protein Kinase on the External Surface of LS-174T Human Colon Carcinoma Cells

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ABSTRACT: The analysis of purified plasma membranes and the surface of intact cells revealed the presence of cyclic adenosine 3′:5′-monophosphate-(cAMP) dependent protein kinase (PKA) on the external surface of LS-174T human colon carcinoma cells. Photoaffinity labeling of intact cells at confluence with 8-azido-[^{32}P]cAMP identified the cAMP-binding proteins on the surface. Immunoprecipitation identified the photoaffinity-labeled cAMP-binding proteins as the RIIα regulatory subunit of PKA. During the logarithmic stage of growth, both the RIα and RIIα subunits of PKA were localized on the cell surface. Intact LS-174T cells catalyzed the phosphorylation of Kemptide in a cAMP-dependent manner; upon substitution of cAMP in the medium with 8-chloroadenosine, which did not compete with cAMP for the binding on intact cells, the ecto-PKA was no longer activated. The specific inhibitory protein for PKA, PKI, abolished the stimulation of phosphorylation by cAMP. Forskolin, which elevates intracellular levels of cAMP, activated ecto-PKA. Moreover, probenecid, which blocks the export of cAMP, inhibited the forskolin-mediated activation of ecto-PKA. These results demonstrate that LS-174T colon carcinoma cells possess an ecto-PKA on the external surface. This ecto-PKA is similar, if not identical, to the soluble intracellular PKA.

The extrusion of cAMP[1] from intact animal cells was discovered by Davoren and Sutherland (*1*) on catecholamine-stimulated pigeon erythrocytes. Since that time, the egress of cAMP from a variety of tissues, cultured cells, and lower forms of organisms, including slime mold *Dictyostelium discoideum* and bacteria, has been described (*2*).

The physiological role of extracellular cAMP is partly known for some lower forms of organisms. In *D. discoideum*, chemotaxis and cell differentiation appear to be regulated by extracellular cAMP pulses (*3*). It was shown that chemotaxis and cAMP signaling are mediated by special cAMP-binding receptor proteins located on the surface of the cells (*4, 5*).

Functions of extracellular cAMP, especially for animal cells, still remain obscure. Under some conditions the cumulative extracellular quantity of the cAMP appears to reflect the influence of various agents on cAMP generation and cell damage (*6*). However, cAMP efflux from the cells cannot be explained only by destruction of cells. It has been shown that, in avian erythrocytes and cultured mammalian cells, cAMP is released by an energy-dependent mechanism that has some properties of active transport (*1, 2, 7).* A number of pharmacological and hormonal agents inhibit this process (*7, 8*). The action of some of them is not related to alterations of adenylate cyclase activity or the level of cellular ATP (*7*).

Because the effects of cAMP in mammalian cells are largely mediated by cAMP-dependent protein kinase (PKA) (*9*), it is conceivable that the effluxed cAMP from the cell may have some physiological significance in regulating ecto-PKA that phosphorylates essential protein substrates on the cell surface or in the intercellular space. Furthermore, cell surface-located PKA may play an important role in functions such as cell motility, cell adhesion, cell−cell interaction, or cell reception and transduction of external signals.

A number of reports have provided evidence for the potential existence of cell surface protein kinases mediating surface protein phosphorylation in a variety of cells (*10*). It was established that at least two types of ecto-protein kinases, which are cAMP-independent, are located on the surface of a wide range of vertebrate cells; CK-1- and CK-2-like enzymes (*11*−*14*) phosphorylate a variety of cell surface proteins and also extrinsic proteins such as phosvitin, casein, and fibrinogen (*11*−*14*). Although the first evidence showing the existence of PKA on the external surface of C-6 rat glioma cells was reported 20 years ago (*15*), the presence of ecto-protein kinase that is cAMP-dependent (PKA) has not been rigorously demonstrated in mammalian cells. Most published studies have utilized histone as a substrate for PKA. However, it has been shown that extracellular histone may cause very rapid membrane leakage in some cells and therefore renders the assay unsuitable for determination of ectokinase activity (*16*). Thus, the data obtained with this

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[1] Abbreviations: cAMP, cyclic adenosine 3′:5′-monophosphate: PKA, cyclic adenosine 3′:5′-monophosphate-dependent protein kinase; PKA-I, protein kinase A type I; PKA-II, protein kinase A type II; RI and RII, regulatory subunits of protein kinase A types I and II, respectively; PBS, phosphate-buffered saline; PKI, protein kinase inhibitor protein (Walsh−Krebs inhibitor).

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substrate are not definitive. By using the synthetic substrate Kemptide, which does not cause membrane damage and which carries the recognition and phosphorylation sites for PKA, Kübler et al. (*17*) presented the most convincing evidence for the existence of ecto-PKA in several mammalian cells. These investigators demonstrated that Kemptide added to cultured, intact cells was phosphorylated in a cAMP-dependent manner (*17*) and that Kemptide preserved cell membrane integrity when added to cultured cells (*17*). However, in the above study, the nature of the protein that binds cAMP to stimulate this ecto-PKA was not identified. Moreover, the question of how this ecto-PKA is regulated by external stimuli was not addressed.

Photoaffinity labeling with 8-N_3-[^{32}P]cAMP (*18*) can identify the cAMP-binding proteins of PKA located on the outer surface of cells because this cAMP analogue appears not to penetrate through the intact plasma membrane barrier. 8-N_3-[^{32}P]cAMP labeling has been used to identify specific cAMP-binding ecto-proteins on the surface of intact cells of slime mold *D. discoideum* (*19*), yeast *Saccharomyces cerevisiae* (*20*, *21*), and rat adipocytes (22). These studies suggest that these cAMP-binding ectoproteins are distinct from the regulatory subunits of the soluble PKA. On the other hand, on the basis of photoaffinity labeling, the cAMP-binding proteins on the external surface of intact BALB 3T3 cells (*23*) and rat epididymal spermatozoa (*24*) were identified as the regulatory subunits of PKA. However, because these studies utilized histone as the substrate for the PKA assay, the direct correlation of the photoaffinity-labeled proteins to the ecto-PKA remains problematic.

