Immunolocalization of the Ecto-ATPase and Ecto-apyrase in Chicken Gizzard and Stomach

PURIFICATION AND N-TERMINAL SEQUENCE OF THE STOMACH ECTO-APYRASE

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We have examined the in vivo localization of extracellular ecto-ATPase and ecto-apyrase (ATPDase) in adult chicken gizzard and stomach by immunofluorescence and laser scanning confocal microscopy. In chicken gizzard, the ecto-ATPase was distributed in discrete clusters restricted to the sarcolemma of the smooth muscle cells. Anti-ecto-apyrase antibody detected a single 80-kDa band (putative apyrase) in Western blots of both chicken gizzard membrane extracts and partially purified anion exchange fractions, but the antibody did not detect ecto-apyrase in immunolabeled gizzard cryosections. In adult chicken stomach, the ecto-apyrase was observed at the apical membrane of the glandular oxynto-peptic cells as described in previous immunoperoxidase studies (Stout, J. G., R. S. Strobel, and T. L. Kirley (1995) Biochem. Mol. Biol. Int. 36, 529–535). However, ecto-ATPase was clustered in the sarcolemma of the organized layer of circular smooth muscle and in smooth muscle cells of the septa surrounding the glandular tissue, but not in the glandular cells containing the ecto-apyrase. The findings indicate compartmentalization of the two related extracellular nucleotide hydrolizing enzymes and suggest differential functions that are specialized for different regions of the chicken stomach.

We also partially purified the ecto-apyrase of chicken stomach, an 80-kDa membrane glycoprotein. Chicken stomach membranes were solubilized in digitonin, glycoproteins were separated from solubilized proteins by lectin chromatography, and nucleotide-binding glycoproteins were selected by immobilized Cibacron blue chromatography. Further purification by size exclusion and anion exchange chromatography yielded purification of 94-fold. The ATPase specific activity of the purified stomach ecto-apyrase was 75,000 μmol of P/sub i/mg of protein/h, and the purified preparation consisted of a major band (55% of total protein) at 80 kDa. The purified enzyme could be deglycosylated with peptide N-glycosidase-F to a core molecular mass of 54 kDa. The N-terminal sequence of the 80-kDa stomach ecto-apyrase band (which reacted with anti-ecto-ATPDase antibodies) was determined to be: MEYKGKVVAGLLTATWV. Immunological cross-reactivity data indicate that the stomach 80-kDa protein isolated is an ecto-apyrase and is related to both the chicken liver and oviduct ecto-ATPDase enzymes characterized earlier, as well as to the human lymphoid cell activation antigen, CD39.

The importance of extracellular nucleotides in biology is just beginning to be appreciated. Extracellular nucleotides have been implicated in many important physiological processes (1), including purinergic signaling in smooth muscle (2, 3), neurotransmission (4, 5), cell adhesion, apoptosis (6), and platelet aggregation and clotting (7). Enzymes for hydrolyzing extracellular nucleotides, including ecto-ATPases and ecto-apyrases (which we call collectively ecto-ATP/Dases), have been implicated in termination of purinergic stimulation of muscle (2, 8), termination of pain transmission (4), parasite adaptations to host defenses (9), as well as important regulators of thrombotic processes and in maintaining hemostasis in the circulatory system (7, 10). Regarding the last putative function, the lymphoid cell activation antigen, CD39, which is also found on vascular endothelial cells, has recently been identified as an ecto-apyrase (11). Soluble forms of apyrases hold promise as being useful clinically as anti-tissue graft rejection agents (12, 13), as well as anti-thrombotic agents acting independently of the aspirin-sensitive pathway (7). Soluble forms of nucleotidases may also be an important mechanism for the termination of the actions of ATP used as a neurotransmitter (14). For a review of the structures and functions of the ecto-ATPases, the reader is referred to a recent review of the subject (15), as well as a very recent book describing the work presented at an international conference on the subject of ecto-ATPases (16).

