Soluble and Membrane-Associated Human Low-Affinity Adenosine Binding Protein (Adenotin): Properties and Homology with Mammalian and Avian Stress Proteins†

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ABSTRACT: A low-affinity adenosine binding protein has recently been distinguished from the adenosine A_2 receptor and purified from human placental membranes. Soluble human placental extracts contain an adenosine binding activity that has properties similar to those of the membrane low-affinity adenosine binding protein. The binding protein was purified from soluble human placental extracts 134-fold to 89% purity with a B_{max} of 2.5 nmol/mg. It comprises 0.7–0.9% of the soluble protein. The major purified soluble protein has a subunit molecular mass of 98 kDa and a Stokes radius identical with that of the membrane-bound adenosine binding protein. Competition analysis of the soluble protein revealed similar affinities and an identical potency order for displacement of 5′-(*N*-ethylcarbamoyl)[2,8-^{3}H]adenosine ([^{3}H]NECA) as follows: NECA > 2-chloroadenosine > adenosine > (*R*)-N^6-(2-phenylisopropyl)adenosine. The soluble binding protein was more acidic than the membrane binding protein as revealed by a comparison of the elution properties during ion exchange chromatography. A second form of soluble adenosine binding activity comprised 17% of the major form and had a charge similar to that of the membrane binding protein, a smaller Stokes radius, and a subunit molecular mass of 74 kDa. Carbohydrate composition analysis revealed that the major soluble form has 4.3% carbohydrate by weight as compared to the membrane-associated form, which has 5.5% carbohydrate by weight. The amino-terminal sequence of each protein is identical and has identity with sequences in other proteins as follows: the glucose-regulated protein (GRP94) from hamster fibroblasts, 83% homology; the endoplasmic reticulum protein from murine plastocytoma tissue (ERp99), 94% homology; the murine tumor rejection antigen precursor (GP96), 94% homology; the chicken heat shock protein (HSP108), 87% homology. The relationship between the membrane and soluble low-affinity adenosine binding proteins is not known at present. The homology of the amino terminus to several stress-induced proteins indicates that the low-affinity adenosine binding proteins have a highly conserved amino-terminal sequence and may be human heat shock proteins. We propose the name *adenotin* to describe these unique low-affinity adenosine binding proteins.

Agonist and antagonist relative binding potencies for the site labeled with 5′-(*N*-ethylcarbamoyl)[2,8-^{3}H]adenosine ([^{3}H]NECA)[1] inconsistently parallel the stimulation of adenylate cyclase (Daly, 1985; Johnson, 1982; Phillis & Barraco, 1985; Schwabe & Trost, 1980; Ukena et al., 1985). In human platelet membranes this dilemma has been explained by the separation of two adenosine binding sites by gel filtration chromatography (Lohse et al., 1988). One binding site comprises 10–25% of the sites and has properties that correlate with adenosine A_2 receptor activation kinetics of adenylate cyclase. The second site represents the majority of the binding sites and has a pharmacological profile that does not consistently correlate with the adenosine A_2 receptor activation kinetics of adenylate cyclase.

The majority of the specific [^{3}H]NECA binding in most tissues studied is to this second ubiquitous binding site (Diocee & Souness, 1987; Florio et al., 1988; Fox & Kurpis, 1983; Hasday & Sitrin, 1987; Hutteman et al., 1984; Lee & Reddington, 1986; Nakata & Fujisawa, 1988; Reddington et al., 1986; Ronca-Testoni et al., 1988; Ukena et al., 1984a,b, 1985). The low-affinity adenosine binding protein is distinguished from the adenosine A_2 receptor by a submicromolar affinity for NECA, a high binding density ranging from 4.2 to 26 pmol/mg of protein, the lack of binding by N^6-substituted adenosine analogues and C^8-substituted xanthine derivatives, and the lack of binding changes with the addition of guanine nucleotides (Bruns et al., 1980, 1986; Diocee & Souness, 1987; Florio et al., 1988; Gavish et al., 1982; Huttemann et al., 1984; Klotz et al., 1986; Lohse et al., 1988; Martini et al., 1985; Nakata & Fujisawa, 1988; Ronca-Testoni et al., 1988; Schwabe & Trost, 1980; Stiles, 1985; Ukena et al., 1985).

