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Production and Characterization of Multiple Antigenic Peptide Antibodies to the Adenosine A_{2b} Receptor

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SUMMARY

A polyclonal antibody to the human adenosine A_{2b} receptor ($A_{2b}R$) was produced by immunizing a chicken with a multiple antigenic peptide consisting of eight copies of a 16-amino acid peptide, corresponding to the presumed second extracellular loop of the $A_{2b}R$, linked to a branched lysine core. Western blotting with affinity-purified antibody revealed the human $A_{2b}R$ to be a protein of approximately 50–55 kDa, found in a variety of tissues including thymus, colon, and small intestine. The antibody also recognized mouse and rat $A_{2b}Rs$ and revealed heterogeneity in size, with a 35-kDa protein being detected in small intestine in addition to the larger 50–52-kDa species in thymus, colon, and placenta. The chicken anti-human $A_{2b}R$ peptide antibody recognized the receptor in both frozen and formalin-fixed tissue sections. In human colon, the $A_{2b}R$ was highly expressed in epithelial cells of the crypts. $A_{2b}R$ immunoreactivity was also apparent in syncytiotrophoblast cells of human placental villi and in the basal zone of murine chorioallantoic placenta. These cell type-specific patterns of expression are consistent with the hypothesized roles of the $A_{2b}R$ in mediating electrogenic Cl^- secretion and the resulting secretory diarrhea caused by colonic crypt abscesses and in regulating morphogenesis of the placenta. Insight into the multiple physiological consequences of $A_{2b}R$ engagement will be forthcoming from an analysis of the cell type-specific expression of this receptor in additional tissues.

ARs are members of the large family of G protein-coupled receptors whose activation on the cell surface can influence signal transduction events through cAMP and ion fluxes (1). These receptors have diverse input into many important physiological responses, including platelet aggregation, cardiac rate, smooth muscle tone, inflammation, neurotransmission, and cell growth and death (2–6). Considerable interest in the classification of AR subtypes in specific tissues derives largely from the therapeutic potential of selective and nonselective AR agonists and antagonists (7). Currently, four AR subtypes are known (A_1, A_{2a}, A_{2b}, and A_3), which differ in their affinity for adenosine and in their tissue distribution (8–11).

The $A_{2b}R$ has a low affinity for adenosine (12) and is poorly characterized pharmacologically and physiologically. Northern blot analysis has demonstrated pronounced expression of $A_{2b}R$ transcripts in tissues such as brain, spinal cord, caecum, large intestine, and urinary bladder (13). More recently, high-level expression of $A_{2b}R$ transcripts has been detected in the T84 human intestinal cell line (14) and in the murine gestation site (15). The T84 cell line displays colon crypt-like features. Stimulation by neutrophil-derived adenosine of the apical membrane $A_{2b}R$ of these cells leads to an electrogenic Cl^- efflux and a secretory response, providing a model for the secretory diarrhea seen in various inflammatory intestinal disorders (14, 16). Dynamic expression of the $A_{2b}R$ has also been demonstrated in the early postimplantation uterus of pregnant mice and in the ectoplacental cone of developing mouse embryos. Engagement of ARs in these tissues may serve as a transitory maternal implantation signal that delays ectoplacental outgrowth until the enzymatic differentiation of the secondary decidua has occurred (15).

Due to the lack of widely available agonists and antagonists specific for the $A_{2b}R$ and the limitations of *in situ* hybridization, we were motivated to generate an immunolog-

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ABBREVIATIONS: AR, adenosine receptor; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; MAP, multiple antigenic peptide; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ADA, adenosine deaminase; PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

ical reagent to detect the cell type-specific tissue distribution of the $A_{2b}R$. Recent development of an anti-peptide antibody to the canine $A_{2a}R$ demonstrated the usefulness of such a reagent for studying the tissue distribution and molecular weight differences of $A_{2a}Rs$ by immunoblotting and immunoprecipitation (17). Therefore, the amino-terminal half of the presumed second extracellular loop of the human $A_{2b}R$ was chosen for the synthesis of a MAP for immunizing chickens. This region of the human $A_{2b}R$ is homologous to the region of the canine $A_{2a}R$ that was previously used successfully to make an anti-peptide antibody recognizing intact $A_{2a}R$ (17). Our MAP consisted of eight copies of a 16-amino acid peptide coupled to an octa-branched lysine core. MAP synthesis results in a complex immunogen that is not conjugated to a carrier protein, avoiding the risk of the carrier protein becoming immunogenic and the risk of generating antibodies to epitopes derived from interactions between the peptide and carrier (18). Here we describe the generation and characterization of a MAP antibody to the human $A_{2b}R$ and demonstrate its utility in analyzing the cell and tissue distribution of the $A_{2b}R$ by immunohistochemistry and Western blotting.

Fig. 1. Proposed structure of the human $A_{2b}R$, showing the location of the amino acid sequence in the MAP. The proposed structure is based upon the model of the rat $A_{2b}R$ described by Stehle *et al.* (13). The amino acids used in making the MAP are shown in *black*. *Y*, potential *N*-linked glycosylation sites.