The objective of this study was to identify and characterize the cAMP-binding proteins and ecto-PKA present on the external surface of cells and to investigate the regulation of this ecto-PKA by external signals. There are two types of PKA, type I (PKA-I) and type II (PKA-II), which share a common C subunit but contain distinct R subunits, RI and RII, respectively (*25*). We used LS-174T human colon carcinoma cells in the present study. This cell line was chosen because it contains an equal amount of PKA-I and PKA-II in the cytosolic fraction (*26*).

MATERIALS AND METHODS

Materials. 8-N_3-[^{32}P]cAMP (60 Ci/mM) and [γ-32P]ATP were obtained from ICN Pharmaceuticals (Irvine, CA). Pepstatin, antipain, aprotinin, and alamethicin were obtained from Sigma Chemical Co. (St. Louis, MO). Protein A−Sepharose was obtained from Pharmacia−LKB (Uppsala, Sweden). All other reagents and chemicals were of the highest grade available.

Cell Cultures. LS-174T colon carcinoma cells were grown in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, 0.2 mM MEM nonessential amino acids (Gibco−BRL, Gaithersburg, MD), pH 7.4, 2 mM glutamine (Gibco−BRL, Gaithersburg, MD), and antibiotic−antimycotic (Gibco−BRL, Gaithersburg, MD) in a humidified atmosphere of 95% air/5% CO_2 at 37 °C. The cell viability was determined by trypan blue exclusion dye (0.4% in PBS, Gibco−BRL), uptake of ethidium bromide (20 μg/mL) (fluorescence microscopy), and cell morphology (phase contact). For efflux, the release of the cytosolic enzyme lactate dehydrogenase into the cell supernatant was assayed accordingly (*27*). The number of damaged cells in the experimental cultures usually did not exceed 2%.

Membrane Purification. Plasma membranes were isolated by the method of Ross et al. (*28*), with minor modifications as previously described (*29*).

Separation of Inside-Out and Outside-Out Plasma Membrane Vesicles. Isolation of the plasma membrane vesicles was carried out as described previously (*20*) with minor modifications (*29*). Briefly, plasma membrane vesicles (125 μg of protein/mL) (fraction II of gradient) (*29*) were suspended in STME buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine, 30 μg/mL leupeptin, 5.0 μg/mL aprotinin, and 5.0 μg/mL pepstatin), sonicated, centrifuged at 200000*g* for 60 min at 4 °C, and resuspended (1 mg of protein/mL) in STME buffer containing 5 mM $CaCl_2$, 75 mM KCl, and 10% glycerol. The suspension was mixed batchwise with 5 mL of concanavalin A−Sepharose (15 mg/mL) in the same buffer and left on ice for 1 h under stirring to allow agglutination. The suspension was then filled into a column with 3 volumes of the same buffer. Outside-out vesicles were eluted with 5 volumes of 0.1 M methyl α-mannopyranoside in 10 mM Tris-HCl (pH 7.5), containing 250 mM Tris-HCl, 10% glycerol, and protease inhibitor cocktail and spun down at 100000*g* for 60 min. Outside-out vesicles of plasma membranes were resuspended in STME buffer and stored at 0−4 °C or used immediately.

Immunoprecipitation of R Subunits of PKA. The identification of R subunits of PKA was carried out by photoaffinity labeling of protein samples with 8-N_3-[^{32}P]cAMP followed by immunoprecipitation with the R antibodies (*30*) as previously described (*31*). Protein concentration was determined by the method of Lowry et al. (*32*) with bovine serum albumin as standard.

Photoaffinity Labeling of Intact Cells. 8-N_3-[^{32}P]cAMP dissolved in methanol was dried under N_2 stream and resuspended in buffer containing 250 mM sucrose and 20 mM Tris-HCl, pH 7.5, to a final concentration of 5 mM. Cells were grown in 3 cm dishes or 6-well plates and rinsed on the dish two times with PBS at room temperature. PBS was aspirated and 1 mL of 250 mM sucrose, 20 mM Tris-HCl, pH 7.5, 100−300 nM 8-N_3-[^{32}P]cAMP, and protease inhibitors was added. The cells were incubated 30 min at room temperature in the dark in the absence or presence of 0.25 mM unlabeled cAMP and then irradiated at 254 nm at a distance of 2 cm for 2 min. After irradiation, the medium was immediately aspirated, and cells were washed 3−5 times with cold PBS containing 0.1 mM unlabeled cAMP. Cells were harvested in PBS, washed two times by centrifugation, and finally resuspended in 100 mL of buffer 10 (Tris-HCl, pH 7.4, 20 mM; NaCl, 100 mM; NP-40, 1%; sodium deoxycholate, 0.5%; $MgCl_2$, 5 mM; pepstatin, 0.1 mM; antipain, 0.1 mM; chymostatin, 0.1 mM; leupeptin, 0.2 mM; aprotinin, 0.4 μg/mL; and soybean trypsin inhibitor, 0.5 μg/mL) (*31*).

cAMP Binding Assay. Binding of nucleosides and nucleotides to proteins was assayed by the method of Gilman (*33*) with minor modifications (*34*).

Protein Kinase A Assay. cAMP-dependent phosphorylation of Kemptide was determined as previously described (*35*).

cAMP-Dependent Ecto Protein Kinase Assay. Cells were cultivated in 24-well plates. Before assay, cells were rinsed twice with cold PBS and reaction was started by the addition of 100 μL of standard assay mixture containing 50 mM Tris-HCl, pH 7.5, 10 μM Kemptide (Gibco−BRL), 0.5 mM [γ-^{32}P]ATP (100−200 cpm/pmol, ICN), 10 mM $MgCl_2$, 75 mM NaCl, and 25% sucrose. After 15 min incubation at 37 °C in the absence or presence of 5 μM cAMP, 50 μL aliquots of reaction mixtures were spotted onto phosphocellulose ion-exchange filter paper P81 (Whatman). Filters were washed three times in 0.5% phosphoric acid, air-dried, and then counted by liquid scintillation.