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[1] Abbreviations: [^{3}H]NECA, 5′-(*N*-ethylcarbamoyl)[2,8-^{3}H]-adenosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ERp99, 99-kDa murine endoplasmic reticulum protein; GP96, 96-kDa murine tumor rejection antigen; GRP94, 94-kDa hamster glucose-regulated protein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HSP108, 108-kDa chicken heat shock protein; NECA, 5′-(*N*-ethylcarbamoyl)adenosine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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To gain insight into the function of this low-affinity adenosine binding protein, we purified it to homogeneity from human placental membranes (Hutchison & Fox, 1989). This ubiquitous protein is a homodimer of M_r 98 000 subunits and is asymmetric with a frictional ratio of 1.5 (Hutchison & Fox, 1989). During the course of our studies we have discovered a similar binding activity in soluble placental extract. We have purified this soluble binding protein and compared its physiochemical properties to those of the membrane-derived low-affinity adenosine binding site. We have demonstrated that these two proteins have similar characteristics and have amino-terminal homology to mammalian and avian stress proteins. Stress proteins are a group of proteins that are synthesized in response to stress or may be expressed in growing cells without exposure to environmental insult (Lindquist, 1986; Pelham, 1986; Schlesinger, 1986).

Materials and Methods

Materials

Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), poly(ethylenimine), glycerol, concanavalin A–Sepharose 4B, DEAE-Sephadex, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Lubrol-PX, phenylmethanesulfonyl fluoride, dithiothreitol, β-mercaptoethanol, methyl α-mannoside, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) protein standards (molecular weight 29 000–200 000) 5′-(*N*-ethylcarbamoyl)adenosine (NECA), (R)-N^6-(2-phenylisopropyl)adenosine, 2-chloroadenosine, adenosine, 3-isobutyl-1-methylxanthine, theophylline, and bovine serum albumin were all from Sigma (St. Louis, MO). 5′-(*N*-Ethylcarbamoyl)[2,8-^{3}H]adenosine (22–31 Ci/mmol) was from Amersham (Arlington Heights, IL). NECA was also from Boehringer-Mannheim (Indianapolis, IN). *N*-Ethylmaleimide was from ICN (Irvine, CA). Sepharose 6B and protein gel filtration standards were from Pharmacia (Piscataway, NJ). Filters for pressure ultrafiltration (YM-10) were from Amicon (Danvers, MA). *o*-Phenanthroline was from Aldrich (Milwaukee, WI). Acrylamide, bis(acrylamide), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, and ammonium persulfate were from Bio-Rad (Richmond, CA). Gf/C glassfiber filters were from Whatman (Maidstone, England). Ecoscint A was from National Diagnostics (Manville, NJ). All other chemicals were reagent grade or better and were from standard suppliers.

Terminology

This low-affinity adenosine binding protein has a confusing history, since it has been identified as the adenosine A_2 receptor in many reports. We have used the term "adenosine A_2-like binding site" in earlier publications (Hutchison & Fox, 1989; Work et al., 1989). This seems to be inappropriate terminology in light of our current findings. We propose the name *adenotin* for this low-affinity adenosine binding protein.

Methods

Tissue Preparation and Purification. Membranes and supernatants were prepared from donor human placentas obtained after normal vaginal delivery or Caesarean section as previously described (Hutchison & Fox, 1989). Five volumes of homogenizing buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride, 0.1 mM *o*-phenanthroline, 0.1 mM EGTA, and 1 mM β-mercaptoethanol was used to homogenize two volumes of placental tissue with three 5-s bursts of a Sorvall Omnimixer. The homogenate was centrifuged at 2000*g* for 10 min and then at 48000*g* for 15 min. The supernatant from this spin was taken and stored at −70 °C.

Soluble adenotin was purified from 1 L of placental supernatant as previously described for the membrane-derived protein (Hutchison & Fox, 1989). The steps of purification included ammonium sulfate precipitation, lectin affinity chromatography with concanvalin A–Sepharose 4B, ion exchange chromatography with DEAE-Sephadex, and gel filtration chromatography with Sepharose 6B (Hutchison & Fox, 1989).

During ion exchange chromatography two peaks of binding activity were eluted: peak A and peak B (Figure 1). Further purification was routinely performed upon peak B, which was the major peak of activity. Throughout this paper, studies of soluble adenotin refer to peak B unless otherwise stated.

Membrane adenotin was purified as previously described (Hutchison & Fox, 1989).

Amino Acid and Carbohydrate Analyses. Amino acid and carbohydrate analyses were performed in the Protein Structure and Design core facility in the University of Michigan Medical School. For amino acid analysis, samples were reduced and alkylated before hydrolysis (Mise & Bahl, 1981). Amino acids were separated by cation exchange chromatography and postcolumn derivatization (Cole et al., 1985). For carbohydrate analyses, samples were prepared as previously described (Fujiwara et al., 1987) with the same system as amino acid analysis. Sialic acid was not measurable.