Our laboratory has been studying the structure and function of the ecto-ATPases and ecto-apyrases (ecto-ATPDases) for the last decade. We have been very involved in the purification of these enzymes because it is critical to the understanding of the structure and function of these integral membrane glycoproteins, as well as being necessary for the development of probes that are specific for ecto-ATPases and ecto-apyrases. We are interested in determining structural and functional similarities and differences between the ecto-ATPases (which do not hydrolyze nucleoside diphosphates) and ecto-apyrases (ecto-ATPDases), which hydrolyze nucleoside diphosphates at similar rates as nucleoside triphosphates. Previously (17, 18), we

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purified and characterized the ecto-ATPase from chicken smooth muscle (gizzard). In addition, we showed that chicken stomach was a good source of ecto-ATPase (19) that was immunologically indistinguishable from that purified by others from chicken oviduct and liver (20).

To gain insight into the function of both the ecto-ATPases and ecto-apyrases, we have immunolocalized both enzymes in adult chicken gizzard and stomach. In gizzard, the ecto-ATPase is localized to the surface of the smooth muscle cells, and the ecto-apyrase is not detected. In contrast, in stomach, the ecto-apyrase is found predominantly in the glandular region (consistent with previous results; Ref. 19), while the ecto-ATPase is found predominantly on the surface of the smooth muscle cells. This mutually exclusive localization of the two enzymes indicates that these two structurally related enzymes perform very different functions in these tissues.

We also purified the chicken stomach 80-kDa ecto-apyrase glycoprotein, showed that it is immunologically related to human CD39, and showed that both are likely to exist as intermolecularly disulfide-linked homooligomers. The chicken gizzard (ecto-ATPase) and stomach (ecto-apyrase) constitute an easily available system, which is amenable to further characterization of the comparative structure and function of the ecto-ATPase/apyrase systems on the protein biochemical level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue from adult chickens (female Ross Avian 24) was obtained fresh from a local poultry slaughterhouse. All reagent grade chemicals were acquired from Fisher Scientific. Digitonin, concanavalin A-Sepharose 4B, gum tragacanth, sucrose, Tris, and MOPS were from Sigma. Goat anti-mouse TRITC and goat anti-mouse HRP were purchased from Jackson Immunochemicals and Calbiochem, respectively. Size exclusion matrix Sephacryl S-300 was obtained from Pharmacia. Mem-Sep quaternary ammonium ion (QMA) 1000 membrane anion exchanger was ordered from Millipore-Waters. All electrophoresis reagents, chemicals, standards, and the Econo-Pac Cibacron blue column were purchased from Bio-Rad. The enhanced chemiluminescence (ECL) reagent kit was from DuPont. Beckman rotors were used in all centrifugation preparations.

**Microsome Preparation and Partial Purification**—Approximately 200 grams of tissue from gizzard or stomach were trimmed of connective tissue and fat and cut into small pieces. The tissue was homogenized in a 7.0-amp commercial Waring blender in 2 liters of tissue homogenization buffer (buffer: 30 mM MOPS, 2 mM EDTA, 250 mM sucrose, pH 7.4) for 2 min at full speed. The homogenate was centrifuged for 35 min at 10,000 rpm in a JA-14 rotor (15,000 × g). The supernatant was poured through two layers of cheesecloth and centrifuged for 20 min at 20,000 rpm in a SW28 rotor (20,000 × g). The supernatant was then centrifuged for 60 min at 44,000 rpm in a 45Ti rotor (150,000 × g). The pellet was homogenized in approximately 20 ml of 30 mM MOPS and 2 mM EDTA, pH 7.4, and loaded on a discontinuous sucrose gradient of 15 and 45% sucrose dissolved in 30 mM MOPS and 2 mM EDTA, pH 7.4, and loaded on a discontinuous sucrose gradient of 15 and 45% sucrose dissolved in 30 mM MOPS and 2 mM EDTA, pH 7.4. After centrifugation for 16 h at 25,000 rpm in a SW28 rotor (85,000 × g), the white membrane bands at the 15–45% interface were collected, diluted, and selected by centrifugation at 150,000 × g for 90 min. Protein concentrations were determined using the Bio-Rad CB-250 dye binding technique according to the modifications of Stoscheck (21, 22), using bovine serum albumin as the standard protein. The Mg-ATPase (ecto-ATPase) activity was measured using a modification (23) of the technique of Fiske and Subbarow (24). The unstimulated Mg-ATPase specific activity of the gizzard membranes was 100–300 μmol/mg/h, and the unstimulated specific activity of the stomach membranes was 700–1300 μmol/mg/h.

