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Selective Cloning of Cell Surface Proteins Involved in Organ Development: Epithelial Glycoprotein Is Involved in Normal Epithelial Differentiation

JEAN-PHILIPPE STEPHAN, PENELOPE E. ROBERTS*, LAURA BALD*, JAMES LEE, QIMIN GU, BRIGITTE DEVAUX, AND JENNIE P. MATHER*

Departments of Protein Chemistry (J.-P.S., P.E.R., L.B., B.D., J.P.M.) and Molecular Biology (J.L., Q.G.), Genentech, Inc., South San Francisco, California 94080-4990

ABSTRACT

Coordinating the activities of neighboring cells during development in multicellular organisms requires complex cellular interactions involving secreted, cell surface, and extracellular matrix components. Although most cloning efforts have concentrated on secreted molecules, recent work has emphasized the importance of membrane-bound molecules during development. To identify developmental genes, we raised antibodies to normal embryonic pancreatic epithelial cell surface proteins. These antibodies were characterized and used to clone the genes coding for the proteins by a panning strategy. Using this approach, we cloned the rat homologue of the mouse epithelial glycoprotein (EGP). Our immunohistochemistry data, describing the expression of EGP during rat development, as well as our *in vitro* data, looking at the effect of the anti-EGP antibody and the extracellular domain of EGP on embryonic pancreatic epithelial cell number and volume, strongly suggest a role for EGP during pancreatic development. (*Endocrinology* **140:** 5841–5854, 1999)

THE NEED TO coordinate the activities of one cell with those of its neighbors during development in multicellular organisms has resulted in the evolution of complex cellular interactions that involve secreted polypeptides, cell surface proteins, and extracellular matrix components. Cellular activities are modulated in response to signals received from neighboring cells and the immediate environment (1). Among the cellular interactions, cell-cell and cell-matrix interactions represent complex and dynamic forms of communication that provide information to the cells for controlling morphogenesis, cell fate specification, gain or loss of tissue-specific functions, cell migration, tissue regeneration, and cell death (2). Normal epithelial cell surfaces present a diverse and multifunctional array of membrane proteins that mediate cell-cell and cell-matrix interactions (3, 4).

With the entire sequence of the human (and other) genome likely to be available in the near future, one of the challenges for the next century will become identifying the subset of known genes that are important in regulating the development of a specific organ. Once these genes are identified, one would wish to have relevant model systems to rapidly assess the function of individual genes. Most cloning efforts to date have dealt with secreted extracellular signaling molecules such as hormones, growth factors, and cytokines. However, more recently, proteins that are either wholly or partially membrane bound [such as the neuregulins (5) and patched and hedgehog families of proteins (6, 7)] have been recognized as playing a crucial role in development. An approach biased toward discovering cell surface proteins present during the development of an organ would, therefore, be expected to provide new information on the regulation of that development.

The pancreas develops from the gut endodermal epithelium, starting at e11 in the rat embryo. A portion of the gut becomes committed to pancreatic differentiation, thickens to form a structure called the pancreatic bud, and then undergoes branching morphogenesis to elaborate the complete pancreatic duct system (8). It has long been recognized that epithelial-mesenchyme interactions are crucial for the initiation and early progression of pancreatic development (9). The ductal epithelium continues to differentiate late in development, and postnatally, to form the endocrine islets and the exocrine acinar tissue (10). This process is of particular interest because new islets can be formed even in the adult, during pregnancy in the female (11), or in response to injury. This process seems to be largely controlled by paracrine and autocrine interactions within the pancreas (12), because regeneration after injury to one section of the pancreas does not affect the opposite lobe (13). Cell surface proteins are known to be involved in many instances of tissue remodeling. It is, therefore, of particular interest to ascertain which cell surface proteins are present on early pancreatic ductal epithelium.

Recent work has shown that serum-free culture conditions can be used to establish continuous cell lines from rodent tissues containing mitotic cells from embryonic or newborn animals (14–16). Several of these cell lines exhibit the characteristics of cells frozen in a predifferentiated phenotype (17, 18). One important aspect, in which some of these lines differ from most cell lines isolated by conventional means, is their dependence on cell-cell contact for survival (17). Serum-free defined media can also be used to specifically select for

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Address all correspondence and requests for reprints to: Dr. Jean-Philippe Stephan, Research Immunochemistry-AAT, Genentech, Inc., South San Francisco, California 94080-4990. E-mail: stephanj@gene.com.
* Present address: Raven Biotechnology, Inc., San Carlos, California 94070.

the growth of a single cell type in a mixed cell population (15, 16).

The aim of the present study was to use this approach to obtain cell lines derived from embryonic epithelial cells early in the process of pancreatic differentiation. We could then use these cells to raise monoclonal antibodies (MAbs) that would recognize cell surface proteins that might be involved in pancreatic differentiation and the differentiation of developmentally related organs derived from the embryonic endoderm (*e.g.* gut, lung, kidney, stomach, and bladder). The cell surface localization of the antigens made a strategy of panning for cells expressing the gene coding for these antigens particularly appropriate.

In the present study, we highlight our results relative to the cloning and characterization of one of these genes, which codes for the 2160 antigen. This protein is the rat homologue of a previously cloned mouse epithelial cell surface glycoprotein (mEGP) (19) and the human proteins EGP-1 and EGP-2, originally described as colon tumor antigens (20) and also known as GA733–2, KSA, KS1/4 Ag (19), and Ep-CAM (epithelial cell adhesion molecule) (21). However, the biological role of this family of membrane glycoproteins in regulating normal cell function remained unclear. Our immunochemistry data, as well as *in vitro* data, strongly suggest that the rat protein 2160 (rEGP) is a membrane-bound signaling protein involved in regulating the growth of epithelial cells during normal pancreatic development.

Furthermore, the strategy used here to study the 2160 antigen provides a rapid and effective method of identifying developmental functions of proteins of interest and constitutes a useful approach to determine which of the increasing number of proteins whose gene sequences are in public databases are involved in the differentiation and function of a specific tissue.

Materials and Methods

Culture of embryonic pancreatic ductal epithelial cells in serum-free medium

Embryonic ducts were isolated using a modification of a previously described procedure (22). To generate the RED cell line, 2 to 3 18-day pregnant Sprague Dawley rats were killed by CO_2 asphyxiation. The embryos were transferred to ice-cold HBSS containing 20 μg/ml gentamycin. The embryonic pancreati were removed on ice under a dissecting microscope and placed in F12/DMEM. To each 12–15 pancreati in 1 ml of F12/DMEM, 25 μl of a collagenase-dispase solution (50 mg/ml), containing soybean trypsin inhibitor (1 mg/ml), was added. The dish was then incubated at 37 C for 30 min, with frequent pipetting to break up tissue into smaller fragments. The digest was washed by centrifugation through a 5% BSA gradient. Further dissociation was accomplished by filtration through a tissue sieve or through 200-mesh Nitex cloth.

Tissue fragments, mostly ducts, were washed by centrifugation at 800 $\times$ *g* for 6 min in F12/DMEM and then resuspended in growth medium, which consisted of F12/DMEM supplemented with 14F: rhu-insulin (10 μg/ml), transferrin (10 μg/ml), epidermal growth factor (EGF) (10 ng/ml), ethanolamine (1 μM), aprotinin (25 μg/ml), glucose (5 mg/ml), phosphoethanolamine (1 μM), triiodothyronine (5 pM), selenium (25 nM), hydrocortisone (0.5 μM), progesterone (10 nM), forskolin (1 μM), heregulin β177–244 (10 nM), and bovine pituitary extract (5 μl/ml, 75 μg/ml protein). The cell suspension was then distributed evenly to either fibronectin-coated or collagen-coated 24-well plates. Cyst-like structures formed within 48–72 h in culture. These were removed with the supernatant, washed, resuspended in the 14F growth medium, and replated onto either collagen- or fibronectin-coated plates. The cyst-like structures attached and began to spread within 24 h. After 5–7 days, these cultures were 75% confluent, whereupon they were subcultured at a 1:2 split ratio by dissociation in trypsin-EDTA, neutralized with 1 mg/ml soybean trypsin inhibitor, washed by centrifugation, resuspended in 14F growth medium, and plated onto fibronectin-coated plates. Thereafter, the cultures were split every 3–4 days at a high split ratio (1:3 to 1:5). Fibroblast contamination was minimal and completely eliminated by serial cloning in 15% self-conditioned medium in 96-well microtiter plates (15, 23).

BUD cultures were established from 12-day pregnant Sprague Dawley rats. After dissecting out the embryos, the dorsal and ventral pancreatic evaginations were surgically dissected and cultured in separate wells of a 48-well dish without initial enzymatic dissociation of the tissue. The dorsal pancreatic cells, carried as described above, were used to establish the BUD cell line. The BUD (dorsal pancreas) and RED (total pancreas) cells have both been in continuous culture for at least 80 population doublings. They have maintained a normal karyotype, are confirmed to be of rat origin, and are free of mycoplasma (data not shown).