Assay of Marker Enzyme Activities. Na^+,K^+-Dependent ATPase activity (EC 3.6.1.3) was determined as described previously (*36*) with minor modifications (*29*). Briefly, the incubation mixtures contained 30 mM Tris-HCl, pH 7.5, 70 mM NaCl, 20 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 3 mM ATP, and alamethicin (0.15 mg/mg of protein). Ionophore alamethicin was added to the reaction mixture to make both sides of the plasma membrane vesicles, which may be formed during disruption of cells, freely accessible to the added substrates and ions (*3*). Released inorganic phosphate was estimated by the method of Ames (*37*). The ATPase activity was determined as the increase in the initial rate of ATP hydrolysis after 20 min preincubation with 3 mM ouabain and alamethicin. Succinate cytochrome *c* reductase (EC 1.3.99.1) as a marker for mitochondria was determined by the method of Avruch and Wallach (*38*). NADPH-cytochrome *c* reductase as a marker for microsomes (EC 1.6.2.4) (*39*), UDP-galactose:ovalbumin galactosyltransferase as a marker for Golgi apparatus (*40*), β-glucuronidase as a marker for lysosomes (*41*), and lactate dehydrogenase as a cytosolic enzyme (*27*) were determined as previously described (*29*).

Statistics. Statistical significance was assessed where appropriate by a paired Student's *t* test with a significant difference taken as $P < 0.05$.

RESULTS

Photoaffinity Binding of 8-N_3-[^{32}P]cAMP by Intact Cells. The time dependence of covalent incorporation of 8-N_3-[^{32}P]-cAMP into intact cells and whole cell homogenates is shown in Figure 1A. In homogenates, the radioactivity incorporation reached its maximal level within 30 min of incubation. In contrast, in intact cells, the incorporation of radioactivity continued to rise up to 2 h of measurement. Unlabeled cAMP, in a concentration-dependent manner, abolished the 8-N_3-[^{32}P]cAMP binding by intact cells (Figure 1A, lanes 7−9). Importantly, one major species of the radioactive band, that migrated faster than the 54−56 kDa bovine RIIα (lane RIIα), was found in the intact cells (Figure 1A, lanes 3−6), whereas two major bands were present in the homogenates (Figure 1A, lanes 1 and 2). These two species of cAMP-binding proteins in the homogenates were previously identified as the 48 kDa RIα and 50 kDa RIIα regulatory subunits of PKA in LS-174T colon carcinoma cells (*26*). In the presence of the ionophore alamethicin, which stimulates plasma membrane conductivity for nucleotides, the intact cells exhibited one additional 8-N_3-[^{32}P]cAMP-binding protein that was minimally shown in the absence of alamethicin (Figure 1A, lane 10). Thus, in the presence of alamethicin,

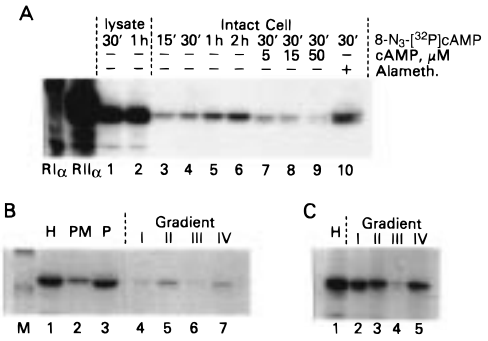

FIGURE 1: Detection of cAMP-binding proteins on the external surface and subcellular membranes of LS-174T cells. (A) Time dependence of incorporation of 8-N_3-[^{32}P]cAMP into whole cell lysate (lanes 1 and 2) and onto intact cells (lanes 3−10) in the absence and presence of unlabeled cAMP or ionophore alamethicin. RIα and RIIα are photoaffinity-labeled 48 kDa rabbit skeletal muscle RIα and 56 kDa bovine heart RIIα, respectively (Sigma). Due to the inconsistency of the product, the 48 kDa RIα protein band is only minimally shown. (B) Analysis of subcellular membrane fractions of photoaffinity-labeled intact cells on sucrose density gradient. H, homogenate; PM, plasma membranes of the first step purification; P, pellet before PM preparation; I → IV, membrane fractions of the second step purification (*29*). (C) The total lysate (lane 1) and subcellular membranes (lanes 2−5) fractionated by sucrose density gradient were photoaffinity-labeled with 8-N_3-[^{32}P]cAMP and analyzed by SDS−PAGE followed by autoradiography. Data represent one of 3−5 independent experiments that gave similar results.

the intact cell labeling with 8-N_3-[^{32}P]cAMP exhibited two species of cAMP-binding proteins, as was in the homogenate labeling.

Influx and efflux of specific molecules has been used to determine cell membrane integrity or damage (*16*, *17*). In parallel with the intact cell labeling experiments, we measured the release of lactate dehydrogenase into cell supernatant as an efflux and uptake of the fluorescent stain ethidium bromide as an influx. The results showed a low background level of lactate dehydrogenase activity and a low percentage (<2%) of cells with instant uptake of fluorescent dye (data not shown). These data support that the binding of 8-N_3-[^{32}P]cAMP by intact cells was not the result of the ligand's penetration through the plasma membrane barriers inside cells or into damaged cells.

Localization of PKA Regulatory Subunits on the Outer Surface of Plasma Membranes. The above results suggest that cAMP-binding proteins are present on the external surface of LS-174T cells. To examine the association of these cAMP-binding proteins with the plasma membranes, we first photoaffinity-labeled intact cells with 8-N_3-[^{32}P]cAMP and then isolated plasma membranes by two-step discontinuous sucrose gradient centrifugation (Figure 1B) (*29*). The specific activity of Na^+,K^+-ATPase was 4.2-fold higher (in comparison to crude homogenate) in plasma membranes obtained at the first step of purification (Figure 1B, lane 2) and 12.6-fold higher in fraction II (Figure 1B, lane 5) obtained at the second step of purification, as was shown previously (*29*).

A single radioactive band was present in the homogenate of the photoaffinity-labeled intact cells (Figure 1B, lane 1). The photoaffinity-labeled proteins were recovered in the plasma membranes of the first step purification (Figure 1B, lane 2) and in the plasma membrane-enriched fraction II (Figure 1B, lane 5) of the second step purification (*29*).

In parallel experiments, cells unlabeled with 8-N_3-[^{32}P]-cAMP were subjected to fractionation by discontinuous sucrose density gradient centrifugation, and cAMP-binding proteins in each fraction were identified by photoaffinity labeling with 8-N_3-[^{32}P]cAMP (Figure 1C). The homogenate exhibited two cAMP-binding proteins, the RIα and RIIα regulatory subunits of PKA, as previously identified (*26*) (Figure 1C, lane 1). The plasma membrane-enriched fraction II exhibited a major cAMP-binding protein corresponding to the RIIα of homogenate and a minor band corresponding to the RIα of homogenate.