In partial purification experiments, 5 mg of adult chicken gizzard or stomach membrane protein was solubilized at 1.0 mg/ml with 1% digitonin in 20 mM MOPS and 2 mM MgCl₂, pH 7.4. The solution was gently mixed at room temperature for 15 min, centrifuged at 48,000 rpm in a 50Ti rotor for 45 min, and the supernatants were diluted to 0.1% digitonin. The solubilized proteins were then loaded onto a QMA Mem-Sep membrane anion exchanger (size 1000) equilibrated in 0.1% digitonin in 10 mM Tris and 2 mM MgCl₂, pH 8.2 (buffer B), at 2 ml/min. The ion exchanger was then washed in 10 ml of buffer B and eluted with a gradient of 0–200 mM NaCl in buffer B. Fractions (1 ml) were assayed for ATPase or ADPase activity and Western-blotted with various antibodies to correlate antibody detection with measured enzyme activities in individual chromatographic fractions.

**Purification of Chicken Stomach Ecto-ATPase**—Adult chicken stomach membranes (1.0 mg/ml) were solubilized in 1% digitonin in 20 mM MOPS and 2 mM MgCl₂, pH 7.4. The solution was gently mixed at room temperature for 15 min and centrifuged at 48,000 rpm in a 50Ti rotor for 45 min, and the supernatant was diluted to 0.1% digitonin with the MOPS/MgCl₂ buffer. The solubilized solution was incubated with 5 ml of concanavalin A-Sepharose 4B overnight at 4 °C. All of the following steps were performed at room temperature. The solubilized stomach-chorus mixture was poured into a column and washed with 10 column bed volumes of 0.1% digitonin in 20 mM MOPS and 2 mM MgCl₂, pH 7.4 (buffer A). The ConA column was incubated for 10 min in 300 mM methyl-a-D-mannopyranoside in buffer A and eluted in 30 ml of the same solution at a flow rate of 1 ml/min. The entire 30-ml fraction was loaded directly onto a Bio-Rad Econo-Pac Cibacron blue column. The blue column was washed with 10 ml of 1 M NaCl in buffer A, and the ecto-ATPase and other tightly bound proteins were eluted using a gradient of 0–8 M urea in buffer A. A flow rate of 1 ml/min was used throughout binding, washing, and elution of the Cibacron blue column. The eluted fractions were immediately put on ice, and ATPase activity was determined. The most active fractions were pooled and concentrated (at 40°C) to a volume of 1 ml in a Centrerp 50 centrifugal concentrator and washed with five volumes of 0.1% digitonin in 10 mM Tris and 2 mM MgCl₂, pH 8.2 (buffer B) to remove excess urea. The 1-ml washed fraction was loaded onto a Sephacryl S300 size exclusion column equilibrated in buffer B. Fractions (2 ml) were collected at 0.5 ml/min, placed on ice, and assayed for ATPase activity. The most active fractions were pooled and directly loaded onto a QMA Mem-Sep 1000 anion exchanger cartridge. The cartridge was washed with 5 ml of buffer B, and the bound proteins were eluted by a 0–200 mM NaCl in buffer B salt gradient. The flow rate of loading, washing, and elution of the cartridge was 1 ml/min. Fractions (1 ml) were collected, kept on ice, and assayed for ATPase and ADPase activity, and the most active fractions were pooled to be used in deglycosylation and N-terminal sequencing experiments or run on SDS-PAGE gels (25).

**N-terminal Sequencing**—The purified chicken stomach proteins were concentrated using a Centrerp 50 centrifugal concentrator, precipitated with four volumes of –20 °C acetone (26), and run on an SDS-PAGE gel according to Laemmli (25) with or without prior deglycosylation with PNGase F (27). The gel was either stained with Coomassie Blue or electroblotted onto PVDF membrane (28) for N-terminal sequencing on a Porton 2090E protein sequence located in the Department of Pharmacology and Cell Biophysics at the University of Cincinnati.