Amino Acid Sequence Determination. Automated Edman degradation was performed directly upon purified protein with a Model 470A Applied Biosystems gas-phase sequencer equipped with a 120A PTH analyzer (Henzel et al., 1987). PTH amino acids were integrated with a Nelson Analytical Model 3000 data system. Sequence interpretation was performed on a Vax computer (Digital). Homology to other proteins was examined by a search of the Dayhoff protein database (Release 19.0, December 31, 1988).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). The subunit molecular weights for membranes and supernatants were determined by SDS–PAGE by the method of Laemmli (1970) using 8% running and 4% stacking polyacrylamide gels. All samples and standards (100 μL each) were reduced and alkylated before electrophoresis as previously described (Evans et al., 1986; Hutchison & Fox, 1989). Molecular weights of unknown proteins were estimated by constructing a standard curve of log (molecular weight) versus R_f (ratio of distance migrated by each protein as compared to the distance migrated by the bromophenol blue dye front). A second-order polynomial regression of log (molecular weight) of the standards on R_f was performed. The resulting equation was used to convert R_f values of molecular weight values for the unknown proteins.

Binding Assay. Assays were performed in triplicate in a total volume of 250 or 425 μL in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM *o*-phenanthroline, and 1 mM β-mercaptoethanol containing 10–20 nM [^{3}H]NECA, adenosine analogues in binding buffer for displacement analysis, or 100 μM NECA for nonspecific binding and 50–200 μL of extracts. Binding was initiated by the addition of radioactive ligand. Incubations were terminated after 30 min by the addition of 3 mL of ice-cold binding buffer and rapid vacuum filtration over Whatmann Gf/C filters previously soaked in 0.3% poly(ethylenimine) (Bruns et al., 1983). The filters were placed into scintillation vials with 5 mL of Ecoscint A and

Table I: Purification of Soluble Adenotin

step	vol (mL)	protein (mg)	binding (pmol)	sp act. (pmol/mg)[a]	recovery (%)	x-fold
supernatant	1000	4710.0	10000	2.1	100	1
ammonium sulfate precipitation	150	1675.5	9635	5.8	96	2.8
concanavalin A chromatography	259	207.2	5381	26.0	54	12.4
DEAE-Sephadex chromatography	3.0	25.1	5311	211	53	101
Sepharose 6B gel filtraton chromatography	5.0	10.6	2960	281[b]	30	134

[a] Assayed at 20 nM [^{3}H]NECA. [b] B_{max} is 2.5 nmol/mg as determined by saturation analysis (Figure 3).

allowed to stand overnight before being counted in an LKB Racbeta liquid scintillation spectrometer system at 50% efficiency.

Specific binding represented from 60% to greater than 90% of the total binding for extracts. Binding was linear with protein to at least 700 μg/assay. Nonspecific binding for extracts was from 100 to 300 cpm.

Protein Assay. Protein was measured by the method of Bradford (1986) using bovine serum albumin as the standard. CHAPS at 0.015% (w/v) or Lubrol-PX at 0.005% (v/v) did not modify the assay. When lower dilutions of detergents were necessary, the appropriate detergent blank was subtracted from protein assays.

Data Analysis. Saturation analyses were fit to a rectangular hyperbola with the program GRAPHPAD (ISI Software). IC_{50} values were obtained from displacement experiments by fitting the raw data to a single-site competitive model. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (1973). The saturation analysis and competition analysis indicated a one-site model.

RESULTS

Soluble Binding Site

Preliminary studies indicated the existence of an adenosine binding activity in the 48000*g* placental supernatant (data not shown). The binding had an IC_{50} of 190 nM for displacement of NECA by itself. This activity was precipitated with ammonium sulfate, bound to concanavalin A–Sepharose, and eluted from DE-52 (aminoethyl)cellulose in a manner similar to that of membrane adenotin (Hutchison & Fox, 1989). This binding activity also coeluted with membrane adenotin during gel permeation HPLC. To test the solubility of this binding site, placental supernatant was centrifuged for 75 min at 110000*g*. The NECA binding activity in the supernatant was 93% of the total binding activity found in placental 48000*g* supernatants.

Purification. Soluble adenotin was purified 134-fold to a final specific activity of 2.5 nmol/mg (B_{max}) with a yield of 30% from the initial supernatant (Table I). In another purification soluble adenotin was purified 94-fold to a final specific activity of 1.4 nmol/mg (B_{max}) with an 18% recovery. On the basis of the *x*-fold purification, we estimate that adenotin comprises 0.7–0.9% of the soluble placental protein.