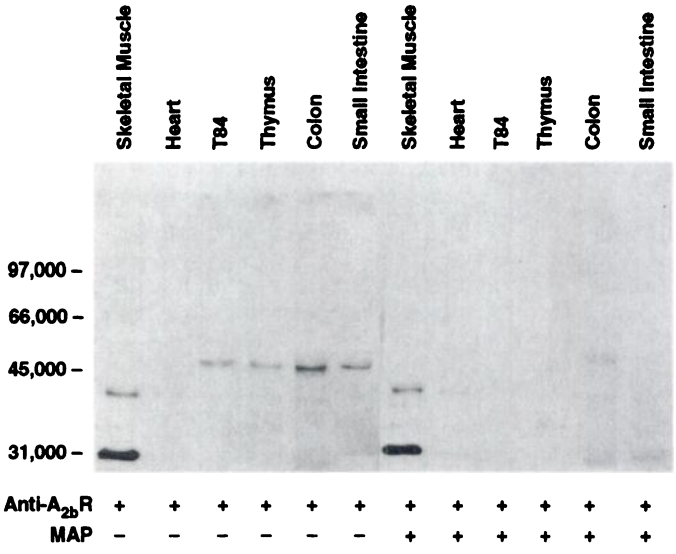

Fig. 2. Western blot analysis of human tissues with anti-$A_{2b}R$ peptide antibody. Detergent extracts of human tissues and the human T84 cell line were subjected to 9% SDS-PAGE under reducing conditions and analyzed for $A_{2b}R$ immunoreactivity by Western blotting. The quantities of protein loaded from each tissue were as follows: skeletal muscle and heart, 15 μg; T84 cells, thymus, and colon, 10 μg; small intestine, 20 μg. *Right half*, before development of the blot, the affinity-purified anti-human $A_{2b}R$ peptide antibody was incubated with a 20-fold excess of the immunizing MAP construct.

Materials and Methods

Cells and tissues. Fresh human colon was obtained from the operating room of University Hospital (Oklahoma City, OK) during resection for adenocarcinoma. Only histologically normal tissue remote from the cancer was utilized. Adult mouse tissues were obtained from 6–8-week-old BALB/c mice euthanized by ether overdose and/or cervical dislocation. Fetal mouse tissues were obtained from pregnant CD-1 mice at 15 days of gestation, with day 0 being defined as the day the cervical plug was observed. Rat colon was obtained from an anesthetized, 10-week-old, male, Sprague-Dawley rat gavaged with Co-lyte. The human colon adenocarcinoma cell line T84 (CCL 248) was obtained from the American Type Culture Collection.

Antibody generation and affinity purification. A MAP was engineered for immunization by synthesizing eight copies of a 16-amino acid peptide (ATNNSTEPWDGTTNES), corresponding to a portion of the deduced amino acid sequence (10) from the putative second extracellular loop of the human $A_{2b}R$ (Fig. 1), on an octa-branched lysine core (18). A single serine was substituted for cysteine at position 5 from the amino terminus of the authentic sequence, to avoid the formation of disulfide bonds during MAP synthesis. Anti-peptide antibodies were generated by immunizing a hen with 1 mg of the MAP in complete Freund's adjuvant. The hen was boosted 28 days later with 1 mg of MAP in incomplete Freund's adjuvant, and eggs were collected daily after an additional 10 days. Antibodies were isolated from egg yolks by two polyethylene glycol precipitations, as described by Gassmann *et al.* (19). Approximately 70 mg of partially purified IgY were recovered from each egg yolk. Final purification of antibodies was achieved by affinity chromatography, using the immunizing MAP coupled to CNBr-activated Sepharose 4B (Pharmacia), according to the manufacturer's instructions. Approximately 17 mg of affinity-purified antibody were recovered from 25 egg yolks.

The anti-peptide antibodies were assayed in a solid-phase ELISA as described previously (20). Briefly, 96-well polyvinyl microtiter plates were coated with the MAP at 10 μg/ml, blocked, and incubated with various dilutions of the anti-$A_{2b}R$ antibody. The antibodies were detected with alkaline phosphatase-conjugated, affinity-purified, bovine anti-chicken IgY (Sigma). Reactivity could be detected in the ELISA down to 30 ng of affinity-purified antibody. The affinity-purified anti-$A_{2b}R$ MAP antibodies failed to recognize, by ELISA, a MAP construct made from the corresponding region of the human $A_{2a}R$ (data not shown).

Preparation of $A_{2b}R$ cDNA expression vector and transfectants. Reverse transcription-PCR with primers specific for human $A_{2b}R$ sequences (10) (5′-GAGGAATTCGCGGCCGCGCTGGCCCGGCCATG and 5′-GAGAAGCTTGGATCCTCATAGGCCCACACCGA) were used to amplify a 1011-bp cDNA from human placenta poly(A)$^+$ RNA. Each PCR cycle (30 cycles) consisted of incubations at 95° for 1 min, at 56° for 1 min, and at 72° for 80 sec, with *Thermus aquaticus* DNA polymerase (Promega). The 1011-bp PCR product was cloned into pGEM-T (Promega), sequenced, and found to be identical to the published human sequence (10). The cDNA was then cloned into the expression vector pSVK3 (Pharmacia) and used to transiently transfect MG, a human Epstein-Barr virus-transformed B lymphoblastoid cell line established in our laboratory, by electroporation (960 μF, 280 V) in a Bio-Rad gene pulser.