**Western Blot Analysis**—Microsomes were diluted with Laemmli (25) SDS sample buffer (containing 8 μg/ml) to a final protein concentration of 1 mg/ml. The samples were vortexed and boiled for 5 min. Ten micrograms of each sample was resolved by 5–15% SDS-PAGE gradient gels according to Laemmli (25). The separated proteins were transferred to PVDF membrane (28) and electroblotted in 20 mM MOPS, pH 7.4, for 3 h at 20 °C. The PVDF membranes were blocked for 1 h in 5% milk and 0.02% NaN₃ in Tris-buffered saline and incubated overnight at room temperature in monoclonal antibodies Ab 15 (anti-chicken gizzard ecto-ATPase antibody; Ref. 18), or MC18 (anti-chicken oviduct ecto-ATPDase (apyrase); Ref. 20). The PVDF membranes were washed, incubated in goat anti-mouse IgG HRP conjugated secondary antibody, washed again, and developed in NEN Life Science Products ECL reagent as described by the manufacturer.

**Immunolocalization**—Chicken gizzards and stomachs were immediately removed from sacrificed animals and rinsed in ice-cold phosphate-buffered saline (PBS), pH 7.3. Tissue was oriented and cut into 5 × 5-mm blocks, mounted on cork with 10% gum tragacanth, and frozen in liquid N₂-cooled isopentane. Cryosections (5 μm thick) were cut using a Zeiss Microm HM 500 OM cryostat, collected on Fisher Superfrost/Plus glass slides, and stored at –20 °C. The immunolabeling of tissue samples was performed at room temperature. Tissue sections were blocked in 1% bovine serum albumin (BSA)-PBS to prevent nonspecific binding of the probes. The slides were incubated for 2 h in primary monoclonal antibody (mAb 15 or MC18), washed in 1% BSA-PBS, and exposed to goat anti-mouse IgG (H+L) conjugated to rhodamine (TRITC) for 1 h. Control slides substituted 1% BSA-PBS for the primary antibodies. The slides were cover-slipped in a dinazoacyclo(2.2.2)-octane 70% glycerol mounting media and examined using a Bio-Rad MRC 600 laser scanning confocal microscope and CoMOS image analysis software.
RESULTS

Characterization of Antibodies—In previous studies, a bank of 18 different monoclonal antibodies, including mAb 15, raised against the partially purified 66-kDa chicken gizzard ecto-ATPase, were evaluated (18). In this study, immunoblots of various adult chicken tissue crude membrane extracts indicated that mAb 15 reacts strongly with a 66-kDa protein, the ecto-ATPase, in gizzard (Fig. 1, lane 1) as well as faint bands of a similar molecular size in chicken skeletal muscle, brain, and stomach (Fig. 1, lanes 3, 4, and 6, respectively).

A monoclonal antibody against the native chicken oviduct ecto-apyrase, MC18, was used previously to characterize the chicken stomach ecto-apyrase in Western blot, immunoprecipitation, and immunocytochemistry experiments (20, 29). Unlike our initial study (19), we observed that MC18 recognized a faint 80-kDa band in chicken gizzard membrane extracts (Fig. 2, lane 3) in addition to the abundant 80-kDa band normally observed in stomach and the starch epithelial lining (Fig. 2, lanes 1 and 2). The presence of the 80-kDa protein in chicken gizzard was confirmed by immunoblots of QMA Mem-Sep partially purified gizzard proteins in which the distribution of MC18 binding to a 80-kDa immunoreactive band corresponded with ATPase/ADPase activity (data not shown). The observed specificity of these monoclonal antibodies for the respective antigens indicated that mAb 15 and MC 18 were appropriate probes for immunocytochemical experiments in which the distribution of both the ecto-ATPase and the ecto-apyrase could be examined in serial cryosections of chicken gizzard and stomach tissue.