To exclude the possible contribution of the other intracellular particles contaminated in the plasma membrane preparation and at the same time to determine the topology of the cAMP-binding proteins in the plasma membranes, we prepared outside-out vesicles by concanavalin A affinity chromatography (*20*) using the plasma membrane-enriched fraction II (*29*) (see Materials and Methods).

The purity of prepared vesicles was determined by measuring the microsomal and lysosomal marker enzyme activities. The efficiency of the separation of the outside-out vesicles from inside-out ones was determined by measurement of Na^+,K^+-ATPase. The ATP-binding site is located on the cytoplasmic surface of plasma membrane. Since these enzyme activities were at undetectable levels in the vesicle preparations (data not shown), the results shown in Figure 2A (lane 2) appear to represent the cAMP-binding proteins of the outer surface of plasma membranes. The upper band has the same electrophoretic mobility (corresponding to about 50 kDa) as the band of starting material (plasma membrane-enriched fraction II [*29*]) (Figure 2A, lane 1). A minor band with a lower MW was also detected in the preparation of outside-out vesicles. Since such a band was not apparent in the starting material (Figure 2A, lane 1), it may represent a degraded protein of the major band protein or other protein that was not readily detected in the starting material. Photoaffinity labeling of membrane vesicles in the presence of ionophore alamethicin, which forms large channels within the plasma membrane (*42*) and thus allows the nucleotide to penetrate into the intravesicular space, only slightly increased the band intensity (Figure 2A, lane 3). The same results were obtained when the photoaffinity labeling of vesicles was carried out in the presence of 0.1% deoxycholate. These results indicate that the cAMP-binding proteins were present at the outer surface of the plasma membranes of the cell.

As shown in Figure 2B, two major bands were photoaffinity-labeled in the homogenates with molecular masses of 48 and 50 kDa, respectively. These proteins were immunoprecipitated with antibodies to RIα and RIIα, respectively (Figure 2B, lanes 2 and 3) (*26*, *29*). In homogenates, the photoaffinity-labeled RIα and RIIα proteins were present in nearly equal amounts (Figure 2B, lanes 2 and 3) (*26*). In contrast, in the plasma membranes, RIIα was predominantly present (Figure 2A) (*29*).

Figure 2C shows the results of immunoprecipitation of surface-located cAMP-binding proteins with antibodies specific to RIα and RIIα. Only antibodies to RIIα recognized the labeled ectoprotein (lane 3).

The above results were obtained with intact LS-174T cells at confluence. We next examined the cAMP-binding protein distribution for the same cells but in the logarithmic stage of growth. The results showed two bands of cAMP-binding proteins (Figure 2D, lane 4). The immunoprecipitation analysis identified these cAMP-binding proteins to be the RIα and RIIα proteins (data not shown).

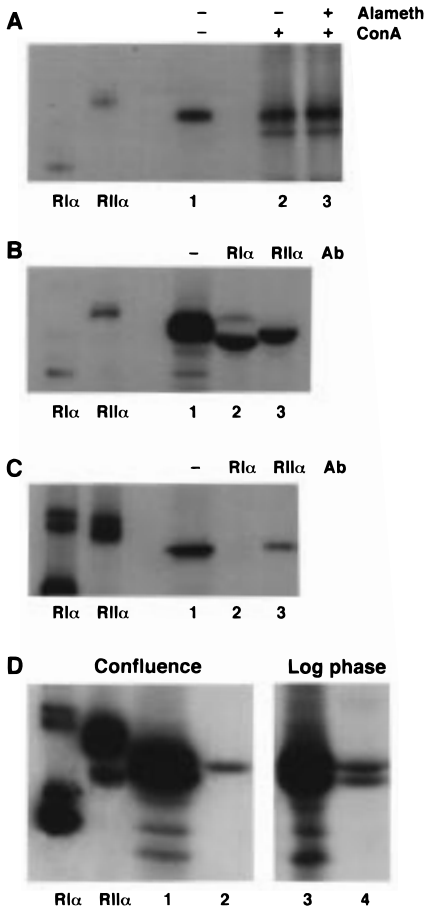

FIGURE 2: Detection of ecto-cAMP-binding proteins in outside-out plasma membrane vesicles and on intact cells at logarithmic versus confluent stage of growth. (A) Photoaffinity labeling of outside-out membrane vesicles prepared by concanavalin A affinity column. Lane 1, purified plasma membrane (fraction II) (*29*); lanes 2 and 3, outside-out membrane vesicles prepared through concanavalin A affinity column. Photoaffinity labeling with 8-N_3-[^{32}P]-cAMP was performed in the absence (lanes 1 and 2) and presence (lane 3) of alamethicin. (B) Photoaffinity labeling with 8-N_3-[^{32}P]-cAMP followed by immunoprecipitation of whole cell homogenate. Lane 1, photoaffinity labeling only; lanes 2 and 3, 8-N_3-[^{32}P]cAMP labeling plus immunoprecipitation with antibodies for RIα and RIIα, respectively. (C) 8-N_3-[^{32}P]cAMP labeling and immunoprecipitation of intact cells. Lane 1, 8-N_3-[^{32}P]cAMP labeling only; lanes 2 and 3, 8-N_3-[^{32}P]cAMP labeling plus immunoprecipitation with RIα and RIIα antibodies, respectively. (D) 8-N_3-[^{32}P]cAMP incorporation onto intact cells at logarithmic versus confluent phase of growth. Lanes 1 and 2, cells at confluent phase; homogenate and intact cells, respectively; lanes 3 and 4, cells at logarithmic phase; homogenate and intact cells, respectively. Data represent one of 3−5 independent experiments that gave similar results. Lanes RIα and RIIα in panels A−D, the photoaffinity-labeled 48 kDa rabbit skeletal muscle RIα and 54−56 kDa bovine heart RIIα (Sigma). Due to the inconsistency of the product, the 48 kDa RIα protein band is only minimally shown.