Generation of MAbs raised to BUD/RED cell surface proteins

Balb/c mice were immunized alternatively with either 5×10^6 intact BUD or RED cells, without adjuvant, weekly for 10–15 weeks. Sera from the immunized mice were tested for antibodies to BUD and RED cells by fluorescence analysis cell sorting (FACS) analysis of binding, as described below. The mice with the highest titers were given an additional boost of 5×10^6 cells. Three days later, the lymphocytes from the mouse spleen were fused with the mouse myeloma line X63-Ag8.653, using 50% polyethylene glycol 4000, according to the procedure described elsewhere (24). Fused cells were plated at a density of 200,000 cells per well in 96-well tissue culture plates, and hybridomas were selected using HAT medium supplement (Sigma, St. Louis, MO). On day 10 after the fusion, the hybridoma supernatants were screened, by FACS, for the presence of BUD/RED-specific Abs. The hybrids producing MAbs that bound to BUD and RED cell lines were then screened against the TR-1 rat endothelial cell line. Selected hybridomas were cloned by limiting dilution to produce stable hybridomas. MAbs were produced in ascites, and the antibodies were purified on protein A-Sepharose columns (Fermentech, Inc., Edinburgh, Scotland, UK) and stored in sterile PBS at 4 C.

FACS analysis

Cells were detached from tissue culture flasks in the presence of 0.5 mM EDTA for 15 min, treated for 10 min with collagenase/dispase (Roche Molecular Biochemicals, Indianapolis, IN), centrifuged at 1400 rpm for 5 min, and resuspended in PBS containing 1% BSA and 2 mM EDTA (FACS diluent). The cells were counted, adjusted to 10^7 cells/ml, and 0.1 ml of cells was incubated with 1 μg of purified MAbs in 100 μl FACS diluent for 30 min at 4 C. The samples were washed, resuspended in 0.1 ml diluent, and incubated with 1 μg of fluorescein isothiocyanate-conjugated $F(ab')_2$ fragment of goat antimouse IgG for 30 min at 4 C. The cells were washed, resuspended in 0.5 ml FACS diluent, and analyzed using a FACScan cell sorter (Becton Dickinson and Co., Mountain View, CA).

Cell lines

The antibodies were screened for binding to various other cell lines in addition to the BUD and RED lines. These include the following: RIN-M and RIN-F rat insulinoma cell lines (25); ARIP rat acinar tumor cell line (26); NODD mouse adult pancreatic ductal cell line (established in this lab by the same method used for RED cells but starting from adult NOD mouse pancreas); BR516 lung epithelial cell line (15, 27); rat adult (ASC) and embryonic (ESC) Schwann cell lines (16), RAT-1 rat fibroblast cell line (28); TR-1 rat capillary endothelial cell line (29); TRM rat peritubular myoid cell line (29), and primary neonatal rat cardiomyocyte cultures rCM (30). All cell lines were carried in F12/DMEM medium supplemented with 10% FCS (ARIP, RIN-F, RIN-M, RAT-1, TR-1, and TRM) or the published hormone supplements appropriate to the cell line (BR516, NODD, ASC, ESC, and rCM).

Immunochemistry procedure

Embryos were snap-frozen in liquid nitrogen immediately after removal from 9, 10, 12, 15, or 18-day pregnant Sprague Dawley rats and stored at −70 C until sectioning. Sections of 4- to 6-μm thickness were cut on a cryostat, air-dried, fixed in acetone for 5 min, and air-dried overnight. BUD and TR-1 cell monolayers were fixed with 4% paraformaldehyde. After quenching of the endogenous peroxidase using the glucose oxidase/glucose method (31), blocking of the endogenous biotin using an avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA), and blocking the endogenous Ig binding sites with PBS/1% BSA (25 min), either the sections or the cells were overlaid for 2 h with purified MAbs 2160, 2161, 2115 or 2117 (4.8 μg/ml, 1.66 μg/ml, 2.1 μg/ml, and 1.8 μg/ml, respectively, in PBS/1% BSA). Subsequently, samples were incubated with rhodamine-conjugated antimouse IgG (Chemicon, Temecula, CA) or biotin-rat antimouse IgG1 (1:500; Zymed Laboratories, Inc., San Francisco, CA) for 2 h and peroxidase-conjugated streptavidin (4 mg/ml; Jackson ImmunoResearch, West Grove, PA) for 30 min. After several rinses in PBS, immunostaining was developed for 10–15 min with 3-amino-9-ethylcarbazole (DAKO Corp., Carpinteria, CA). Sections were conterstained with Mayer's hematoxylin and mounted in glycergel (DAKO Corp.). For the e9, e10, e12, e15, and e18 embryo immunohistochemistry (IHC), two separate experiments were performed and at least four sections examined from each embryo for each MAb or control.

The gut region was dissected from 12.5-day rat embryos and fixed in 3% paraformaldehyde overnight. After washes, permeabilization with acetone at −20 C for 7 min, and blocking the endogenous Ig binding sites with PBS/1% BSA/1% dimethylsulfoxide/2% goat serum, tissues were incubated overnight either with a rabbit polyclonal antirat PDX1 (1:1000) or the MAb 2160 (1:400). Immunostaining was analyzed after an overnight incubation (either with the secondary antibody, Cy3-conjugated affinity-purified goat antimouse or rabbit IgG).

Messenger RNA (mRNA) isolation and complementary DNA (cDNA) library construction

mRNA was isolated directly from cultured BUD cells using the Invitrogen FastTrack 2.0 mRNA Isolation System. Oriented cDNA transcripts were prepared from 5 mg poly-$(A)^+$ mRNA using the Life Technologies, Inc. SuperScript Plasmid System and fractionated on 5% acrylamide-TBE slab gel. Eluted cDNAs were ligated into the *Xho*I-*Not*I sites of the mammalian expression vector pRK5D and then electroporated into Life Technologies, Inc. DH10B cells, under conditions recommended by the manufacturer.

Recovery of cDNA clones by panning

Screening of the BUD cell library was carried out using a modified version of a technique previously described (32). Briefly, the cDNA library was transfected into COS cells by electroporation (33). After 2 days of culture, transfected COS cells were resuspended, then incubated with a pool of antibodies (of 2 mg/ml each; see Table 2), and replated onto dishes coated with affinity-purified rabbit antimouse IgG and IgM. A Hirt supernatant was prepared from adherent cells and used to transform competent *Escherichia coli*. After amplification, bacterial colonies were harvested, then plasmid cDNA was isolated using the alkaline miniprep method (34) and transfected into COS cells to perform a new round of immunoselection. After 4 rounds of panning with the pooled antibodies, subsequent rounds of panning were performed on the individual purified MAbs.

DNA sequence determination and analysis

ABI Dye-terminator chemistry (PE Applied Biosystems, Foster City, CA) was used to sequence the clone 2160 with a primer walking strategy (35). The sequences were collected with an ABI377 instrument (PE Applied Biosystems). The sequences generated by the different walking primers for both DNA strain were edited and assembled in the Sequencher (Gene Codes Corp, Ann Arbor, MI). All sequence analyses were performed using an in-house sequence analysis program (Genentech, Inc.). The program ALIGN (36) was used to analyze relationships among the clones 2160, mEGP, hEGP-1, and hEGP-2.

Western blots

Untreated, MAb 2160 (10 μg/ml)-treated, or fusion protein 2160 (P2160) (10 μg/ml)-treated BUD and RED cells were either lysed in PBS/1% NP40/0.5% deoxycholate/0.1% SDS/5 mM EDTA and the lysate loaded on a 4–20% Novex Tris-Glycine gel or lysed in a buffer containing 10 mM Tris pH 8.0, 150 mM sodium chloride, 1% sodium deoxycholate, 1% (vol/vol) triton-X-100, 0.1% SDS, 1 mM leupeptin, and 1 mM PMSF, and the lysate immunoprecipitated with an antiphospho-Ser/Thr/Tyr MAb (CLONTECH Laboratories, Inc., Palo Alto, CA) or MAb 2160, boiled and loaded on a 4–12% Novex Tris-Glycine gel. The gel was run at 100 V and electroblotted for 60 min at 0.5 A onto a Protran nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The membrane was blocked in PBS/5% nonfat milk/0.5% Tween 20/0.01% Thimerosal (assay buffer) for 1 h at room temperature. The blot was washed in PBS/0.05% Tween 20, and incubated with each MAb (1 μg/ml), an antiphospho-Ser/Thr/Tyr MAb (CLONTECH Laboratories, Inc.) or antibodies against pancreatic markers [cytokeratin 7 (1:500), PDX1 (1:500), carboxypeptidase A (1:500), tyrosine hydroxylase (1:1000)] for 1 h. The membrane was washed with PBS/0.05% Tween 20 and incubated for an additional 1 h with a 1:5000 dilution of goat antimouse IgG or antirabbit IgG peroxidase. The membrane was washed thoroughly and developed using an ECL chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, VA).

Northern blot analysis

Poly-$(A)^+$ RNA blots from the indicated human and rat adult tissues were purchased from CLONTECH Laboratories, Inc. and hybridized to a 1.29-kb (α-^{32}P)deoxycytidine triphosphate cDNA probe for clone 2160, labeled by random priming (2×10^6 cpm/ml) (37). After a 1-h hybridization, membranes were washed at 65 C in 0.1 × SSC/0.1% SDS and subjected to autoradiography at −70 C.