Immunolocalization of the Ecto-ATPase and Ecto-apyrase—The chicken gizzard is a highly muscular organ composed of numerous parallel bundles of smooth muscle separated by bands of dense connective tissue (Fig. 3C). It is well documented that gizzard smooth muscle is one of the most readily available sources of the ecto-ATPase (17, 30), and enzymology studies have determined that the protein is a membrane bound extracellular protein. However, in vivo immunolocalization of the protein still remains undetermined. Additionally, in contrast to previous data (19), immunoblots with MC18 suggested that an ecto-apyrase-like protein was present in gizzard membranes (Fig. 2, lane 3). To study the distribution of both proteins in the gizzard we used immunocytochemistry and confocal microscopy.

In frozen cryosections of gizzard smooth muscle, binding of mAb 15, specific for the ecto-ATPase, was observed as foci restricted to the sarcolemma of the smooth muscle cells (Fig. 3A). This pattern suggested that the protein is found in clusters rather than being randomly distributed throughout the cell membrane. Faint, diffuse staining was also observed throughout vascular smooth muscle cells. No staining with mAb 15 was detected in the connective tissue bands. Immunocytochemical staining with MC18, the anti-ecto-apyrase antibody, was absent in the chicken gizzard tissue (Fig. 3B).

The chicken stomach (proventriculus) is a secretory organ characterized by numerous concentrically arranged glandular papilla and ducts of oxyntico-peptic cells separated by septa of connective tissue, smooth muscle, and blood vessels. The glandular region is encaused by the submucosa and a triple layer of smooth muscle (Fig. 4C). Stout et al. (19) determined in immunoperoxidase experiments that the distribution of the stomach ecto-apyrase is localized to the apical membranes of the oxyntico-peptic cells, suggesting that the protein is involved in secretory processes. We have observed an identical distribution in this study. In cryosections containing both the glandular region and the outer smooth muscle layers (Fig. 4C), diffuse staining with MC 18 was localized in the apical membrane associated with or near the lumen of ducts (Fig. 4A, lower left corner).

We also examined the distribution of ecto-ATPase in serial cryosections of the approximate region in which MC18 binding was examined. Staining with mAb 15 was observed only in the circular smooth muscle layer that surrounds the glands (Fig. 4B, upper right corner) and in the smooth muscle found between the tubular glands, but not in glandular cells (Fig. 4A). As in the gizzard (Fig. 3A), mAb 15 staining is observed as discrete spots in the cell membrane of the smooth muscle cells.

In control experiments, the primary antibody was omitted, and the cryosections were exposed to only the secondary antibody, goat anti-mouse IgG-TRITC. In both chicken gizzard (Fig. 3D) and stomach tissues (Fig. 4D), no staining was detected (black panels), demonstrating that the secondary antibody did not react nonspecifically with chicken tissue.

Isolation of the Stomach Ecto-ATPase—In previous studies by Strobel and collaborators (20, 29), the chicken oviductal
ecto-apyrase was purified to homogeneity by lectin, ion exchange, and immunoaffinity chromatography. Those investigators determined that MC18, also used in this study, was not a viable choice for immunopurification experiments because purified ecto-apyrase could not be dissociated from the antibody-antigen complex in an active form. Therefore, isolation of the chicken stomach ecto-apyrase in this study was based on previous strategies developed while purifying the ecto-ATPase from chicken gizzard and rabbit T-tubule membranes (17, 27).

The specific Mg-ATPase activity of chicken stomach membranes isolated as described above was 800 μmol of P/mg of protein/h. Solubilization in digitonin resulted in only a 65% recovery of protein and activity, but this is the detergent of choice because it (unlike many other detergents) has no adverse effects on activity. Stomach glycoproteins were isolated by concanavalin A-Sepharose 4B. Bound glycoproteins were eluted with 300 mM methyl-α-D-mannopyranoside and resulted in a 2-fold purification (Table I).