Intact LS-174T Cells Bind cAMP Specifically. As shown in Table 1, binding of [^{3}H]cAMP to intact cells was strongly competed by an excess amount (100-fold over [^{3}H]cAMP) of unlabeled cAMP. cAMP analogues, such as N^6-benzoyl-cAMP, 8-Cl-cAMP, 8-Br-cAMP, and 8-N_3-cAMP, exhibited strong competition (90−75%) for the binding. In contrast, ATP, 5′-AMP, and 8-Cl-adenosine did not compete with the

Table 1: Ligand Specificity of the cAMP-Binding Proteins on Intact LS-174T Cells[a]

unlabeled nucleoside or nucleotide added in 100-fold molar excess	[^{3}H]cAMP retained on cells	
	cpm × 10^{-3}	%
none	7.06	100
ATP	7.10	100
5′-AMP	6.83	96.7
8-Cl-adenosine	6.80	96.4
cGMP	4.11	58.2
8-N_3-cAMP	1.73	24.5
8-Br-cAMP	1.31	18.6
8-Cl-cAMP	0.87	12.2
N^6-benzoyl-cAMP	0.63	8.9
cAMP	1.00	14.1

[a] Cells grown on 12-well plates (5×10^4 cells/well, about 225 μg of protein/well) were washed two times with PBS and incubated at room temperature in isotonic buffer (see Materials and Methods) in the presence of 12 nM [^{3}H]cAMP and absence or presence of a 100-fold molar excess of unlabeled nucleoside/nucleotide. After 50 min of incubation, cells were washed four times with cold PBS, dissolved in buffer containing 4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *N*-lauroylsarcosine, and 0.1% mercaptoethanol, and the radioactivity was determined by a scintillation counter. The results represent the means of four independent experiments.

labeled cAMP for the binding. However, cGMP exhibited a 40% decrease in the [^{3}H]cAMP binding. Cyclic GMP is known to have a low affinity for the cAMP sites(s) (*25*), and this probably explains its effect.

Intact Cells Catalyze Phosphorylation of Kemptide in a cAMP-Dependent Manner. The above results demonstrated the presence of cAMP-binding proteins on the external surface of LS-174T cells. We next examined whether these cells exhibit ecto-PKA activity. As the substrate for ecto-PKA, we used Kemptide, which has been shown to have cytophilic properties (*17*); i.e., it is a molecule preserving cell membrane integrity when added to cultured cells.

The incubation of intact cells with Kemptide in the presence of [γ-^{32}P]ATP resulted in the incorporation of radioactivity, which was dependent on the concentration of cAMP (Figure 3A). The phosphorylating activity of intact cells was detected at nanomolar concentrations of cAMP and was increased linearly with increasing cAMP concentration; the K_m for cAMP was 2×10^{-7} M. At cAMP concentrations exceeding 10^{-5} M, a decrease in its stimulatory effect was observed. Such dependence of the phosphorylation upon the cAMP concentration may indicate the competition between cAMP and ATP for the binding at the active site of the catalytic subunits (*43*). Upon substitution of cAMP in the medium with 8-Cl-adenosine, which did not compete with cAMP for binding on intact cells (Table 1), the ecto-PKA was no longer activated. At Kemptide concentrations up to 7 μM, the initial rate of phosphorylation increased linearly, but at concentrations above 7 μM, the phosphorylation reaction reached a plateau (Figure 3B). The linear curve allowed the estimation of an apparent K_m value of 4.5 μM for Kemptide, which is close to the K_m value for Kemptide obtained with ecto-PKA in HeLa cells (*17*). The substitution of cAMP with cGMP at the same concentration resulted in only a minor stimulation of phosphorylating activity above the control (no cAMP added). This result may reflect a lower affinity of cGMP observed for the surface-located cAMP-binding proteins (see Table 1).

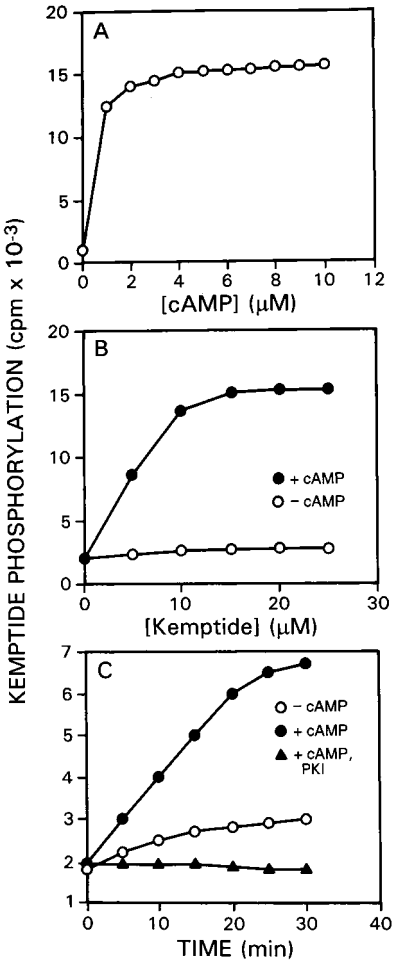

FIGURE 3: Kemptide phosphorylation by intact LS-174T cells. (A) Dependence on cAMP concentration. Cells (1×10^5) were assayed for phosphorylation of Kemptide (10 μM) with cAMP at various concentrations as indicated (see Materials and Methods). (B) Dependence on Kemptide concentration. Cells were incubated for 15 min as in panel A but with different Kemptide concentrations; the reactions were carried out with 5 μM cAMP (●) or in the absence of cAMP (○). The background radioactivity measured 2000 cpm. (C) Time course of Kemptide phosphorylation. The reactions were carried out with 5 μM Kemptide in the absence of cAMP (○), in the presence of 5 μM cAMP (●), and in the presence of cAMP (5 μM) and PKA inhibitor PKI (3 μM) (▲). The data shown are the means of closely agreeing duplicate determinations from 3−5 independent experiments.