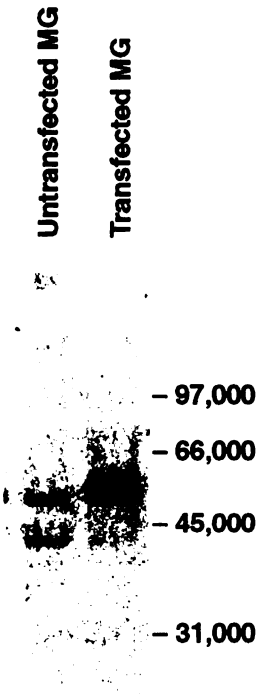

Fig. 3. Western blot analysis of MG cells before and after transfection with $A_{2b}R$ cDNA. MG cells were transiently transfected with $A_{2b}R$ cDNA. At 48 hr, cell lysates of transfected and untransfected MG cells were analyzed for $A_{2b}R$ expression by Western blotting. Fifteen micrograms of protein were loaded onto each lane of the gel.

Human	A	T	N	N	C	T	E	P	W	D	G	T	T	N	E	S
Rat	A	T	S	N	C	T	E	P	G	D	G	I	T	N	K	S
Mouse	A	T	S	N	C	T	E	L	G	D	G	I	A	N	K	S

Fig. 4. Comparison of amino acid sequences in the second extracellular loop of the $A_{2b}R$. The amino acid sequences of the region of the presumed second extracellular loop of the human (10), rat (13), and mouse (23) $A_{2b}Rs$ used to make the immunizing MAP were aligned.

Western blots. Human, mouse, and rat tissues, as well as the human colon adenocarcinoma cell line T84, were homogenized in lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 3 mM EDTA, 1%, v/v, Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 0.25% ϵ-amino-*n*-caproic acid, 1 mg/ml iodoacetamide), incubated for 45 min on ice, and centrifuged at 14,000 rpm for 5 min in a microcentrifuge. The supernatants underwent electrophoresis in duplicate on 9% SDS-polyacrylamide gels under reducing conditions, as described by Laemmli (21). Proteins on the gels were transferred to nitrocellulose membranes (0.45-μm, BA85; Schleicher & Schuell) (22). Nonspecific protein binding sites were blocked by overnight incubation in PBS containing 0.5% (w/v) gelatin and 0.05% (v/v) Tween-20. The blots were incubated with affinity-purified chicken anti-$A_{2b}R$ antibody at 0.6 μg/ml, followed by HRP-conjugated goat anti-chicken IgY (Kirkegaard and Perry Laboratories), for 1 hr each at room temperature. The bands were visualized with LumiGLO chemiluminescent substrate kit (Kirkegaard & Perry Laboratories). The protein concentrations in the extracts were determined by the Lowry method.

Glycosidase digestion. Twenty-five micrograms of T84 cell extract were reduced and denatured by boiling for 3 min in 0.5% SDS/0.1 M 2-mercaptoethanol. This material was incubated overnight at 37° in 15 mM sodium phosphate, pH 7.5, 10 mM 1,10-phenanthroline, 1.25% Nonidet P-40, with 0.4 units of endoglycosidase F plus *N*-glycosidase F (Sigma). The deglycosylated sample was then subjected to SDS-PAGE under reducing conditions, followed by Western blotting.

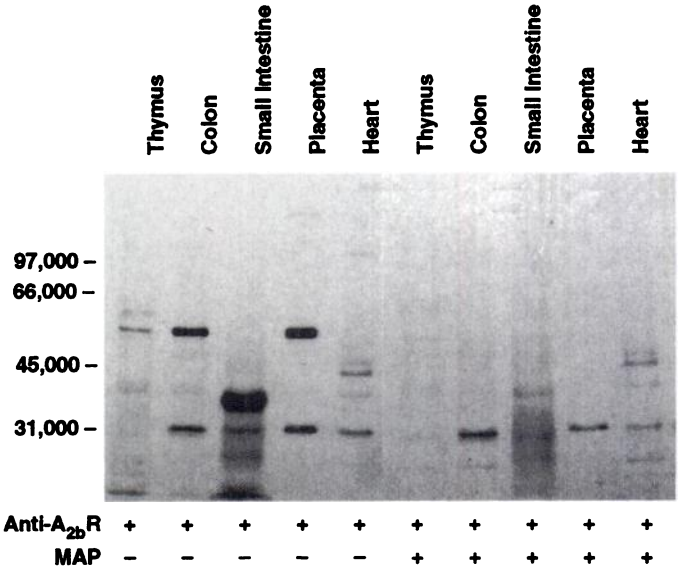

Fig. 5. Western blot analysis of mouse tissues with anti-$A_{2b}R$ peptide antibody. Detergent extracts of adult mouse tissues were subjected to 9% SDS-PAGE under reducing conditions and analyzed for $A_{2b}R$ expression. The quantities of protein loaded from each tissue were as follows: thymus and heart, 15 μg; colon and small intestine, 10 μg; term placenta, 5 μg. *Right half*, before development of the blot, the affinity-purified anti-human $A_{2b}R$ peptide antibody was incubated with a 20-fold excess of the immunizing MAP construct.