Two Species Resolved by Ion Exchange Chromatography. Two peaks of adenosine binding activity, peaks A and B, were eluted from the DEAE-Sephadex column (Figure 1A). Peak A contained 17% of the [^{3}H]NECA binding activity in peak B and eluted between 275 and 325 mM KCl. Membrane adenotin was previously observed to elute under conditions identical with those of peak A (Hutchison & Fox, 1989). Peak B eluted at 375–425 mM KCl. These data indicate that soluble peak B is more acidic than both peak A and membrane adenotin.

The relationship between these two peaks became more apparent during a second purification using the identical placental supernatant that was used in the first purification

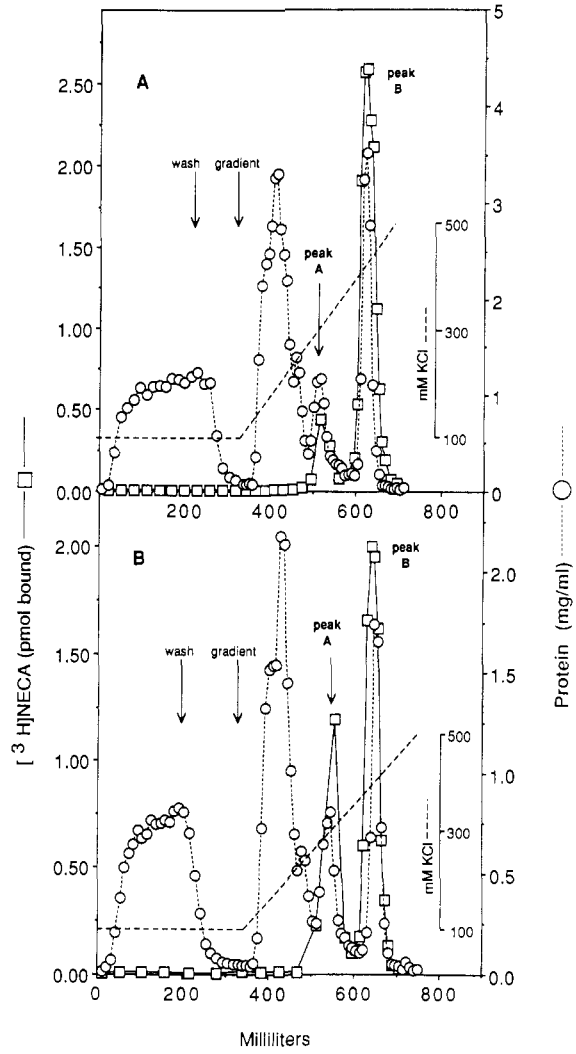

FIGURE 1: Purification of soluble adenotin from human placenta. (A) Ion exchange chromatography I. 258 mL of concanavalin A eluate containing 206 mg of protein was loaded into DEAE-Sephadex (6 × 1.5 cm). The column was washed with 100 mL of buffer A and eluted with a 400-mL gradient of 100–500 mM KCl in buffer A. Fractions 104–111 were pooled (57.6 mL) and concentrated to 3.0 mL. (B) Ion exchange chromatography II. In another purification, 187 mL of concanavalin A eluate containing 160 mg of protein was applied to the same column and eluted as described above. Fractions 51–58 (68 mL, peak A) and fractions 65–69 (42.5 mL, peak B) were pooled and concentrated.

described. Material in this second purification had been subjected to two cycles of freezing and thawing after preparation of the placental homogenate and was stored at −70 °C for 2 weeks. After ion exchange chromatography, peak A was 35% of the [^{3}H]NECA binding activity of peak B (Figure 1B). This suggested a shift in binding activity from peak B to peak A upon storage of placental supernatant. Total activity from the second purification was similar to that of the first purification when adjustments were made for the total protein used. After gel filtration chromatography peak A was purified to

Table II: Saturation Analysis of Membrane-Derived and Soluble Adenotin[a]

source of adenotin	K_d (nM)	B_{max} (nmol/mg)
cytosol[b]	220 (170, 270)	2.0 (1.4, 2.5)
membranes[c]	210 (170, 250)	1.7 (1.5, 1.9)

[a] Purified extracts (1.3–2.9 μg of membrane and 0.5–2.9 μg of soluble protein) were incubated in 425 μL of binding buffer with [^{3}H]-NECA from 5 nM to 1 μM. For total ligand concentrations above 120 nM, the specific activity of [^{3}H]NECA was diluted with NECA to reduce the nonspecific binding. Data were fit by nonlinear least-squares regression with GRAPHPAD program (ISI Software) to a rectangular hyperbolic model. [b] Value represents average of two determinations in triplicate. The values in parentheses represent individual determinations. [c] Data are taken from Hutchison and Fox (1989).