Production of the P2160 extracellular domains (ECD) HIS-6

Specific PCR primers were synthesized on the basis of the DNA sequence of the protein 2160 ECD. A HIS-6 tag sequence was added to each of the C-terminal primers for affinity purification purposes. The p2160 ECD cDNAs were generated by PCR and inserted into pRK5, an expression plasmid using the cytomegalovirus promoter/enhancer with simian virus 40 (SV40) termination and polyadenylation signals located downstream of the inserted cDNA. These constructs were transiently transfected into human embryonic kidney 293 cells using Lipofectamine. The expressed proteins were purified using a chelating Sepharose column charged with nickel (Amersham Pharmacia Biotech, Piscataway, NJ). Protein concentration was determined by OD 280.

Results

BUD and RED embryonic pancreatic ductal epithelial cell lines

Two pancreatic epithelial cell lines were established from primary cultures of dissected rat e12 embryonic pancreatic buds (BUD) and rat e17 ductal epithelium (RED), respectively. The cultures were initiated and carried in a serum-free medium, optimized to select for the growth of the epithelial cells. Each component of the 14F medium contributes to the optimal growth of the cells (Table 1). Under these optimal conditions, the fibroblast and mesenchymal cells are lost from the cultures within two passages, and the remaining cells are uniformly epithelial. The cultures have a log phase population doubling time of 11.4 h and 14 h for BUD and RED cells, respectively. The cells form a contact-inhibited monolayer, have a normal karyotype (data not shown), and have been grown continuously for over 80 population doublings with no obvious change in cell morphology or growth

profile. In accordance with previous work establishing rodent cell lines in this fashion (14, 15), no cell senescence has been observed.

To better characterize the BUD and RED cell lines, the presence of various proteins known to be present at early stages of pancreatic development were investigated by Western blot analysis (Fig. 1). We demonstrate that the BUD cells and, to a lesser extent, the RED cells express cytokeratin 7 (molecular weight, 54 kDa), which is present only in the ductal epithelium in the pancreas (38). BUD and RED cells also express carboxypeptidase A (35 kDa), another ductal marker (39). The procarboxypeptidase (45 kDa) is also present in the two pancreatic cell lines. Both the BUD and RED cell lines also express the homeodomain-containing transcription factor for insulin gene expression PDX1 (42 kDa), which appears in the pancreatic bud epithelium early, before insulin, in the ontogeny of the pancreas (40). In the adult, PDX1 is expressed only in β-cells and not in the mature ductal epithelium. Tyrosine hydroxylase (60 kDa), a marker for early islet progenitor (41) and early ductal cells, was detected in the BUD cells and, to a lesser extent, in the RED cells.

TABLE 1. Medium optimized for the growth of BUD and RED embryonic pancreatic ductal epithelial cells

Factor withdrawn	Cell growth (% of the control ± SEM)
Insulin	33.60 ± 0.83
Transferrin	37.70 ± 0.06
EGF	43.42 ± 3.15
Selenium	59.54 ± 7.62
Triiodothyronine	81.84 ± 8.67
Progesterone	61.80 ± 0.42
Ethanolamine/Phosphoethanolamine	53.63 ± 11.30
Hydrocortisone	54.34 ± 6.08
Forskolin	35.63 ± 13.94
BPE	1.66 ± 0.47
Glucose	68.50 ± 2.78
Fibronectin	54.37 ± 4.94
Heregulin	80.94 ± 4.97
Complete media 14F (control)	100 ± 0.95
F12/DMEM only (no adds)	4.23 ± 0.39

Bud cells were cultured in the complete 14F selective media (control), or omitting a single factor, for 5 days and the cell number determined. Results are expressed as percentage of the control growth SEM. Note that all of the supplements are required for optimal growth.

MAbs to BUD/RED cell surface proteins

Intact, viable BUD and RED cells were used to immunize mice and generate (MAbs). Five thousand hybrid clones were obtained and screened by FACS analysis of binding to intact cells. Of these, approximately 0.3% of the clones recognized the BUD and RED cells. These were recloned to obtain stable clones. Ten MAbs were selected for further study, based on their binding to the RED and BUD cells (Table 2A). MAbs 2101, 2103, and 2104 are of the IgM isotype, and the remainder are IgG. As defined by Western blot analysis, the molecular mass of the different proteins

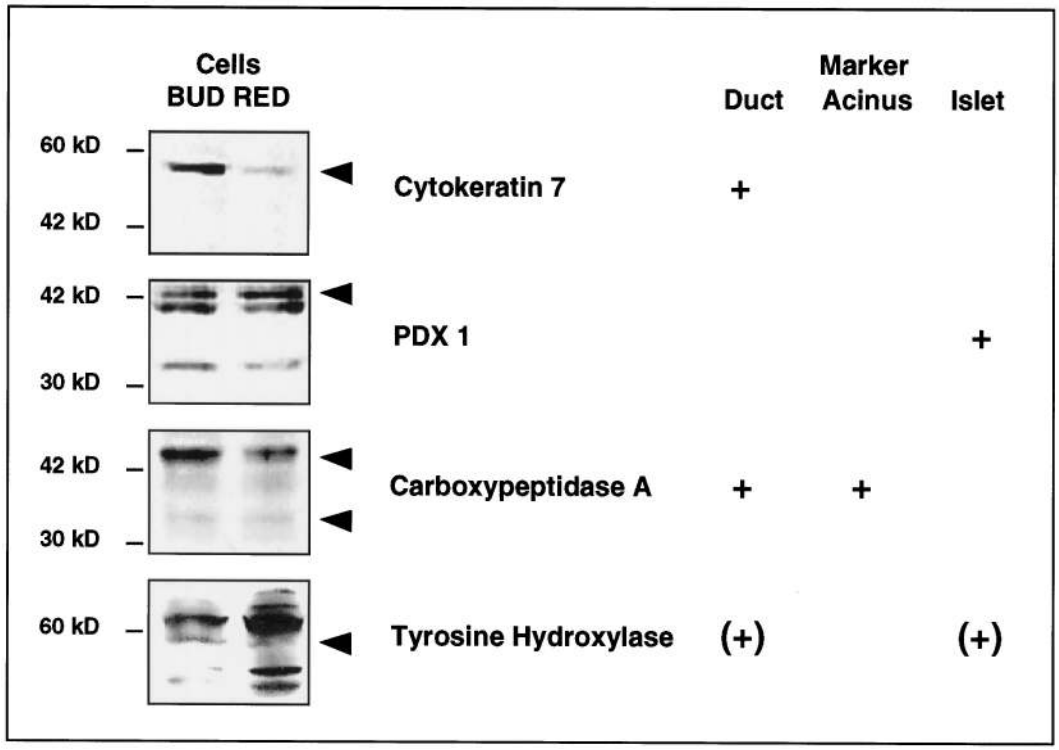

FIG. 1. Characterization of pancreatic cytoplasmic markers in the BUD and RED cells. A 100-μg total protein sample from BUD and RED cells was reduced in Laemmli sample buffer, loaded on 4–20% polyacrylamide gradient gel, and electrophoresis was performed at 100 V for 2–3 h. After transfer, the membrane was blocked and incubated either with mouse monoclonal antihuman cytokeratin 7 (1/500), rabbit polyclonal antirat PDX1 (1/500), rabbit polyclonal antibovine carboxypeptidase A (1/500), or rabbit polyclonal antirat tyrosine hydroxylase (1/1000). These markers are coexpressed only in embryonic ductal tissue. In the adult, the expression segregates to different tissues. +, Expression in adult duct, acinus, or islet; (+), expression in embryonic tissue only and not in adult.

TABLE 2. Description of MAbs raised to BUD and RED cells and FACS characterization of the antigens targeted

A

MAb	2100/01	2103	2104	2160	2161	2115/16	2117	2140
Isotype	IgG3/M	IgM	IgM	IgG1	IgG3	IgG1/2a	IgG1	IgG1
Epitope	A	C	D	G	H	B	E	F
Western (Kd)	60-70	N/D	N/D	40-50	20-30	120	60-70	N/D

B

									Species	Cell line type
BUD	+++	+++	++	+++	+++	++	+	+++	**R**	**e12 pancreatic ductal epith. cell**
RED	+++	+++	+++	+++	+++	++	+	+++	**R**	**e18 pancreatic ductal epith. cell**
NODD	+++	+++	+++	++	++	++	++	++	M/NOD	adult pancreatic ductal cell
BR516	+++	+	+++	++	+	++	+	+++	R	lung epithelial cell
RIN-M	-	-	-	+/-	+/-	+	+	+	R	insulinoma cell
RIN-F	-	-	-	+/-	-	+	+	+	R	insulinoma cell
ASC	+/-	-	+	-	+/-	+	+	+	R	adult Schwann cell
RAT-1	-	-	+/-	-	-	+	+	+	R	fibroblast
TR-1	**-**	**-**	**-**	**-**	**-**	**++**	**+**	**++**	**R**	**capillary endothelial cell**
TRM	-	-	-	-	-	+	+	+	R	peritubular myoid cell
ARIP	-	-	-	-	-	+/-	+/-	+/-	R	acinar tumor cell
ESC	-	-	-	-	-	+/-	+	-	R	e14 Schwann cell
rCM	-	-	-	-	-	-	-	-	R	primary cardiomyocytes

A, The isotype and MW (on Western blots) of the 10 monoclonal antibodies raised to BUD and RED embryonic pancreatic ductal epithelial cell surface proteins. N/D, Not detected. B, Binding of the different anti-BUD/RED MAbs to various normal and tumor cell lines analyzed by FACS. The hybrids producing MAbs that bound to BUD and RED cell lines (*bold*) were then screened against the TR-1 rat endothelial cell line (*bold*). The level of the binding is expressed in order of magnitude differences as: +++, very high; ++ high; + moderate; and +/− weak binding. See Fig. 2 for actual binding data for selected antibodies.

recognized by these MAbs varies between 20 and 120 kDa. (Table 2). MAbs 2103, 2104, and 2140 were not suitable for Western blot analysis. The Western blots and cross-competition for FACS binding suggested that MAb pairs 2100/2101 and 2115/2116 recognize the same antigens. All other antibodies recognize distinct antigenic determinants.