Immunohistochemistry. Fresh samples of human and rat colon were fixed in formalin overnight at 4°, embedded in paraffin, and then sectioned at 6 μm. Fetal mouse *in utero* and term human placenta specimens were fixed either in phosphate-buffered formalin overnight at 4° or in a solution of acetic acid/formalin/ethanol (5:10:85, v/v/v) for 1 hr at room temperature and were processed routinely for paraffin sectioning at 6 μm. The slides were deparaffinized and boiled in 0.01 M citrate buffer, pH 6.0, for antigen retrieval, according to standard procedures. The slides were then washed in PBS for 5 min, treated with 3% hydrogen peroxide to inhibit endogenous peroxidase, and blocked for 15 min using either 10% normal mouse serum (Sigma) and 0.1% (w/v) BSA in PBS or 0.5% (w/v) BSA in PBS. The human and rat colon slides were then incubated for 1 hr at 37°, in a humidified chamber, with 100 μg/ml affinity-purified chicken anti-$A_{2b}R$ peptide antibody, followed by HRP-conjugated goat anti-chicken IgY. The slides were then developed with 3-amino-9-ethylcarbazole reagent (Zymogen 3-amino-9-ethylcarbazole kit) and counterstained with hematoxylin. Fetal mouse and human placenta sections were incubated for 1 hr at room temperature with anti-$A_{2b}R$ peptide antibodies at 25–50 μg/ml in BSA/PBS, followed by biotin-conjugated mouse monoclonal antibody against chicken light chains (Sigma). The slides were then incubated for 30 min with avidin/biotin-conjugated HRP (Vector Laboratories), and color development used diaminobenzadine/urea peroxide tablets (Sigma) enhanced with nickel chloride.

Control reactions included 1) substitution of the primary reagent with similar concentrations of affinity-purified chicken yolk antibodies raised against bovine lipoprotein lipase (a gift from Dr. Jeffrey Gimble, Oklahoma Medical Research Foundation), 2) neutralization of the anti-$A_{2b}R$ antibodies with a 20-fold (w/w) excess of the immunogenic $A_{2b}R$ MAP for 24 hr at 4°, and 3) omission of the primary reagent altogether. Slides were analyzed using a Zeiss light microscope.

Results and Discussion

Western blotting of the $A_{2b}R$ proteins. The affinity-purified chicken anti-$A_{2b}R$ peptide antibody detected a single

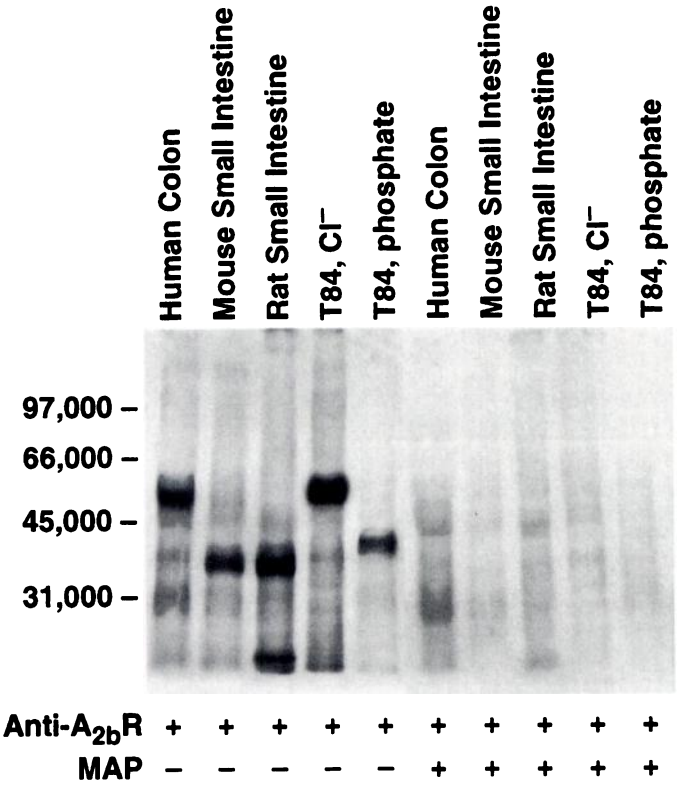

Fig. 6. Size of the T84 cell $A_{2b}R$ in extracts containing Tris·HCl versus phosphate buffer. Detergent extracts of T84 cells prepared in buffer containing either 50 mM Tris·HCl, pH 7.5, or 20 mM potassium phosphate, pH 7.0, were subjected to 9% SDS-PAGE under reducing conditions and analyzed for $A_{2b}R$ immunoreactivity by Western blotting. Extracts of human colon and mouse and rat small intestine prepared with Tris·HCl buffer are included for comparison. *Right half*, before development of the blot, the affinity-purified anti-human $A_{2b}R$ peptide antibody was incubated with a 20-fold excess of the immunizing MAP construct.