Table III: Equilibrium Displacement Analysis of Membrane-Derived and Soluble Adenotin[a]

ligand	K_i (μM) soluble[b]	K_i (μM) membrane[b,c]
NECA	0.18	0.37
2-chloroadenosine	1.5	1.8
adenosine	8.4	20
(*R*)-phenylisopropyladenosine	>1000 (90)[d]	>1000 (70)[d]
isobutylmethylxanthine	10	22
theophylline	170	220

[a] Membrane extracts were purified through DEAE-Sephadex chromatography before assay. Soluble binding was purified through Sepharose 6B gel filtration chromatography before assay. Competition analysis was performed with 5 μg of membrane protein and 1.1 μg of soluble protein in a total volume of 425 μL. The control specific binding at equilibrium ranged from 435 to 594 fmol per tube for the purified membrane site and from 85 to 144 fmol per tube for the purified soluble site. Nonspecific binding was 7.4 fmol per tube. IC_{50} values were obtained by nonlinear least-squares regression with the GRAPHPAD program (ISI Software) fit to a model describing competitive binding at one site. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (1973). [b] The extract has a specific activity of 281 pmol/mg, measured with 20 nM [^{3}H]NECA. [c] Data for placental membranes were described previously by Hutchison and Fox (1989). [d] Value in parentheses represents the percent of control binding obtained in displacement studies using 1 mM (*R*)-phenylisopropyladenosine.

76 pmol/mg of protein, and peak B was purified to 166 pmol/mg of protein.

Gel filtration chromatography was performed upon Sepharose 6B to compare the Stokes radii of the membrane-derived adenotin, peak A from DEAE-Sephadex, and peak B from DEAE-Sephadex. We have previously determined the Stokes radius of placental membrane adenotin to be 70 Å (Hutchison & Fox, 1989). Membrane adenotin eluted in 121 mL, the soluble peak A adenosine binding protein eluted in 132 mL, and the soluble peak B adenosine binding protein eluted in 120 mL from the Sepharose 6B column. This indicates that both the membrane and soluble peak B adenotin have similar Stokes radii, while the binding protein from soluble peak A has a smaller Stokes radius.

The description below of the properties of soluble adenotin refer to peak B unless otherwise indicated.

Binding Properties. The purified soluble site had an average K_d for [^{3}H]NECA of 220 nM with an average B_{max} of 2.0 nmol/mg (Table II). These values are similar to the K_d and B_{max} observed for membrane adenotin of 210 nM and 1.7 nmol/mg, respectively (Hutchison & Fox, 1989). Equilibrium displacement analysis of purified soluble adenotin revealed K_i values for NECA, 2-chloroadenosine, isobutylmethylxanthine, and theophylline of 0.18, 1.5, 10, and 170 μM, respectively (Table III). The K_i values for these ligands were similar to that of purified membrane adenotin (Hutchison & Fox, 1989). Equilibrium displacement analysis of peak A from the

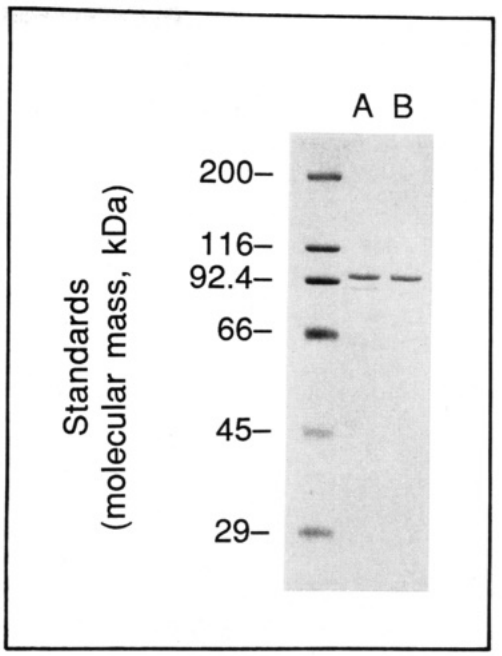

FIGURE 2: SDS–PAGE of soluble and membrane-derived adenotin. 100-μL samples obtained from pooled gel filtration samples were prepared as described under Materials and Methods and then subjected to SDS–PAGE. Proteins were visualized by Coomassie brilliant blue staining. The molecular weight standards used were as follows: myosin, 200 000; β-galactosidase, 116 000; phosphorylase B, 92 400; bovine serum albumin, 66 000; egg albumin, 45 000; carbonic anhydrase, 29 000. (Lane A) 1.5 μg of soluble pool; (lane B) 1.0 μg of membrane pool.