To further characterize the antigens targeted by the different MAbs, FACS analysis was performed using the different anti-BUD/RED MAbs on various normal and tumor-derived cell lines. As expected, the BUD (Fig. 2A1) and RED cells are positive for all the MAbs generated (Table 2). Immunocytochemistry confirmed that the staining is cell-surface in nature (Fig. 2B). All antibodies also bind to the NODD cell line derived from adult nonobese diabetic (NOD) mouse pancreatic ductal epithelial cells and to the normal neonatal lung epithelial line, BR516 (15, 27); although, for 3 of the antibodies (2103, 2161, 2117), the antibody binding to BR516 was only 1–10% of that on the BUD cells. The three antibodies that bound to the TR-1 rat endothelial cell line exhibited fairly broad specificity of binding, because they

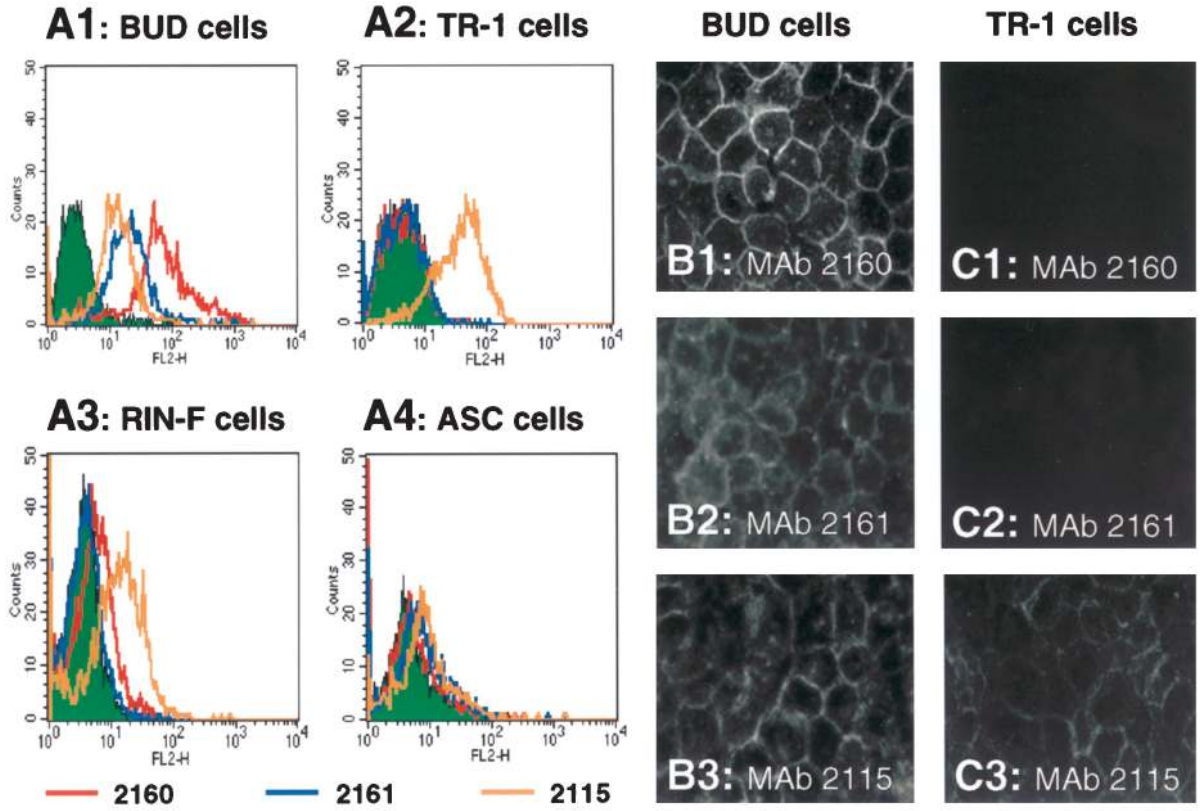

FIG. 2. Expression of different BUD and RED embryonic pancreatic ductal epithelial cell antigens at the surface of various cell lines. A, BUD, TR-1, RIN-F, and ASC cells were analyzed by FACS for the binding of the MAbs 2160 (*red*), 2161 (*blue*), and 2115 (*orange*). Controls (*green*) have no second antibody. B, Immunofluorescence staining with MAbs 2160, 2161, and 2115 on the BUD (B) and TR-1 (C) cell monolayers.

bound, albeit weakly, to most of the other cell types tested, except cardiomyocytes (Table 2, Fig. 2, A3 and A4). These results are consistent with the immunocytochemistry results on the TR-1 cells (Fig. 2C).

In contrast, MAbs 2100/01, 2103, 2104, 2160, and 2161 were more specific and did not bind to most of the other cell types tested, including several insulinoma and acinar tumor-derived cell lines (RIN-M, RIN-F, and ARIP) (Table 2, Fig. 2A).

Immunolocalization of Ag 2101, PDX1, and Ag 2160 along the gut at early stage of pancreatic development

Considering the origin of the BUD and RED cell lines, from primary cultures of dissected rat e12 embryonic pancreatic buds and rat e17 ductal epithelium, respectively, we were interested in studying the expression of the antigens recognized by the different MAbs at early stages of pancreatic development. IHC experiments, performed with the MAb 2101 (and MAb 2100) on e12 rat embryo, revealed the pancreatic specificity of the Ag 2101 at this stage (Fig. 3A). Only sections across this region of the gut present a strong and specific staining on the pancreatic bud. The nonspecific signal visualized in the anal region was present in the controls without first antibody or with mouse isotype IgG (data not shown). The immunoreactivity along e12.5 rat embryonic gut was also studied using MAb 2160 (Fig. 3C) and was compared with the staining visualized using a rabbit polyclonal antirat PDX1 (Fig. 3B). PDX1 immunoreactivity was seen mainly in the dorsal pancreas and in a restricted area along the gut adjacent to the pancreas. A weaker signal was also observed in the ventral pancreas. The MAb 2160 was strongly reactive along a ventral layer of cells from the inferior part of the stomach to the ventral evagination of the pancreas. An intense signal was also visualized along the developing ducts in the dorsal pancreas and, to a lesser extent, in the ventral pancreatic bud.

IHC study

To better characterize the expression of the antigens during embryonic development, an IHC study was done in e9- to e18-day rat embryos, and adult pancreas, using the anti-BUD/RED MAbs. From this analysis, the MAbs raised against the pancreatic epithelial cell lines could be roughly divided into two groups: one group (corresponding to the MAbs 2100/01, 2103, 2104, 2160, and 2161) specifically targets epithelial cells of the gastrointestinal tract and other endodermally-derived epithelia (*e.g.* lung and kidney); and one group (MAbs 2115/16, 2117, and 2140) that bind to these epithelia but also to endothelial cells and neuronal cells (42). Interestingly, though the different anti-BUD/RED MAbs stain similar organs, for example the vibrissa and the rectum, the cell type stained within an organ is, in many cases, quite different. This is particularly well illustrated by comparing the staining of the vibrissa by MAbs 2117, 2160, 2161, and 2115 (Fig. 4).