specific protein, with an apparent molecular mass of 50–55 kDa, in Western blots of human tissues (Fig. 2). Levels of $A_{2b}R$ expression were highest in the colon. The $A_{2b}R$ was also readily detectable in the T84 colon epithelial cell line, as well as in thymus and small intestine. In contrast, heart and skeletal muscle were negative (Fig. 2). With the exception of the thymus, these results are consistent with the tissue distribution of the rat $A_{2b}R$ determined by Reppert and colleagues (13) by Northern blot analysis. The 50–55-kDa immunoreactive band was not apparent when the chicken anti-$A_{2b}R$ antibody was preincubated with a 20-fold excess (by weight) of MAP peptide (Fig. 2) or when blots were developed with second antibody alone (data not shown). To confirm that the 50–55-kDa band was due to the $A_{2b}R$, Western blots were performed with Epstein-Barr virus-transformed B lymphoblasts (MG cells) transiently transfected with $A_{2b}R$ cDNA. As shown in Fig. 3, our affinity-purified anti-human $A_{2b}R$ peptide antibody recognized a specific protein of similar size in transfected MG cells. The intensity of the band was approximately 5-fold higher than with untransfected MG cells when equivalent amounts of protein were analyzed. Again, this band was not seen when the anti-$A_{2b}R$ antibody was preincubated with the MAP peptide or when the blots were developed with secondary antibody alone (data not shown). The mobility of the recombinant protein was the same as that of the native $A_{2b}R$ when extracts of transfected MG cells, T84 cells, and human colon were analyzed on the same gel. Therefore, we conclude that our affinity-purified chicken anti-$A_{2b}R$ peptide antibody can recognize denatured $A_{2b}R$. The fact that we can detect $A_{2b}R$ in human thymus in Western blots, whereas $A_{2b}R$ mRNA was not detected in rat thymus in Northern blots, suggests either a species difference in $A_{2b}R$ distribution or a greater sensitivity of our antibody, compared with Northern blotting. The latter is apparently true, because we are also able to detect $A_{2b}R$ in Western blots of rat thymus (see below).

Because developmental and physiological studies are more easily undertaken with experimental animals, we determined the cross-species reactivity of the anti-human $A_{2b}R$ antibody for the homologous mouse and rat receptors. The human peptide used for immunization is identical in 13 of 16 residues (75% homology) to the corresponding region of the rat $A_{2b}R$ (13) and in 11 of 16 residues (62% homology) to that of the mouse $A_{2b}R$ (23) (Fig. 4). The anti-human $A_{2b}R$ peptide antibody detected a specific protein of 50–52 kDa in Western blots of detergent extracts of mouse thymus, colon, and term placenta, but not of heart (Fig. 5). In addition, a specific immunoreactive protein of approximately 35 kDa was detected in small intestine. Our finding is similar to that of Palmer *et al.* (17) with the canine $A_{2a}R$, where the size of the receptor is 52 kDa in liver but only 34 kDa in striatum. Rat $A_{2b}R$ was also detected as a 50–52-kDa protein in detergent extracts of colon and thymus; the size of the receptor in small intestine was approximately 35 kDa, as seen in the mouse (Fig. 6). As with human tissue extracts, detection of mouse and rat $A_{2b}R$s was inhibited by preincubation of the antibody with a 20-fold excess of the MAP construct (Fig. 5 and data not shown).

One explanation for the difference in molecular mass of the $A_{2b}R$ in mouse and rat small intestine, compared with other tissues, is differential glycosylation. To look for evidence of *N*-linked carbohydrate, extracts of T84 cells were treated with a mixture of endoglycosidase F and *N*-glycosidase F. These enzymes cleave *N*-linked carbohydrates within the chitobiose part of the carbohydrate side chain and between the asparagine and the carbohydrate side chain, respectively. Purified human CD73, which is known to contain *N*-linked sugars (24), was used as a positive control. The electrophoretic mobility of the $A_{2b}R$ was unchanged by this treatment, whereas CD73 showed the expected decrease in molecular mass (data not shown). These results suggest either that the human $A_{2b}R$ lacks *N*-linked sugars or that *N*-linked sugars are inaccessible to endoglycosidase F plus *N*-glycosidase F under standard digestion conditions. Because the amino acid sequence of the $A_{2b}R$ contains only two potential *O*-glycosylation sites, it is unlikely that *O*-linked sugars alone can account for the difference in molecular mass of the $A_{2b}R$ in mouse and rat small intestine, compared with other tissues. Nevertheless, we looked for evidence of *O*-linked carbohydrate by digesting T84 extracts with neuraminidase plus *O*-glycanase, followed by Western blotting. After removal of sialic acid, *O*-glycanase cleaves the core disaccharide galactose-(1,3)-*N*-acetylgalactosamine from serine or threonine residues of glycoproteins. This treatment necessitated preparation of a new T84 extract in sodium phosphate buffer, because the Cl^- ions in our standard lysis buffer would inhibit *O*-glycanase activity. Surprisingly, Western blots of T84 extracts made in phosphate buffer showed an