DEAE-Sephadex column gave an IC_{50} of 0.17 μM for NECA. One millimolar (*R*)-(phenylisopropyl)adenosine caused 64% inhibition of the control [^{3}H]NECA binding. These results are similar to the properties of peak B and indicate that peak A has the properties of adenotin.

In both purified membranes and supernatants 1 M KCl and NaCl stimulated [^{3}H]NECA binding from 130 to 155% of the control value. Ten millimolar $CaCl_2$ and $MgCl_2$ did not substantially alter [^{3}H]NECA binding to crude and purified supernatant extracts.

Subunit Molecular Weight. SDS–PAGE of the extracts from Sepharose 6B gel filtration chromatography revealed a major band of 98 kDa (Figure 2). Scanning gel densitometry revealed that the 98-kDa band comprised 89% of the Coomassie brilliant blue stainable material. There was a minor contaminating band containing 11% of the total stainable protein. The subunit molecular mass was identical with the 98-kDa value for purified membrane adenotin (Figure 2, lane B).

SDS–PAGE of peak A, further purified by gel filtration chromatography, revealed two major bands (Figure 3). The existence of one major contaminating band provides a basis for explaining why the specific activity of peak A was half the specific activity of peak B following purification.

We propose that the lower molecular weight band is adenotin. The basis for our conclusion is as follows. Only one of the two bands had a molecular mass less than the 98-kDa soluble adenotin. Peak A had a smaller Stokes radius than adenotin during gel filtration chromatography. Since peak A had properties of adenotin, we concluded that its subunit is likely to be 98 kDa or less. This proposal is further supported by our observation that the intensity of staining of this 74-kDa band was highly correlated with the quantity of [^{3}H]NECA binding in five separate fractions eluted from the gel filtration column ($r = 0.99$). The latter correlation was

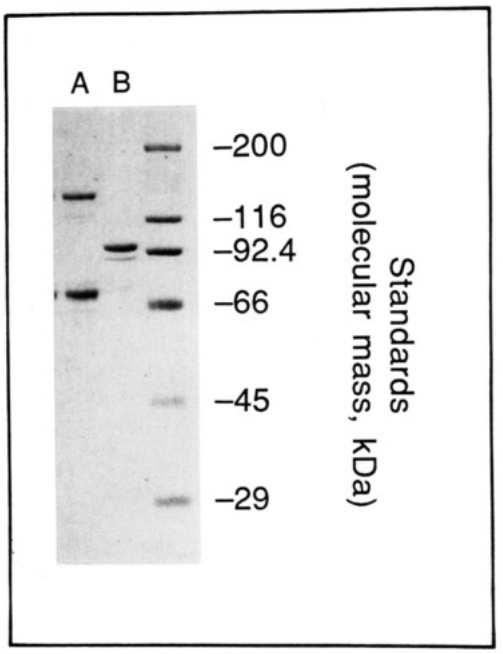

FIGURE 3: **SDS–PAGE of soluble adenotin. Aliquots were prepared** and electrophoresed with molecular mass standards as described in Figure 2. (Lane A) 4.4 μg of purified soluble adenotin (peak A) from DEAE-Sephadex chromatography; (lane B) 3.0 μg of purified soluble adenotin (peak B).

performed according to methods previously described (Hutchison & Fox, 1989).

Structural Studies of Membrane and Soluble Binding Proteins

Amino Acid and Carbohydrate Composition. Amino acid composition analysis revealed that the membrane-bound site has an amino acid composition similiar to that of the soluble binding site (Table IV). Both sites contain a large amount of potentially acidic amino acids and basic amino acids. Carbohydrate analysis revealed that the membrane-derived protein was 5.5% carbohydrate by weight and the soluble protein was 4.3% carbohydrate by weight. Membrane and soluble adenosine A_2-like binding sites appeared to have both O-linked and N-linked carbohydrate chains on the basis of the carbohydrate composition (Table IV). The oligosaccharide chain may have been processed or degraded, thus explaining the unusual ratio of carbohydrates in the two forms.