In the e9 rat embryo (Fig. 5A), the protein recognized by

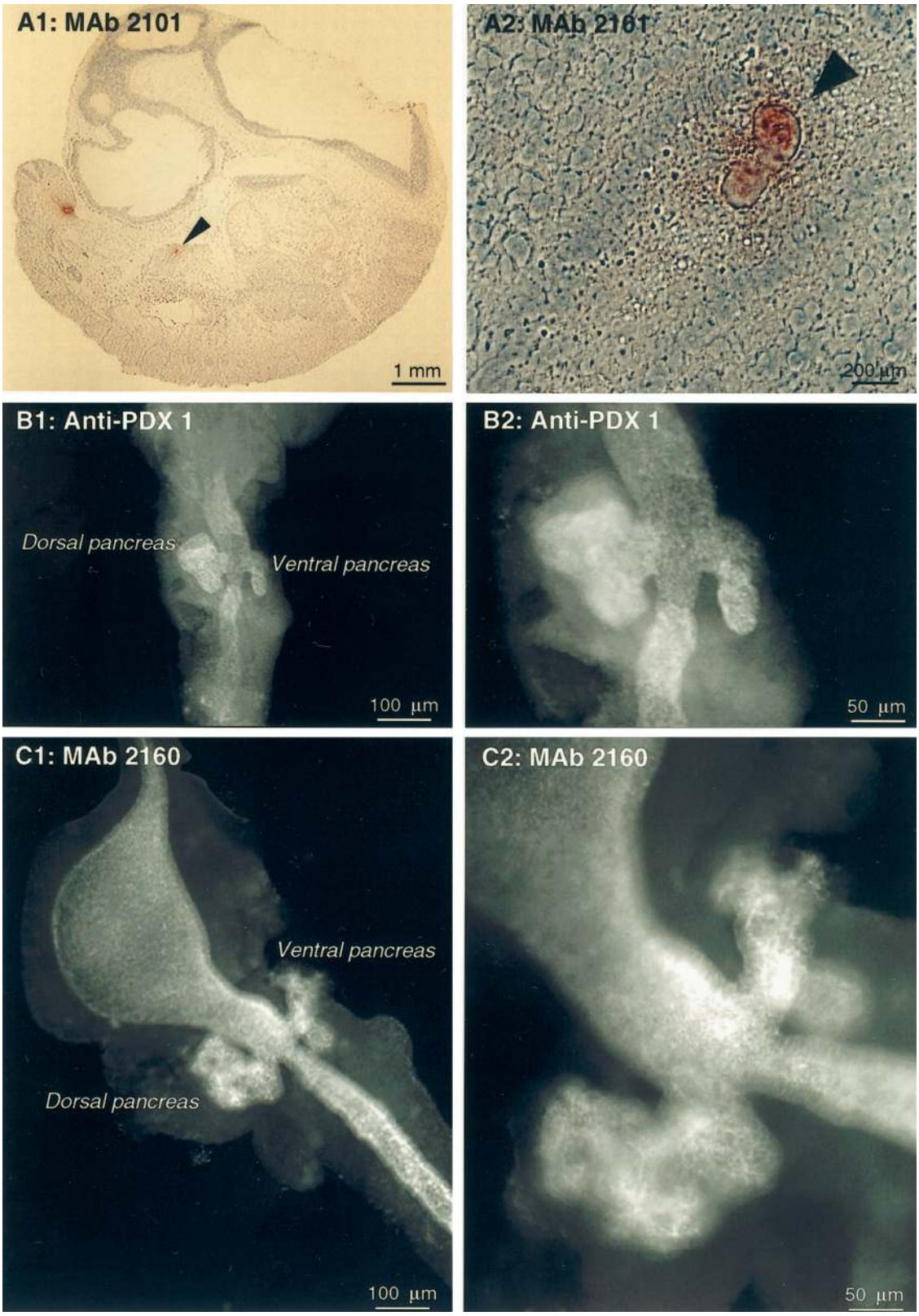

FIG. 3. Immunolocalization of Ag 2101, PDX1, and Ag 2160 along the rat embryo gut. Staining of 12-day rat embryo frozen section with MAb 2101. *Arrows* indicate the specific staining in the pancreatic bud (A). Shown is the staining of dissected 12.5-day rat embryo viscera with a rabbit polyclonal antirat PDX1 (B) and MAb 2160 (C), respectively.

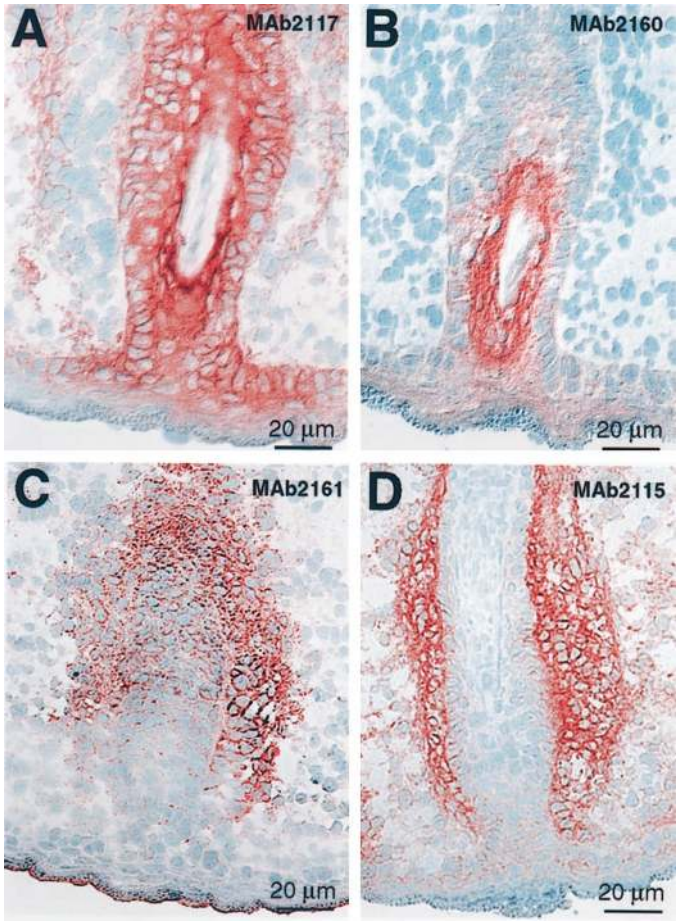

FIG. 4. Immunoperoxidase staining with MAbs 2117, 2160, 2161, and 2115 of frozen sections from 18-day rat embryo vibrissa. High magnification of the staining of the Vibrissa from an e18 embryo is shown for MAbs 2117 (A), 2160 (B), 2161 (C), and 2115 (D). Note that, even though this structure is recognized by all four antibodies, each stains a different subset of cells within the structure.

MAb 2160 is clearly present in a layer of cells corresponding to the visceral endoderm. A weak staining at the level of the extraembryonic endoderm is also observed. In the e10 rat embryo (Fig. 5B), visceral and parietal endoderm are stained. At days e12, e15, and e18 (Fig. 5, C–E) of development, epithelia of multiple organs are stained. The MAb 2160 was strongly reactive with epithelial cells in the olfactory sinus, the lung, the intestine, and the colon at e12. These structures are still positive at e15 and e18. At e15, a positive signal was also detected in the epithelium of the developing ear and pancreas. The stratified epithelium covering the olfactory sinus, oral cavity, tongue, pharynx, and trachea showed moderate-to-strong staining in the e18 embryo (Fig. 5E). The submandibular gland and thymus were also stained. Weak-to-moderate immunoreactivity was observed in the lung, liver, and kidney epithelium. The epithelial lining of the small and large intestine, as well as the epithelium of the urinary bladder and urethra, were strongly reactive. At 18–20 days, a clear staining was observed on the membrane of the epithelial cells in the ear (Fig. 5F), in the vibrissa (Fig. 4B), and in the anal canal including the rectum (Fig. 5G). Whereas very intense staining was detected in the ductal epithelium of developing pancreas; in the adult (Fig. 5H), strong staining was seen only in occasional pancreatic ducts adjacent to the islets. Acinar cells of the adult pancreas exhibited little or no staining, and no specific signal was observed in the islets of adult pancreas except for a few cells at the periphery of the islets (Fig. 5H). No staining was observed in the muscular, skeletal, or nervous tissues at any age studied.

Cloning the genes coding for the antigens

Because all of the MAbs recognized the native configuration of cell surface antigens, the antibodies were used in an expression-cloning strategy to clone the genes coding for the antigenic proteins. A cDNA library was prepared from BUD cells and expressed in COS cells. The antibodies were then used to pan for cells expressing the cell surface molecules of interest. Pure clones were obtained after 6–10 rounds of panning, starting with a pool of 10 antibodies and using individual antibodies by the fifth round.

Using high-efficiency COS cell expression, we have purified, sequenced, and expressed the cDNA clone encoding the proteins recognized by the MAb 2160, called antigen 2160 (Ag 2160). COS cells expressing the genes for Ag 2160 showed a high level of binding of MAb 2160 when analyzed by FACS, but no specific binding to mock transfected COS cells (data not shown).

cDNA sequence for Ag 2160

The DNA sequence encoding Ag 2160, shown in Fig. 6A, predicted an open reading frame of 315 amino acids, with a molecular mass of 35 kDa, in accordance with the estimated molecular mass on Western blot (Table 2A). The hydrophobicity plot of the predicted protein suggests an integral membrane protein (Fig. 6B). A putative signal sequence of 11 hydrophobic amino acids is observed in the sequence core. If the signal peptidase cleavage site is before the Glu-Lys-Asp sequence (43), the ECD of Ag 2160 would contain 243 amino acids. The cysteine-rich ECD of the protein contains two potential N-linked glycosylation sites (NXT/S) at asparagine 111 and 198, which may explain the broad band, between 40 and 50 kDa, observed by Western blot. Ag 2160 is anchored to the membrane by a hydrophobic 23-amino acid sequence that separates the ECD from a highly charged 26-residue cytoplasmic domain.

The protein recognized by the MAb 2160 is homologous to mouse (mEGP) and human pan-EGPs (hEGP-1 and hEGP-2). Comparison of the amino acid sequences reveals a 93% homology with mEGP (19) (88% nucleic acid homology), 88% homology with hEGP-2 (44) (77% nucleic acid homology), and 63% homology with hEGP-1 (20) (43% nucleic acid homology). The highest homology between Ag 2160 and EGPs is in the regions of the 12 cysteine residues, the 2 potential N-linked glycosylation sites, and the signal and transmembrane sequences (Fig. 6C).