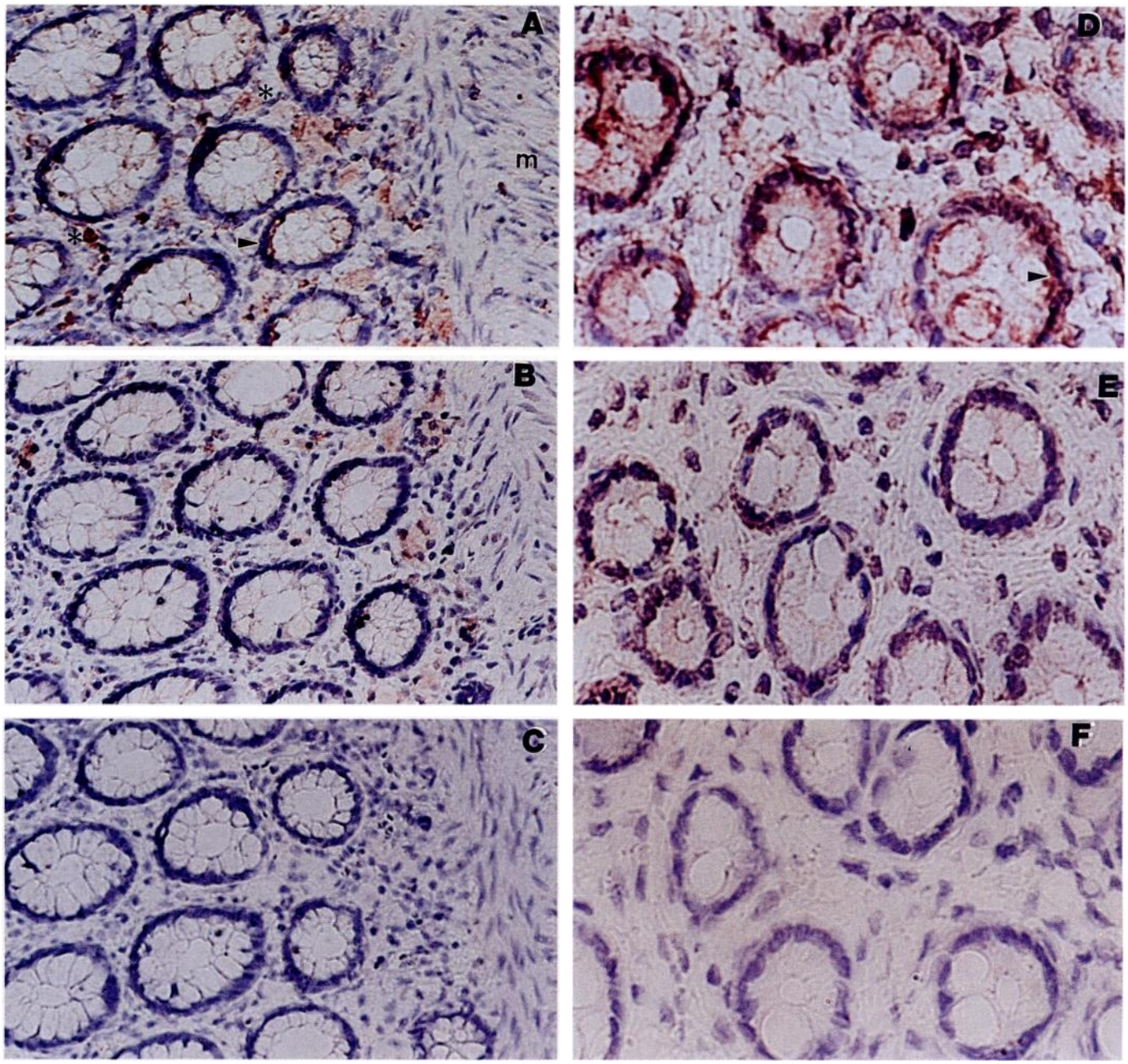

Fig. 7. Immunohistochemical localization of the $A_{2b}R$ in human and rat colon. Formalin-fixed sections of human (A-C) (magnification, 63×) and rat (D-F) (magnification, 100×) colon were stained with affinity-purified chicken anti-human $A_{2b}R$ peptide antibody at 100 μg/ml (A and D), anti-$A_{2b}R$ peptide antibody at 100 μg/ml plus MAP at 2 mg/ml (B and E), or HRP-anti-chicken antibody alone (C and F) and were counterstained with hematoxylin. Immunoreactivity was seen in the epithelial cells of the mucosa (*arrowheads*), macrophages of the lamina propria (*), and the muscularis (*m*).

immunoreactive $A_{2b}R$ protein of 40 kDa (Fig. 6), slightly larger than the $A_{2b}R$ in mouse and rat small intestine (35 kDa). These data suggest the presence of an endogenous protease (or glycosidase) in our extracts whose activity is influenced by the ionic composition of the lysis buffer. Further experimentation will be necessary to determine whether the 35-kDa size of the $A_{2b}R$ in mouse and rat small intestine is also the result of enzymatic degradation during extract preparation or whether this is the size of the native receptor in these tissues. If the 35-kDa $A_{2b}R$ is a naturally occurring isoform of the receptor, it will also be important to determine whether it is functionally active.