Amino-Terminal Sequence. Twenty-six of the first 29 amino acids of membrane adenotin were identical with the first 29 amino acids of soluble adenotin (Table V). The other three amino acids from soluble adenotin could not be identified. These adenotin amino-terminal peptides had 94% identity with residues from a highly conserved endoplasmic reticulum protein from mouse plastocytoma tissue (ERp99) (Mazzarella & Green, 1987). Eighty-three percent of the sequenced residues of the 94-kDa glucose-regulated protein (GRP94) from hamster fibroblasts were also identical with the sequenced residues of adenotin (Lee et al., 1984). There was 94% homology of the total identified residues in the amino terminus of adenotin with residues 22–68 of the murine tumor rejection antigen (GP96) (Srivastava et al., 1987). There was 87% homology in the amino terminus of adenotin with residues 21–67 of the chicken 108-kDa heat shock protein (HSP108) (Kulomaa et al., 1986).

Table IV: Amino Acid and Carbohydrate Composition of Membrane-Derived and Soluble Adenotin[a]

	mol % of residue per subunit	
amino acid	membrane	soluble
As(x)	12.7	12.8
Thr	6.1	6.1
Ser	7.2	7.3
Gl(x)	17.6	16.8
Pro	3.5	4.3
Gly	6.1	6.0
Ala	6.0	6.0
Val	4.3	4.2
Met	1.6	1.8
Ile	4.2	4.0
Leu	7.9	8.1
Tyr	3.8	3.7
Phe	3.8	4.0
Lys	8.3	8.0
His	0.9	0.5
Arg	4.3	4.8
CMC[b]	1.7	1.6
Trp	nd[c]	0.1
	residues per 98-kDa protein	
carbohydrate[d]	membrane	soluble
$GlcNH_2$	6.3	7.5
$GalNH_2$	2.0	2.2
Man	9.5	6.1
Gal	3.7	4.5
Fuc	0.4	0.6

[a] 39.4 or 41.7 μg of soluble and 156 or 163 μg of membrane protein were analyzed for amino acids or carbohydrates, respectively, as described under Methods. [b] (Carbomethyl)cysteine. [c] nd = not determined.

Table V: Sequences of the Amino Terminus of Soluble and Membrane-Derived Placental Adenotin and Homologous Residues of GRP94, ERp99, GP96, and HSP108[b]

	1 5 10 15 20 25 30 35 40 45
Human Adenosine A2-like site (soluble):	DDEVDVDGTVEEDLGKSREGSRTDDEVVQRXEEAIQLDGLNASQXR
Human Adenosine A2-like site (membrane):	DDEVDVDGTVEEDLGKSREGXRTXDXVVQ §
Hamster Glucose Regulated Protein (GRP94):	DDEVDVDGTVEEDLXXK # §
Murine Endoplasmic Reticulum Protein (ERp99)[a]:	DDEVDVDGTVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIR
Murine Tumor Rejection antigen (GP96)[a]:	DDEVDVDGTVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIR
Chicken Heat Shock Protein (HSP108)[a]:	• # # • AEEVDVDATVEEDLGKSREGSRTDDEVVQREEEAIQLDGLNASQIK

[a] Residues are numbered from the amino terminus of the mature protein: GP96, residues 22–68; HSP108, residues 21–67. [b] Symbols: (*) indicates that the residue is a conservative substitution from adenotin; (#) indicates that the residue is a nonconservative substitution from adenotin; (X) designates an undetermined amino acid; (§) designates an uncertain amino acid.

DISCUSSION

A low-affinity adenosine binding protein has only recently been distinguished from the adenosine A_2 receptor in human platelet membranes (Lohse et al., 1988). The purified low-affinity adenosine binding protein is a homodimer of 98 kDa comprising approximately 1% of the membrane protein (Hutchison & Fox, 1989). Our results indicate the existence of a soluble low-affinity adenosine binding protein that is ubiquitous and comprises approximately 0.7–0.9% of the soluble protein from human placenta. This soluble binding site is virtually identical with the human placental membrane