The phosphorylation reaction in the presence of cAMP was linear up to 20 min of incubation time (Figure 3C). At this time point, cAMP stimulated the incorporation of radioactivity into Kemptide 4-fold over that in the absence of cAMP (Figure 3C). The specific inhibitory protein for PKA, Walsh−Krebs inhibitor (PKI), completely abolished the cAMP stimulation of phosphorylation (Figure 3C).

cAMP and cAMP Analogue That Are Membrane-Impermeable Activate Ecto-PKA but Not Soluble PKA. We next examined whether intracellular soluble PKA contributed to the ecto-PKA activity detected in these cells. The phosphorylation experiments were performed with cells treated with various agents that cause varying degrees of membrane damage. As shown in Table 2, the cAMP stimulation of Kemptide phosphorylation increased 5−10-fold in cells treated with membrane-damaging agents, and PKI completely abolished the cAMP-stimulated phosphorylation. However, the incubation of the cells up to 20 min with the ionophore

Table 2: Kemptide Phosphorylation by Intact LS-174T Cells Pretreated with Membrane-Damaging Agents[a]

addition in preincubation	Kemptide phosphorylation (cpm × 10^{-3})		
	no cAMP added	cAMP	cAMP + PKI
none	8.0	26.6	5.4
0.1% saponin	8.3	225.8	4.8
alamethicin	7.6	27.1	5.1
20% ethanol	8.1	161.2	5.2

[a] Cells (24 well plates, 5 × 10^4 cells/well) were washed twice with cold PBS and treated with membrane-damaging agents at 37 °C for 30 min. Kemptide (5 μM) phosphorylation was carried out either in the absence or presence of cAMP (5 μM), and presence of cAMP (5 μM) plus Walsh−Krebs inhibitor (PKI) (3 μM).

Table 3: Kemptide Phosphorylation by Intact Cells and Cell Lysates after Pretreatment of Intact Cells with cAMP and cAMP Analogues[a]

addition in preincubation	Kemptide phosphorylation (cpm × 10^{-3})	
	cell lysates	intact cells
none	5.6 ± 0.4	2.0 ± 0.3
cAMP	6.2 ± 0.7	22.5 ± 1.2
8-N_3-cAMP	5.7 ± 0.5	24.2 ± 0.9
8-Cl-cAMP	25.4 ± 1.5	25.6 ± 0.8
8-Cl-adenosine	5.1 ± 0.8	2.0 ± 0.5

[a] Cells were pretreated with 20 μM cAMP or its analogues for 30 min at 37 °C, then medium was removed and cells were washed four times with PBS, and then cells were assayed for Kemptide phosphorylation in the absence of cAMP (see Materials and Methods). Cell lysates were prepared from pretreated cells and assayed for Kemptide phosphorylation in the absence of cAMP.

alamethicin did not affect the Kemptide phosphorylation in the absence and presence of cAMP. Thus, even under conditions when the cell membrane becomes permeable for both nucleotides (ATP or cAMP), Kemptide probably remains incapable of penetrating through the membrane barrier. A clear distinction of ecto-PKA from intracellular soluble PKA was made by the experiments with cAMP versus 8-Cl-cAMP, a membrane-permeable analogue of cAMP. Cells were preincubated with either cAMP or 8-Cl-cAMP, cells were washed, and then ecto-PKA was assayed on intact cells. In the same experiment, the preincubated cells were lysed, and PKA was assayed on the lysates. Pretreatment of cells with cAMP, which is impermeable to membrane, brought about activation of ecto-PKA without activating intracellular PKA (Table 3). In contrast, pretreatment with 8-Cl-cAMP, which is membrane-permeable, resulted in activation of both ecto-PKA and intracellular PKA (Table 3). In addition, membrane-impermeable 8-N_3-cAMP activated only ecto-PKA without affecting soluble PKA, and 8-Cl-adenosine had no effect on either ecto-PKA or soluble-PKA (Table 3).

Ecto-PKA Regulation by Excreted cAMP. Considering that cAMP is known to be actively secreted by some cells into extracellular medium (*8*), we next examined whether these cells can provide cAMP for ecto-PKA in an autocrine fashion. We used forskolin as an agent elevating intracellular levels of cAMP. Forskolin stimulated Kemptide phosphorylation of intact cells to a level similar to that achieved by cAMP addition (Figure 4A). An inactive analogue of forskolin (1,9-dideoxyforskolin) did not stimulate the PKA

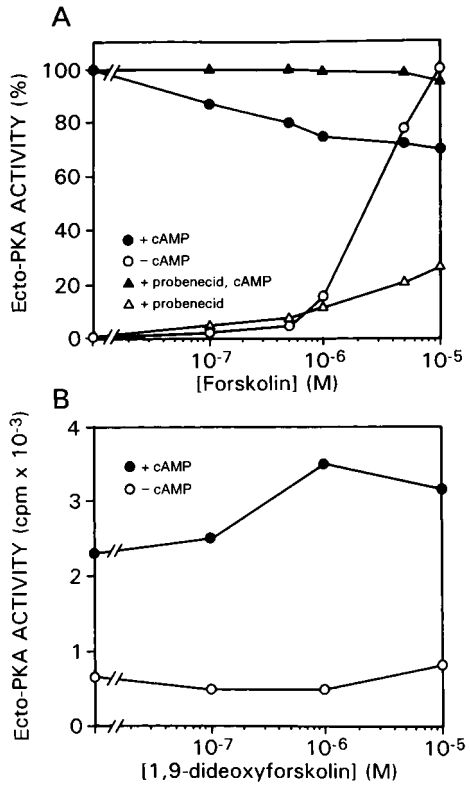

FIGURE 4: Forskolin stimulation of Kemptide phosphorylation by intact LS-174 cells. (A) Cells were incubated in MEM with different concentrations of forskolin in the absence (○) or presence of 5 μM cAMP (●), in the presence of 10 mM probenecid with 5 μM cAMP (▲) or without cAMP (△). (B) Cells were incubated in MEM with different concentrations of 1,9-dideoxyforskolin, with cAMP (●) or without cAMP (○). The results shown are the means of closely agreeing duplicate determinations from three independent experiments.

activity (Figure 4B) (*44*). The use of the renal transport inhibitor probenecid, which prevents cAMP extrusion, completely abolished the forskolin effect (Figure 4A). Thus, by blocking the export of cAMP, the activation of ecto-PKA was inhibited.