Immunohistochemical localization of the $A_{2b}R$. Immunohistochemical staining of formalin-fixed, paraffin-embedded sections of human and rat colon with the affinity-purified chicken anti-$A_{2b}R$ peptide antibody revealed strong reactivity in the mucosa (Fig. 7, A and D). This staining was largely inhibited by preincubation of the antibody with the MAP construct (Fig. 7, B and E). $A_{2b}R$ immunoreactivity was located primarily in the epithelial cells, giving additional credence to the notion that T84 cells are an appropriate model for studying the physiological consequences of $A_{2b}R$ engagement in colonic mucosa (14, 15). Significant immunoreactivity was also found in both small and large macro-

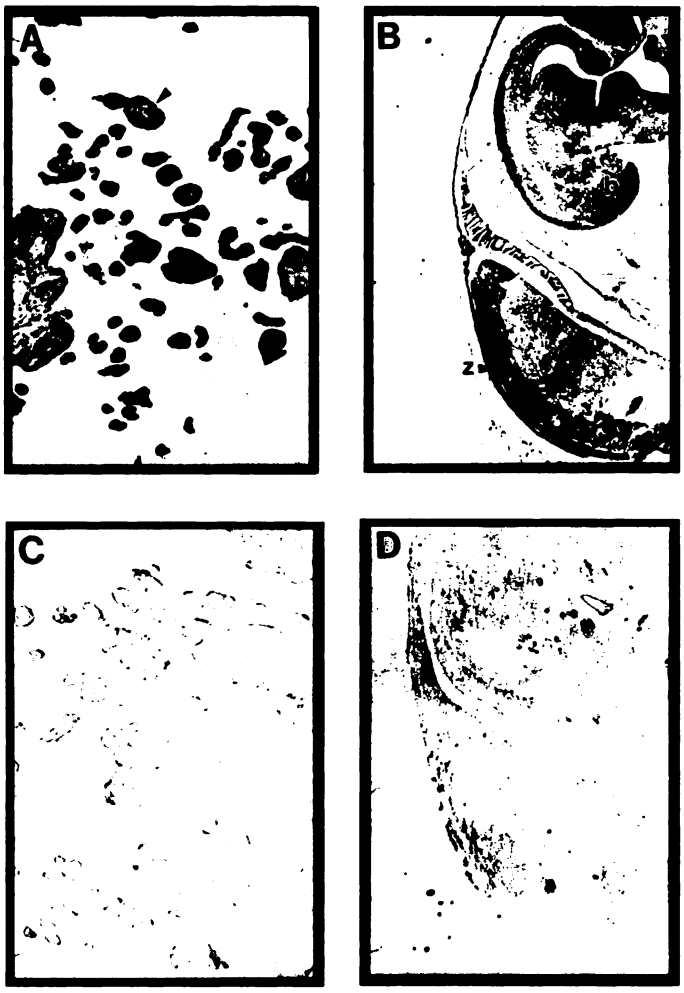

Fig. 8. Immunohistochemical localization of the $A_{2b}R$ in human placenta and 15-day mouse embryo. Sections of human term placental villi (A and C) (magnification, 75×) and mouse fetus *in utero* on day 15 of gestation (B and D) (magnification, 15×) were reacted with 50 μg/ml chicken anti-$A_{2b}R$ peptide antibody (A and B) or the same concentration of chicken anti-lipoprotein lipase antibody (C and D) and were visualized by avidin/biotin-HRP staining without counterstaining. Specific immunoreactivity for the $A_{2b}R$ was apparent in the syncytiotrophoblast cells lining the human placental villi (*arrowhead*) and in the giant trophoblast and spongiotrophoblast cells comprising the basal zone (*z*) of the murine chorioallantoic placenta. Intermediate labeling was apparent in the fetal brain (*b*).