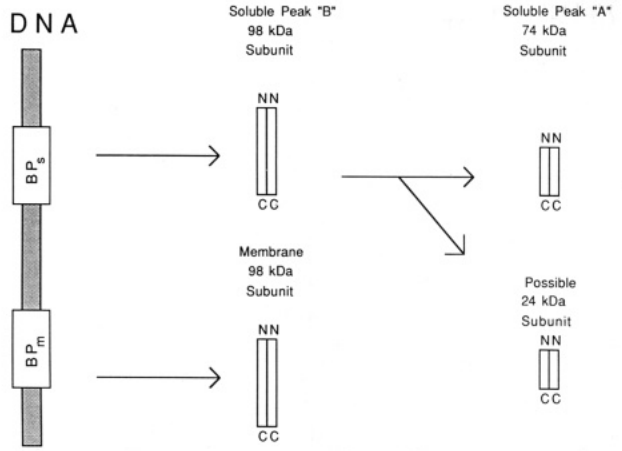

FIGURE 4: Hypothetical model to explain different charge and molecular mass forms of adenotin in human placenta. This diagram proposes a model to explain the three forms of adenotin that we have isolated. Two highly homologous genes (BP_s and BP_m) or alternative splicing of the same gene may lead to the synthesis of the membrane-derived protein or the soluble protein (peak B). By proteolysis, soluble peak B is converted to soluble peak A. We propose that a silent 24-kDa acidic polypeptide is released. The adenosine binding site is located on the 74-kDa protein. BP_s and BP_m designate the genes for the soluble and membrane-derived adenotin, respectively. Amino termini are shown as N and carboxy termini as C. Adenotin is shown as a linear side-by-side dimer, as conformational information is not known at present. Soluble peaks A and B refer to soluble adenotin eluted during ion exchange chromatography (Figure 1).

low-affinity adenosine binding protein in terms of specific activity, agonist and antagonist binding, monovalent and divalent cation effects, subunit molecular mass, Stokes radius, and amino-terminal sequence. We have named this membrane and soluble binding protein adenotin to clearly differentiate it from adenosine receptors.

Despite the high degree of similarity between membrane and soluble adenotin, these two proteins have a different isoelectric pH as shown by their elution properties during ion exchange chromatography. We propose a hypothetical model to explain the relationships between the charge and molecular mass of the three distinct adenotins (Figure 4). The stronger negative charge of the soluble protein as compared to the membrane protein is a structural difference, which may result from alterations in aspartate or glutamate content. Therefore, we propose that these proteins are the products of two highly homologous genes, or alternative splicing of the same gene. However, other hypotheses, involving posttranslational modifications of sialic acid content or different states of phosphorylation, remain highly plausible explanations.

Our model also proposes that soluble adenotin peak B (98-kDa subunit molecular mass) is converted to peak A (74-kDa subunit molecular mass) by proteolytic degradation during storage of placental supernatants (Figure 4). The proposed 24-kDa second protein fragment cannot be detected at present, since it does not contain a functional NECA binding site. Whether degradation of soluble adenotin occurs and is an artifact of preparation and storage, or occurs to a more limited extent in vivo, cannot be distinguished by our studies. Further studies will be necessary to establish the true relationship among these three proteins.

The physiological role of adenotin may relate to its striking amino-terminal homology with stress protein sequences in chicken HSP108, murine tumor rejection antigen GP96, murine ERp99, and hamster GRP94 (Kulomaa et al., 1986; Lee et al., 1984; Mazzarella & Green, 1987; Srivastava et al., 1987). The activities of heat shock proteins include protection during thermal shock, glucose starvation, viral infection, metabolic insult, prevention of transformation to the DNA binding state of steroid receptors, and regulation of protein synthesis initiation (Koyasu et al., 1986; Pratt, 1987; Szyszka et al., 1989). HSP100 and -90 are highly homologous to ERp99, GRP94, and HSP108 and complex with actin (Koyasu et al., 1989). In addition, HSP90 forms a complex with the steroid hormone receptor (Denis et al., 1988; Sanchez et al., 1987a,b) and avian sarcoma virus transforming genes (Adkins et al., 1982; Brugge et al., 1981; Lipsich et al., 1982; Oppermann et al., 1981). It seems likely that adenotin belongs to this class of proteins and has a similar biological role in addition to adenosine binding.

Our recent studies indicate that adenotin occurs together with adenosine A_1 or A_2 receptors in placenta, platelets, rat pheochromocytoma PC-12 cells, and JAR choriocarcinoma cells (Work et al., 1989; C. Work, K. A. Hutchison, M. Prasad, S. Zolnierowicz, and I. H. Fox, unpublished results). It is possible that the relationship between the 90-kDa heat shock protein and the mammalian steroid receptor (Denis et al., 1988; Sanchez et al., 1987a,b) is a model for an as yet unknown protein to protein interaction of other mammalian stress proteins such as adenotin. Whether there is a role for adenosine in regulating the function of these proteins remains to be determined.

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