DISCUSSION

We have shown in the present study that the external surface of LS-174T colon carcinoma cells contains cAMP-binding proteins and exhibits protein kinase activity that is cAMP-dependent. The protein kinase appears to be an ecto-cAMP-dependent kinase (PKA) for the following reasons: (i) Photoaffinity labeling of intact cells with 8-N_3-[^{32}P]cAMP, which does not penetrate through plasma membranes, identified the surface-located cAMP-binding proteins. (ii) The 8-N_3-[^{32}P]cAMP-bound surface proteins were recovered in purified preparations of outside-out plasma membrane vesicles. (iii) Intact cells catalyzed phosphorylation of Kemptide, a phosphate acceptor substrate for PKA, in a cAMP-dependent manner. (iv) The specific inhibitory protein for PKA, PKI, abolished the cAMP-stimulated phosphorylation of Kemptide. (v) Forskolin, which elevates intracellular levels of cAMP, activated the Kemptide phosphorylation by intact cells. (vi) Agents that block cAMP extrusion abolished the activation of ecto-PKA.

The extracellular presence of PKA was previously found in HeLa, Chinese hamster ovary, and mouse lymphoma S49

cells (*17*). This ecto-PKA phosphorylated Kemptide in a cAMP-dependent manner and was inhibited by PKI (*17*). However, the identities of the cAMP-binding proteins were not determined.

In the present study, we used the photoaffinity labeling with 8-N_3-[^{32}P]cAMP to identify the surface-bound cAMP-receptor proteins taking advantage of the fact that 8-N_3-cAMP does not penetrate through plasma membranes of intact cells (Table 3) (*19*−*22*). We previously reported that purified preparation of plasma membranes from LS-174T cells in confluent growth phase contained the RIIα regulatory subunit of soluble PKA (*29*). If this protein is located on the outer surface of the cell membrane, it would be detected with 8-N_3-[^{32}P]cAMP by the photoaffinity labeling of intact cells. In fact, in intact cells at the confluent stage of growth, photoaffinity labeling with 8-N_3-[^{32}P]cAMP identified only one type of ecto-cAMP-binding protein, and this protein was immunoprecipitated with anti-RIIα, but not anti-RIα, antibody (Figure 2C, lane 3). In contrast, the homogenate of the same cells exhibited two types of cAMP-binding proteins, the RIα and RIIα regulatory subunits of soluble PKA (Figure 2B) (*26*). These results strongly indicate that the conditions used in the photoaffinity labeling of intact cells did not permit 8-N_3-[^{32}P]cAMP to penetrate inside the cell.

Outside-out plasma membranes are characterized by their binding to concanavalin A−Sepharose (*20*). The 8-N_3-[^{32}P]-cAMP-labeled surface proteins were recovered in the purified plasma membranes (Figure 1B) and the cAMP-binding proteins were detected in membrane vesicles prepared by concanavalin A−Sepharose (Figure 2A). These results provide strong evidence that the surface cAMP-binding proteins were oriented toward the external cellular space.

Importantly, photoaffinity labeling of intact cells in the logarithmic phase of growth exhibited two types of the surface-located cAMP-binding proteins that were immunoprecipitated with anti-RIα and anti-RIIα antibodies, respectively. Thus, when cells are in the logarithmic phase of growth, a new species of ecto-cAMP-binding protein, RIα, which was not found on cells during the confluent stage of growth, appeared. Taken together, our results demonstrated that LS-174T cells on the external surface express ecto-cAMP-binding proteins similar, if not identical, to the RIα and RIIα cAMP-binding proteins of soluble PKA, and that expression of these ecto-cAMP-binding proteins appears to be determined by the intracellular signals that regulate cell growth.

Intact LS-174T cells catalyzed the phosphorylation of Kemptide in a cAMP-dependent manner; this PKA is referred to as ecto-PKA. The conditions of phosphorylation were physiological as was shown previously (*17*) and did not cause cell damage as evidenced by the lack of permeability of the plasma membrane barrier; the uptake of the viability stain ethidium bromide or release of the cytoplasmic marker lactate dehydrogenase into the cell supernatant measured after the assay was as low as prereaction levels.

Several approaches were utilized to distinguish the intracellular/extracellular PKA. In cells treated with cell-damaging agents, the Kemptide phosphorylation in the presence of cAMP markedly increased, exceeding that of intact cells (Table 2). A clear distinction of ecto-PKA from intracellular soluble PKA was obtained in the experiments with pretreatment with cAMP versus 8-Cl-cAMP, a membrane-permeable analogue of cAMP. The results showed that after pretreatment of cells with cAMP, which is membrane-impermeable, ectokinase was highly activated, but intracellular soluble PKA was inactive and needed additional cAMP for Kemptide phosphorylation. In the case of 8-Cl-cAMP treatment, both ecto- and intracellular soluble PKA were activated. During treatment of cells with the ionophore alamethicin, which opens up cell membranes (*42*), the Kemptide phosphorylation by intact cells did not change either in the presence or in the absence of cAMP. The results show that even under conditions in which the cell membranes become permeable for both nucleotides (ATP and cAMP), Kemptide remained incapable of penetrating through the plasma membrane barrier. These results support the conclusion that the ecto-PKA detected in intact LS-174T colon carcinoma cells represent mainly, if not solely, the PKA located on the external surface of the cell.

Considering that cAMP is actively secreted by some cells into the extracellular medium (*8*), the question arises whether LS-174T cells are able to provide cAMP for ecto-protein kinase in an autocrine fashion. We used forskolin to elevate intracellular levels of cAMP. Our results show that forskolin stimulated Kemptide phosphorylation of intact cells to a level similar to that achieved by cAMP addition (Figure 4). An inactive analogue of forskolin (1,9-dideoxyforskolin) did not stimulate the PKA activity. The results indicate that cAMP is exported from the cell in an amount sufficient to activate ecto-protein kinase. However, the observed stimulation of Kemptide phosphorylation by forskolin may be the result of activation of soluble PKA inside the cell. To answer this question, we used the renal transport inhibitor probenecid, which prevents cAMP extrusion. This agent completely reversed the action of forskolin. Thus, by blocking the export of cAMP, the activation of ecto-protein kinase was inhibited.

We demonstrated for the first time that LS-174T colon carcinoma cells possess on their external cell surface ecto-cAMP-binding proteins and ecto-PKA. The ecto-PKA is very similar, if not identical, to the intracellular soluble PKA. This enzyme may phosphorylate specific substrates at the cell surface using intracellular ATP and cAMP extruded from inside the cell. This may occur in response to an increase in the intracellular concentrations of these nucleotides. Further studies are required to elucidate the physiological role of ecto-PKA.