Ag 2160 mRNA expression in normal adult tissues

The expression of Ag 2160 mRNA was analyzed in various normal human and rat adult tissues, by Northern blotting,

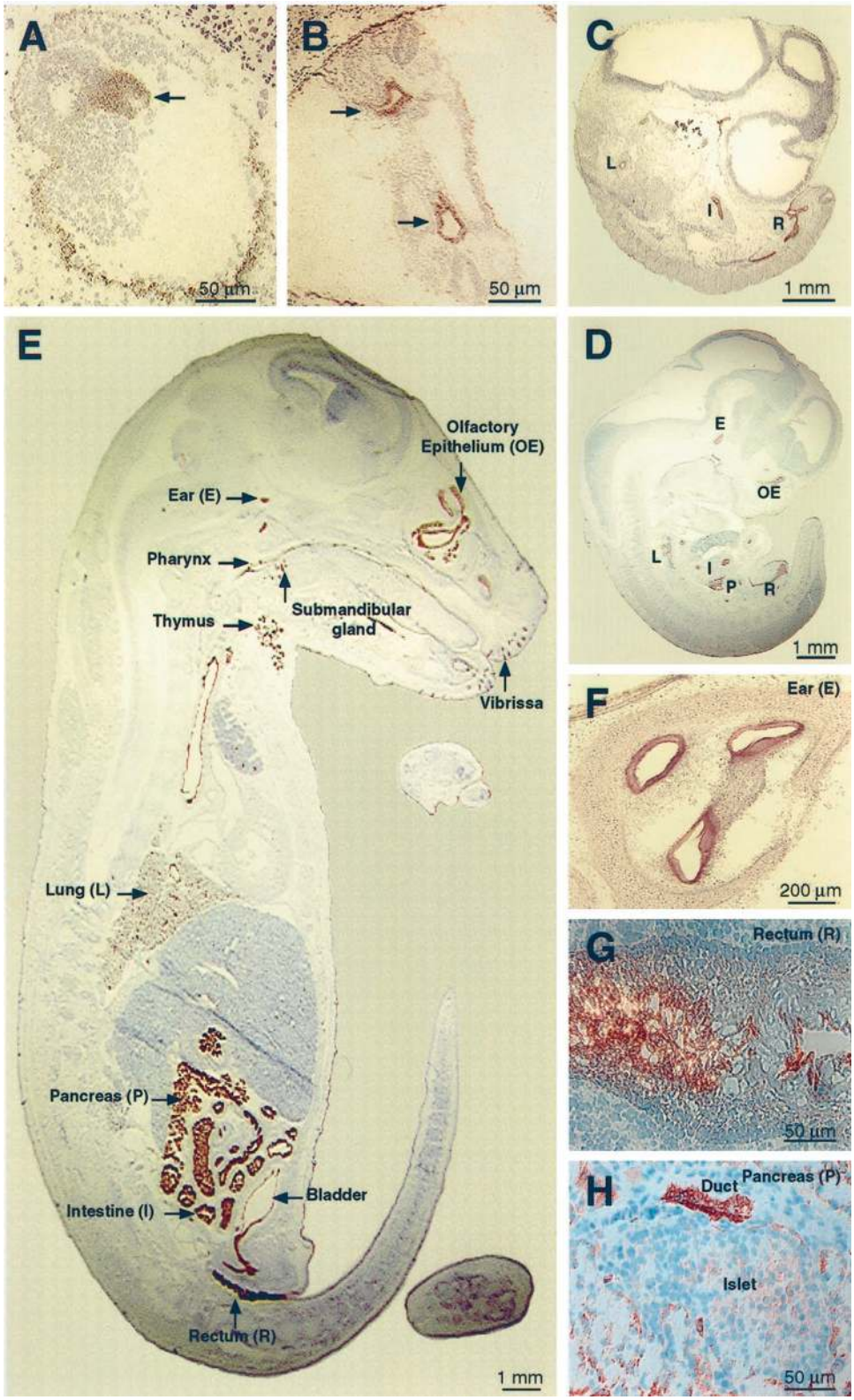

FIG. 5. Immunoperoxidase staining with MAb 2160 of frozen sections from rat embryos and tissues during development. Staining of 9- (A), 10- (B), 12- (C), 15- (D), and 18-day (E) rat embryo frozen sections with MAb 2160. *Arrows* indicate the location of the staining of endoderm in e9 (A) and e10 (B) rat embryos. Note staining of epithelial cells in the vibrissa, olfactory epithelium (OE), ear (E), submandibular gland, pharynx, lung (L), pancreas (P), intestine (I), bladder, and rectum (R). Higher magnification sections show staining of e20 rat embryo ear (F), rectum (e18) (G), and adult pancreas (H), showing positive ductal epithelial cell and negative islet staining.

using the full-length cDNA clones 2160 (Fig. 6; D1 and 6D2, respectively). In human, expression of a 1.7-kb Ag 2160 mRNA was detected in the pancreas, kidney, lung, small intestine, colon, thyroid, and (to a lesser extent) in the stomach and trachea (Fig. 6D1). In rat, expression of Ag 2160 mRNA was detected in heart, lung, liver, kidney, and testis, where two sizes of transcript were detected (Fig. 6D2). These results are in good agreement with the distribution of the antigen seen with IHC in the rat embryo, except for the heart, where no staining was observed in the embryo. No signal

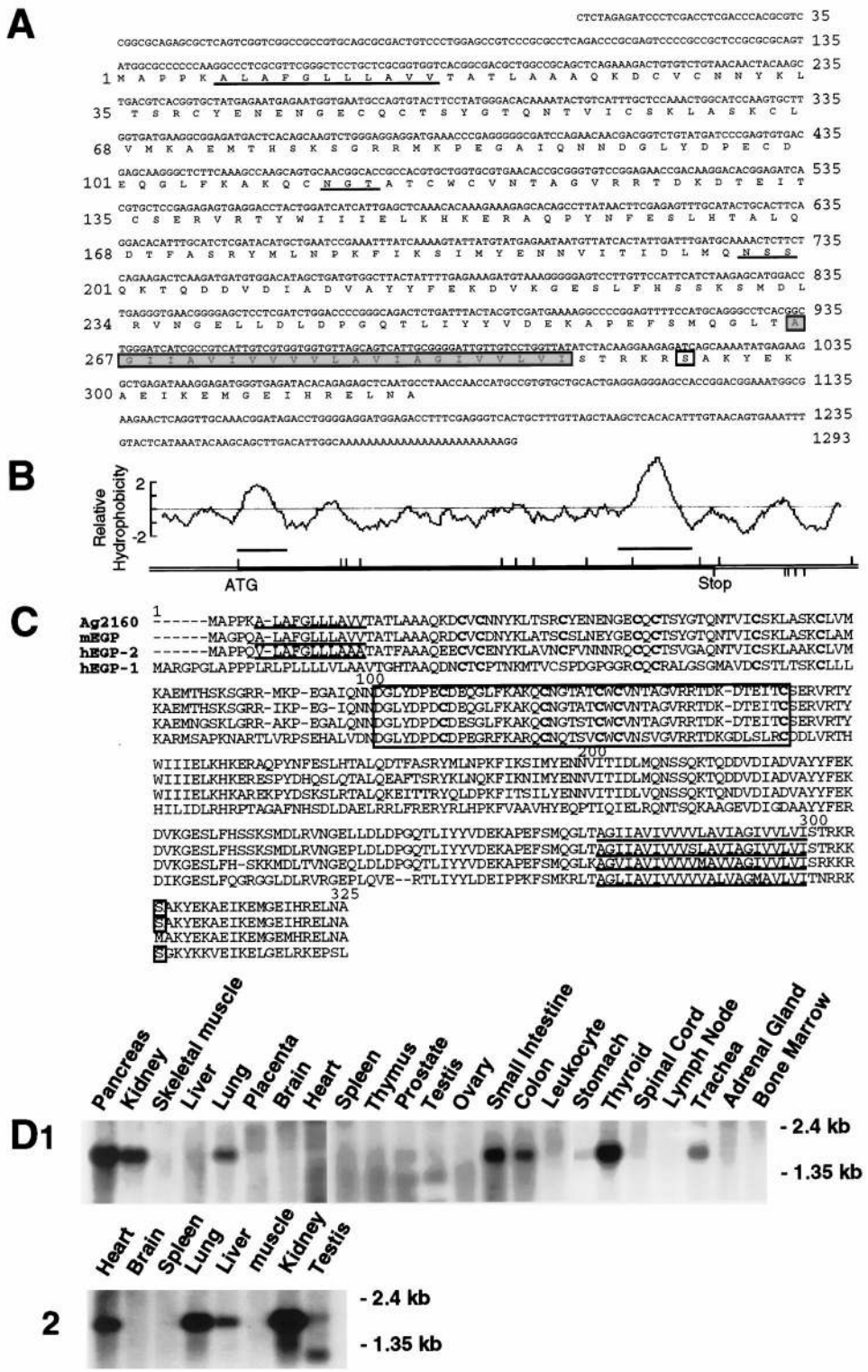

FIG. 6. Characterization of clone 2160. Complete DNA sequence and deduced amino acid sequence of Ag 2160 is shown (A). The nucleotide numbering is at the *right* and amino acid numbering at the *left*. The predicted protein is characterized by a potential signal peptide (*black underline*), 2 potential N-linked glycosylation sites (*gray underline*), and a single 23-amino acid transmembrane domain (*gray frame*). B, The Kyte-Doolittle plot of the deduced amino acid sequence of Ag 2160. The predicted start and stop codons are indicated. The putative hydrophobic signal peptide and the hydrophobic transmembrane domain are *underlined*. C, Homology of Ag 2160 with the previous described related proteins mEGP, hEGP-2, and hEGP-1. The hydrophobic signal peptide and the hydrophobic transmembrane domain are *underlined*. The protein sequences are aligned with the type I thyroglobulin sequence repeat (*framed*). Conserved cysteine residues are in *bold type*, and highly conserved regions are indicated. D, Analysis of human (1) and rat (2) tissue distribution of the message coding for Ag 2160. Poly-(A+) mRNA blots from a variety of adult tissues were hybridized to the cDNA probe.

was detected in muscular, skeletal, lymphoid, and nervous tissues in either human or rat tissues.