Fig. 9. Immunohistochemical localization of the $A_{2b}R$ in the nervous system of day 15 mouse embryo. Higher magnification (220×) photographs of the tissue sections in Fig. 8, B and D, are shown. A and B, Staining with chicken anti-$A_{2b}R$ peptide antibody; C and D, staining with chicken anti-lipoprotein lipase. A and C, Large α motor neurons (*AMN*) of the ventral horn of the spinal cord were clearly labeled, whereas many smaller cells of the dorsal horn were not. The processes from labeled cells (*arrowhead*) can be seen growing out through the white matter (*w*) of the spinal cord *en route* to the ventral root of the developing spinal nerve. B and D, $A_{2b}R$ immunoreactivity was associated with cell bodies of a subpopulation of autonomic (sympathetic) ganglion cells (*g*) in the cervical region. The apparent intensity of labeling increased as cell size increased, indicating an association with the state of cell differentiation.

phages in the lamina propria. Although the intense staining of the small macrophages was markedly reduced by the MAP peptide, the staining of the large, mucin-filled macrophages adjacent to the muscularis was less effectively inhibited, making the specificity of the reaction more difficult to evaluate. Weak staining for $A_{2b}R$ expression was also observed in muscularis mucosae, although the identity of the positive cells was not resolved. No reactivity was observed when the primary antibody was omitted (Fig. 7, C and F). Similar staining patterns were obtained with frozen sections of human colon and mouse colon and ileum (data not shown).

Analysis of the human placenta at term (Fig. 8A) and the murine gestation site on day 15 (Figs. 8B and 9, A and B) revealed specific immunoreactivity with the anti-$A_{2b}R$ antibody. Little or no signal was detected in either specimen when the primary reagent was replaced with an equivalent concentration of chicken antibodies directed against bovine lipoprotein lipase (Figs. 8, C and D, and 9, C and D) or with anti-$A_{2b}R$ antibodies that had been preincubated with a 20-fold (w/w) excess of the immunogenic MAP or when the primary reagent was omitted altogether (data not shown). Moderate immunoreactivity for the $A_{2b}R$ protein was observed in the syncytiotrophoblast cells lining the human placental villi (Fig. 8A). In the mouse placenta, immunoreactivity for the $A_{2b}R$ protein was heavily localized to giant trophoblast cells and the spongiotrophoblasts comprising the basal zone (Fig. 8B). It is interesting to note that both of these cell types, which are derived from the ectoplacental cone of the early embryo, also express ADA at unusually high levels (25). ADA is a major degradative enzyme of adenosine metabolism and irreversibly deaminates the nucleoside at the C6 position of the purine ring, to form inosine. Because the $A_{2b}R$ requires relatively high concentrations of adenosine for activation (12), it is possible that the placental $A_{2b}R$ is hypoactivated under normal homeostatic conditions. Alternatively, adenosine may be produced at a high rate in the placenta, requiring a high level of ADA to prevent receptor hyperactivity. Studies are in progress to examine the role of adenosine and $A_{2b}R$ engagement during prenatal development.

In addition to the strong staining in the placenta, we observed specific $A_{2b}R$ immunoreactivity of weak to moderate intensity in multiple regions of the fetal nervous system. Labeled cells were present in fetal brain (Fig. 8B), the α motor neurons of the ventral horn of the spinal cord (Fig. 9A), and spinal and autonomic ganglia (Fig. 9B). Moderate immu-

noreactivity was found in the septal area of the brain and in the peripheral cerebral cortex (Fig. 8B). Because these regions of the nervous system are not fully differentiated in 15-day mouse fetuses, $A_{2b}R$ expression may be more involved with neuronal development than with neuronal physiology *per se*. Supernumerary cells of the developing nervous system are normally deleted by apoptosis, so it is possible that $A_{2b}R$ activity plays a role in regulating susceptibility to cell death or survival signals that act through second messenger systems (26). The distribution of the $A_{2b}R$ in the mouse fetus and placenta is consistent with pleiotropic roles of this receptor during embryogenesis.

In summary, we have described the production of a polyclonal chicken anti-human $A_{2b}R$ antibody that recognizes the $A_{2b}R$ protein in human, mouse, and rat tissues by Western blotting and immunohistochemistry. If the portion of the native receptor that corresponds to the immunizing MAP has undergone disulfide bond formation, this modification does not appear to interfere with the recognition of the denatured $A_{2b}R$ by our anti-peptide antibody. However, as is the case with many anti-peptide antibodies, our antibody does not recognize the native $A_{2b}R$, because neither T84 cells nor $A_{2b}R$-transfected COS cells were positive for cell surface staining using our antibody followed by fluorescein isothiocyanate-mouse anti-chicken IgY (data not shown). Nevertheless, this antibody will allow further elucidation of the tissue- and cell type-specific distribution of the $A_{2b}R$ in human, mouse, and rat tissues by Western blotting and immunohistochemistry. Immunohistochemistry has several advantages over *in situ* hybridization for the cellular localization of $A_{2b}R$ expression in tissues. In addition, in the absence of a selective agonist for the $A_{2b}R$, this reagent will allow a better understanding of the physiological roles of $A_{2b}Rs$, as illustrated above for the human colon and mouse embryo. The MAP strategy may also be successful for the production of antibodies to the remaining human AR subtypes, i.e., A_1R, $A_{2a}R$, and A_3R, enabling a comparison of the tissue distribution of the various subtypes with that of the $A_{2b}R$. The availability of subtype-specific antibodies will reinforce or refute current agonist-based tissue distribution profiles for the various AR subtypes and thus lead to greater insight into AR function.

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