REFERENCES

1. Davoren, P. R., and Sutherland, E. M. (1963) *J. Biol. Chem. 238*, 5296−5303.
2. Barber, R., and Butcher, R. W. (1983) in *Advances in Cyclic Nucleotides Research* (Greengard, P., and Robison, G. A., Eds.) pp 119−138, Raven Press, New York.
3. Darmon, M., Bachtel, P., and Periera da Silva, L. H. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3163−3166.
4. Bonner, J. T. (1982) in *The Development of Dictyostelium discoideum* (Lommis, W. F., Ed.) pp 1−33, Academic Press, New York.
5. Theibert, A., Klein, P., and Devreotes, P. N. (1984) *J. Biol. Chem. 259*, 12318−12321.
6. Broadus, A. E., Hardman, J. G., Kaminsky, N. I., Ball, J. H., Sutherland, E. W., and Liddle, G. W. (1971) *Ann. N.Y. Acad. Sci. 185*, 5−65.
7. Ridler, M. J., Bashor, M. M., Spitzer, N., and Saier, M. H., Jr. (1978) *J. Biol. Chem. 253*, 5431−5436.

8. Heasley, L. E., and Brunton, L. L. (1985) *J. Biol. Chem. 260*, 11514−11519.
9. Krebs, E. G., and Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923−939.
10. Ehrlich, Y. N., Hogan, M. V., Pawlowska, Z., Naik, U., and Kornecki, E. (1989) *Ann. N.Y. Acad. Sci. 550*, 401−416.
11. Walter, J., Kinzel, V., and Kübler, D. (1994) *Cell. Mol. Biol. Res. 40*, 473−480.
12. Kübler, D., Pyerin, W., and Kinzel, V. (1982) *J. Biol. Chem. 257*, 322−329.
13. Kübler, D., Pyerin, W., Burow, E., and Kinzel, V. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4021−4025.
14. Pyerin, W., Burow, E., Michaely, K., Kübler, D., and Kinzel, V. (1987) *Biol. Chem. Hoppe-Seyler 368*, 215−227.
15. Schlager, E. J., and Kohler, G. (1976) *Nature 260*, 705−707.
16. Kübler, D., Pyerin, W., and Kinzel, V. (1982) *Eur. J. Cell Biol. 26*, 306−309.
17. Kübler, D., Pyerin, W., Bill, O., Hots, A., Sonka, J., and Kinzel, V. (1989) *J. Biol. Chem. 264*, 14549−14555.
18. Walter, U., Uno, I., Liu, A. Y.-C., and Greengard, P. (1977) *J. Biol. Chem. 252*, 6494−6500.
19. Klein, P., Theibert, A., Fontana, D., and Devreotes, P. N. (1985) *J. Biol. Chem. 260*, 1757−1764.
20. Muller, G., and Bandlow, W. (1991) *Biochemistry 30*, 10181−10190.
21. Muller, G., and Bandlow, W. (1991) *Eur. J. Biochem. 202*, 299−308.
22. Muller, G., Wetekam, E.-M., Jund, C., and Bandlow, W. (1994) *Biochemistry 33*, 12149−12159.
23. Boman, B. M., Zschunke, M. A., and Scott, R. E. (1984) *J. Cell. Physiol. 121*, 357−367.
24. Atherton, R. W., Khatoon, S., Schoff, P. K., and Haley, B. E. (1985) *Biol. Reprod. 32*, 155−171.
25. Beebe, S. J., and Corbin, J. D. (1986) in *The Enzymes: Control by Phosphorylation* (Boyer, P. D., and Krebs, E. G., Eds.) pp 43−111, Academic Press, New York.
26. Nesterova, M. V., Yokozaki, H., McDuffie, E., and Cho-Chung, Y. S. (1996) *Eur. J. Biochem. 235*, 486-494.
27. Bergmeyer, H. V., Grassl, M., and Walter, H. E. (1983) in *Methods of Enzymatic Analysis*, (Bergmeyer, H. V., Ed.) 3rd ed., Vol. 2, pp 232−233, Verlag Chemie mbH, Weinheim, Germany.
28. Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L., and Gilman, A. G. (1977) *J. Biol. Chem. 252*, 5761−5775.
29. Kondrashin, A. A., Nesterova, M. V., and Cho-Chung, Y. S. (1998) *Biochem. Mol. Biol. Int. 45*, 237−244.
30. Lee, G. R., Kim, S. N., Noguchi, K., Park, S. D., Hong, S. H., and Cho-Chung, Y. S. (1998) *Mol. Cell. Biochem.* (in press).
31. Tortora, G., Clair, T., and Cho-Chung, Y. S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 705−708.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
33. Gilman, A. G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 305−312.
34. Tagliaferri, P., Katsaros, D., Clair, T., Neckers, L., Robins, R. K., and Cho-Chung, Y. S. (1988) *J. Biol. Chem. 263*, 409−416.
35. Rohlff, C., Clair, T., and Cho-Chung, Y. S. (1993) *J. Biol. Chem. 268*, 5774−5782.
36. Lever, J. (1982) *J. Biol. Chem. 257*, 8680−8686.
37. Ames, B. N. (1966) in *Methods in Enzymology* (Neufeld, E. F., and Ginsburg, V., Eds.) Vol. 8, pp 115−118, Academic Press, New York.
38. Avruch, J., and Wallach, D. F. H. (1971) *Biochim. Biophys. Acta 233*, 334−347.
39. Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstand, A. (1967) *J. Cell Biol. 32*, 415−438.
40. Hino, Y., Asano, A., Sato, R., and Shimuzu, S. (1978) *J. Biochem. (Tokyo) 83*, 909−923.
41. Gianetto, R., and DeDuve, C. (1955) *Biochem. J. 59*, 433−438.
42. Xie, Z., Wang, Y., Ganjeizadeh, M., Mebec, R., Jr., and Askari, A. (1989) *Anal. Biochem. 183*, 215−219.
43. Nesterova, M. V., Sashchenko, L. P., Vasiliev, V. Y., and Severin, E. S. (1975) *Biochim. Biophys. Acta 377*, 271−281.
44. Graves, L. M., Bornfeldt, K. E., Raines, E. W., Potts, B. C., Macdonald, S. G., Ross, R., and Krebs, E. G. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10300−10304.

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