In vitro effect of the MAb 2160 and P2160

To understand the biological role of Ag 2160, we constructed, expressed, and partially purified a fusion protein combining the Ag 2160 ECD with a HIS-6 tag (called P2160). To test the *in vitro* effects of these reagents on cells expressing the antigens on their cell surface, BUD cells were cultured in the presence of increasing concentrations of MAbs 2160, P2160, or a nonrelevant control antibody or control HIS-tagged protein. After 5 days of culture, cells were

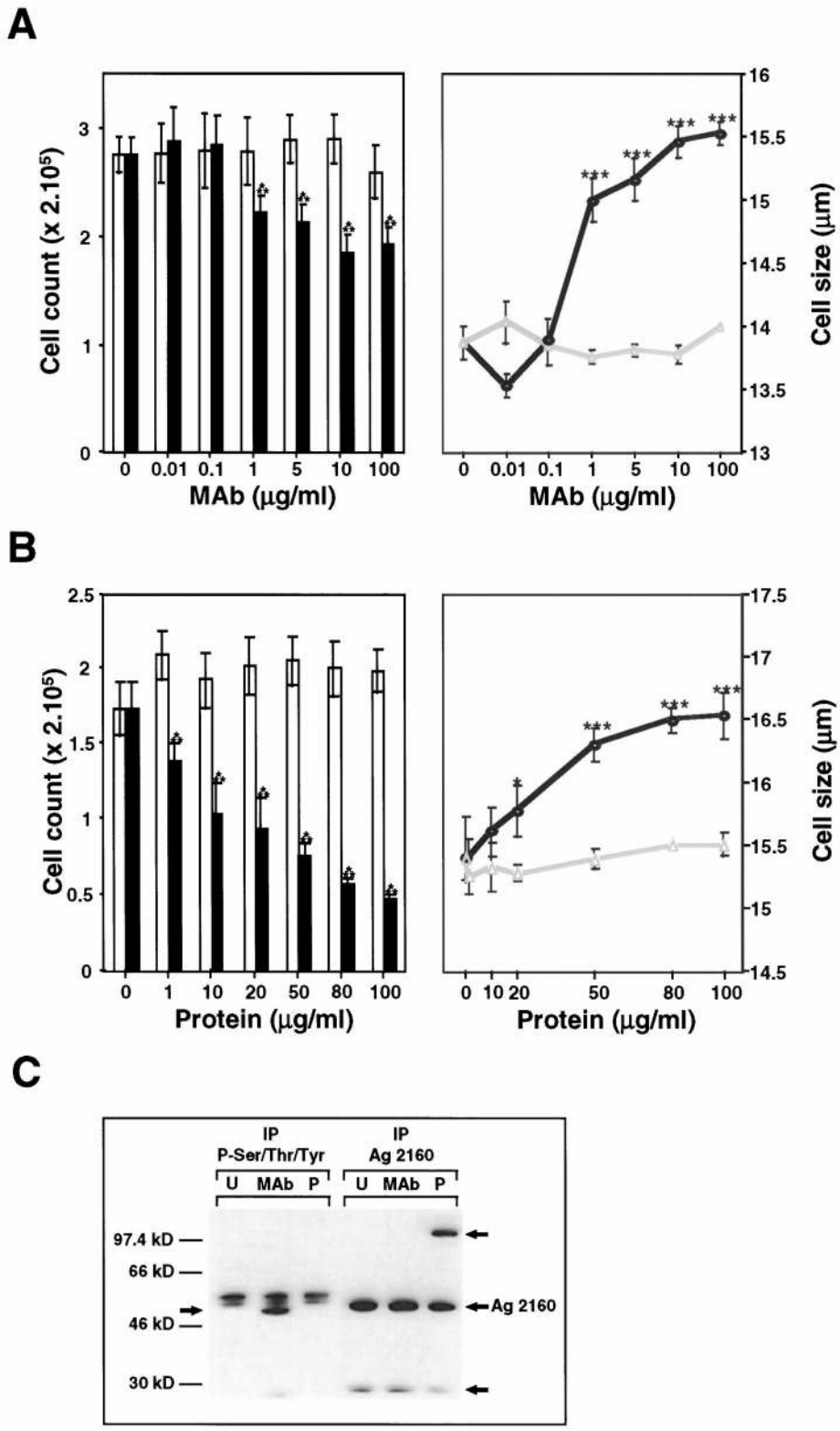

FIG. 7. Effect of the MAb 2160 and P2160 on BUD cell number and volume. BUD cells were plated and cultured for 5 days with the addition of 0–100 μg/ml of MAb 2160 (*black, circles*) or a nonrelevant Ab (*white, triangles*) (A). On day 5, cell number and volume were determined. B, Cells were cultured with or without 0–100 μg/ml of P2160 (*black, circles*) or a nonrelevant fusion protein with an HIS-6 tag (*white, triangles*) and were analyzed as in A. Each value represents mean ± SEM of three (A) or two (B) independent experiments, each run in triplicate. **, $P < 0.01$; ***, $P < 0.001$. C, Immunoblot analysis of phosphorylation in BUD cells after treatment with MAb 2160 or P2160. After 4 days of culture, BUD cells were untreated or treated with either 10 μg/ml of MAb 2160 or 10 μg/ml of P2160. After 2 h of treatment, cells were lysed and phosphorylated proteins or Ag 2160 were immunoprecipitated using an anti-Phospho-Ser/Thr/Tyr (IP P-Ser/Thr/Tyr) or MAb 2160 (IP Ag 2160), respectively. A volume of immunoprecipitated proteins equivalent to 8 μg was reduced in Laemmli sample buffer and loaded on 4–12% polyacrylamide gradient gel, and electrophoresis was performed at 100 V for 2–3 h. After transfer, the membrane was treated as described in *Materials and Methods*.

trypsinized, and cell number and volume were determined. As shown in Fig. 7, culture of the BUD cells in the presence of increasing concentrations of MAb 2160 resulted in a dose-dependent inhibition of cell growth, as well as a dose-dependent increase in the cell volume. The inhibitory effect of MAb 2160 was seen with as little as 1 μg/ml antibody (6.25 nM). The maximal effect of MAb 2160 was seen at 10 μg/ml: 33% growth inhibition and 12% increase of the BUD cell volume. The MAb 2160 had no effect on TR-1 cell growth or volume (data not shown), consistent with the lack of MAb

2160 binding in the FACS analysis. In addition, culture of BUD cells for 5 days in the presence of concentrations up to 100 μg/ml of nonrelevant control antibody had no effect on either cell growth or cell volume.

Similarly, BUD cells were plated in the presence of increasing concentrations of P2160. Culture of the BUD cells in the presence of P2160 also resulted in a dose-dependent inhibition of cell growth. The minimal concentration of P2160 required to inhibit growth was 1 μg/ml (28.6 nM). A dramatic inhibition (>70%) of the proliferation of the cells and an increase in cell volume (7%) were observed when 100 μg/ml of P2160 was added to the culture media. Similar to the effect of the MAb, increased cell volume correlated with the decrease in cell number after treatment with P2160. As before, BUD cell number and volume were unaffected by the addition of the nonrelevant HIS-6 fusion protein (Fig. 7B).

Considering the effect of MAb 2160 and P2160 on BUD cell growth and volume, it seemed possible that Ag 2160 might signal through changes in protein phosphorylation of the cytoplasmic domain of the protein and/or other associated cytoplasmic proteins. To determine the influence of treatment of BUD cells, either with MAb 2160 or P2160 on phosphorylation status, confluent cell cultures were lysed, immunoprecipitated either with antiphospho-Ser/Thr/Tyr MAb or with MAb 2160, separated by gel electrophoresis, transferred, and immunoblotted with antiphospho-Ser/Thr/Tyr MAb. As shown in Figure 6C, a 2-h treatment of the cells with MAb 2160 resulted in the appearance of a 50-kDa phosphorylated protein. The phosphorylation of this protein occurs on a tyrosine, because the corresponding band is also present when the membrane was probed with an antiphosphotyrosine MAb. No significant change was seen in the phophorylation levels when the cells were treated for 2 h with P2160 after immunoprecipitation of the phosphorylated proteins. However, the appearance of a 100-kDa phosphorylated protein and a decrease of the phosphorylation of a 28-kDa protein were observed when P2160-treated cell lysate was immunoprecipitated with MAb 2160. Parallel immunoblots with specific antiphospho-tyr suggest that the 100-kDa protein was phosphorylated on a serine or a threonine, and the 28-kDa protein on a tyrosine. In addition, the immunoprecipitated Ag 2160 itself seems to be phosphorylated on a tyrosine.

Discussion

A large body of evidence suggests that the epithelial cells in the pancreatic bud and the ductal epithelial cells undergoing branching morphogenesis in the early pancreas eventually give rise to the ductal, islet, and acinar cells in the adult (45). In addition, it is becoming increasingly clear that the adult pancreas retains the ability to increase islet cell number by neogenesis of new β-cells from a cell that resides within the ductal compartment of the adult pancreas. It is unclear, at this time, whether the adult pancreatic ducts contain a small percentage of undifferentiated stem cells or whether most ductal cells (3–4% of the pancreas) are capable of differentiation to islet and acinar cells. We established 2 cell lines from these early stages of pancreatic differentiation, which have markers consistent with those expected for embryonic pancreatic epithelium. These markers (cytokeratin 7, PDX1, carboxypeptidase A, and tyrosine hydroxylase) would be expected to be coexpressed in the same cell only during embryonic development. In the adult, they are each expressed in a distinct cell type. In addition, all 10 antibodies raised to the BUD and RED cell lines (shown in Table 2) recognized the epithelium of the developing pancreas. These thus provide a further set of markers for the developing pancreas. Moreover, the fact that all of the antibodies raised to the BUD/RED cells recognize embryonic pancreatic epithelial cells *in vivo* suggest that these cell lines have retained an appropriate expression of cell surface antigens, as well as cytoplasmic markers. These antibodies specifically recognized pancreatic epithelial cells of e12.5 embryos and the epithelia of developmentally related organs at later stages of development.

Using this strategy, we generated more than 15 MAbs, including the 10 presented here, which were specific for cell surface proteins, with minimal cross-reactivity to embryologically unrelated cells (*e.g.* mesodermally derived tissues). All of the MAbs that we raised using this method recognize the ECD of transmembrane proteins. These MAbs proved to be useful in IHC analysis of frozen tissues, including the IgM MAbs. Only a subset of the antibodies were useful for Western blots or IHC of fixed tissues. Thus, this method selectively yields antibodies that recognize the native configuration of the proteins.

None of the antigenic determinants are found exclusively on pancreatic epithelium in older (e18) embryos or adult tissues. This is not surprising, given the early stage of development (e12) from which the BUD cell line was established. The simple epithelia seen at this stage is known to give rise to all of the endocrine and exocrine tissues of the pancreas. Other endodermally derived organs undergo similar elaboration during development. Two groups of antigens are targeted by these MAbs: antigens present on the surface of epithelial cells of the gastrointestinal tract and embryologically related organs; and antigens more broadly distributed and present on the surface of epithelial cells, endothelial cells, and neuronal cells (*e.g.* Ag 2117 (42). The IHC study on e9–e18 embryos confirmed the distribution of antigens predicted from the FACS analysis of the cell lines, and it revealed the largely epithelial distribution of the antigens targeted.

Recently, an inherited syndrome, called multiple endocrine neoplasia type 1 (MEN-1), was demonstrated to be related to a new type of tumor suppressor gene called μ (46). MEN-1 is characterized by the occurrence of neoplastic lesions in the pancreas, parathyroid, duodenum, anterior pituitary, stomach, thymus, and lung, a distribution similar to that seen with the antigens recognized by the antibodies from the first group (MAbs 2100/01, 2103, 2104, 2160, and 2161). Interestingly, even though the antibodies were raised to cloned cell lines, different MAbs recognize different cell layers in many of the same structures, such as the developing vibrissae shown in Fig. 4.

In accordance with the derivation of the BUD and RED cells from pancreatic ductal epithelial cells of the embryo, all of the antigens recognized by the antibodies were expressed in the pancreatic ductal epithelium in embryos, and many in ductal cells in the adult. Some of the antigens were expressed

at varying levels in the acinar cells derived from the ducts, whereas none were expressed in the islets of the adult pancreas. Because islets also derive from the pancreatic bud ductal epithelial cells (13, 47–49), these results suggest that these antigens disappear from the surface of these cells during the differentiation of e12 epithelial cells of the pancreatic bud into endocrine pancreas.

The expression of a BUD cell line cDNA library in COS cells, in combination with panning with the MAbs (32, 50), allowed us to clone and sequence the genes coding for Ag 2160, Ag 2117, the rat homologue of the chicken neural adhesion molecule BEN/SC1/DM-GRASP (42) and Ag 2103, the rat homologue of the α 1,2-fucosyltransferase. Sequencing of several more clones is in progress. Analysis of sequences revealed that Ag 2160 is most probably the rat homologue of the mouse EGP (19). Even though the human EGP was cloned in 1989 (20, 44), the biological function of this cell surface glycoprotein is still being elicudated. Like the mouse and human EGP, rat EGP contains a 45-amino acid type I thyroglobulin repeat, which is found in thyroglobulin, nidogen, laminin B1, and IGFBP-3. Furthermore, the thyroglobulin type-1 repeats bind protease (51) and might therefore act as a protease inhibitor (52).

Both rat EGP-ECD (P2160) and MAb 2160 inhibited the growth and increased the volume of the BUD cell line. Both biological events would be consistent with a cytoplasmic signaling cascade leading to cellular differentiation of the BUD cells. Furthermore, we hypothesize that the binding of MAb 2160 to Ag 2160, as well as the binding of P2160 to the binding site of EGP, blocks the normal interaction between the rat EGP and the EGP binding site, resulting in the inhibition of cell growth. It should be noted that the BUD cells, which are rapidly dividing cells, express a high level of EGP on their surface, as demonstrated by the FACS and immunocytochemistry studies. In addition, we demonstrate that both the MAb 2160 and P2160 treatment are able to modify the phosphorylation status of several proteins, supporting the hypothesis that EGP is an active component of a signal transduction pathway. The identification of the proteins that are phosphorylated in response to P2160 binding is currently under investigation. Rat EGP shares a serine residue with mEGP and hEGP-1, which has been recently been shown to be phosphorylated by protein kinase C (53), confirming that EGP may have the capacity to transduce a signal across the cell membrane. We demonstrate the presence of a phosphotyrosine in Ag 2160 immunoprecipitated from BUD cell lysates. The intracellular domain of Ag 2160 contains a unique potential phosphotyrosine site at tyrosine 297.

The results of the Western blot experiments, observed after treatment of the BUD cells with P2160, strongly suggest the existence of a binding site for Ag 2160 (EGP) on the surface of the BUD cells. The characterization of this molecule will most probably be important in understanding Ag 2160 activity. These results strongly suggests that EGP plays a role in regulating normal epithelial cell growth, in addition to tumor cell growth (54, 55).

Although EGP is not a tumor-specific antigen (56), it has been studied predominately in tumors where it is overexpressed (20). In this study, we confirm previous results concerning the expression of EGP in normal epithelial cells in several organs (such as pancreas, intestine, and colon) and extend these studies to the rat embryo, as well as in the adult. Furthermore, we demonstrate the presence of EGP on the surface of endodermal cells in e9 and e10 rat embryos, suggesting a fundamental role for this glycoprotein in the establishment of the early endoderm. Though our data suggest that EGP functions as a signaling transmembrane mitogen, it may also act as a protease inhibitor and a cell-cell adhesion molecule (21) with a morphoregulatory role during development, as recently suggested by Cirulli *et al*. (57), who proposed that EGP (or Ep-Cam) delivers a specific developmental signal at key stages of pancreatic islet morphogenesis. Our data complement those of Cirulli *et al*., using human embryonic organ cultures. They demonstrate an up-regulation of Ep-Cam during epithelial cell growth *in vitro* and a down-regulation during endocrine differentiation of fetal pancreatic epithelial cells *in vivo*. In this study, we show effects of the MAb and the protein-ECD on growth of a cloned cell line, as well as modification of phosphorylation status of several proteins in response to EGP-ECD and MAb 2160, supporting the suggested signaling role for EGP (Ep-Cam).

As more and more sequences become available, with the increase in partial ESTs in public databases and the sequencing of the human genome, the ability to determine a biological role for proteins becomes increasingly important. In the approach described here, we first defined conditions to selectively culture one cell type from the embryo, obtained antibodies to a select subset of proteins on these cells (those expressed on the cell surface of a defined cell type), then used these to study the biology of related cells *in vitro*, as well as cloning the genes coding for the proteins. The complete approach taken here, with a combination of expression pattern analysis and effects on the growth of a target cell population *in vitro*, provides a rapid method of investigating the biological effects of proteins involved in specific stages of the development of individual organs or tissue types.

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