The nucleotide-binding glycoproteins were isolated by loading the sample onto a Bio-Rad Econo-Pac Cibacron blue dye column. The Econo-blue column was washed to remove unbound proteins and eluted with a gradient of 0–1 M NaCl in digitonin buffer to release the more loosely bound nucleotide-binding glycoproteins. After assaying the eluted fractions, the unbound fraction, and the washes, it was apparent that the ATPase activity remained bound to the Cibacron blue column. After trying unsuccessfully to elute the ecto-apyrase with various combinations of salt gradients and low concentrations of nucleotides (i.e., 5 mM ATP), the ATPase activity was eluted by using a 0–8 M urea gradient. The ecto-ATPase was eluted as a single peak in approximately 2 M urea (data not shown). The presence of urea was found to have no measurable effect on the activity of the eluted enzymes, as long as the temperature and time of exposure of the ecto-ATPDase to urea were minimized. The increase in purity after this step was 17-fold (see Table I).

The sample was further purified by size exclusion chromatography using a Sephacryl S-300 matrix. The most active fractions from the Econo-blue columns were concentrated, washed in buffer B to rid the sample of urea, and loaded unto the Sephacryl column. The most active fractions from the size exclusion column were then directly loaded unto a QMA Mem-Sep cartridge. Size exclusion chromatography only increased the purity by an additional factor of 2 (Table I); however, it was an essential step because it quickly separated the ecto-apyrase from the urea, which both stabilized the enzyme, and allowed it to bind to the QMA Mem-Sep cartridge.

Fractions eluted in 0–200 mM NaCl from the QMA Mem-Sep ion exchange cartridge were assayed for ATPase and ADPase activity in the presence and absence of ecto-apyrase inhibitors NaF and NaN₃ (data not shown). The purified stomach ecto-apyrase responded to the two inhibitors in a manner seen previously for other ecto-apyrases found in chicken liver and oviduct (20). The specific activity was 75,000 μmol of P/mg of protein/h, resulting in a final purification of 94-fold (Table I).

It is interesting to note that the ATPase/ADPase ratio of 6.7 reported here for chicken stomach ecto-ATPDase is somewhat higher than the ratio reported previously for other ecto-apyrases.

Aliquots of eluted fractions from the size exclusion column and ion exchange cartridge were immunoblotted and screened with MC 18 (anti-chicken oviduct ecto-apyrase). The distribution of MC 18 reactivity with a 80-kDa protein paralleled that of the ATPase/ADPase activity in the chromatographic fractions (data not shown). The most active ion exchange fractions

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**Fig. 3. Localization of the ecto-ATPase in adult chicken gizzard.** Cryosections of gizzard tissue were blocked to prevent nonspecific antibody reactions, incubated in mAb 15 or MC 18, washed, and then visualized with secondary antibody conjugated to rhodamine. Immunofluorescence was detected by confocal microscopy. Tissue exposed to mAb 15 was observed to have a punctate staining pattern, restricted to the cell membrane of the smooth muscle cells (A) but absent in dense bands of connective tissue surrounding the smooth muscle bundles (A, *). Tissue incubated in MC18 did not generate a visible staining pattern (B). C is representative of hematoxylin- and eosin-stained chicken gizzard cryosections. The asterisk denotes a band of dense connective tissue similar to that seen in A (*). In secondary antibody control experiments nonspecific reactions in gizzard tissue were not detected (D). A, B, and D, magnification ×600; scale bar = 50 μm. C, magnification ×500; scale bar = 10 μm.
were pooled, subjected to SDS-PAGE, transferred to PVDF membrane, and stained with Coomassie Blue. Densitometric scans indicated that the major band (55% of total protein) from the four-step purification was a 80-kDa protein equivalent to the MC18 immunoreactive band. Minor bands of 120, 66, and 43 kDa were also present in the partially purified preparation (results similar to those shown in Fig. 5).

Deglycosylation—Pooled purified chicken stomach (Mem-Sep) fractions from a single purification were acetone-precipitated (26), dissolved in 0.2% SDS, and then divided into two equal parts. Half of the protein was treated with PNGase-F overnight at 37 °C, and the remaining, untreated half was stored at 220 °C to be used as a control. In both Coomassie Blue-stained gels and PVDF membranes after Western blotting, the major band in the untreated controls was observed at 80 kDa (Fig. 5, lane 2). Incubation in PNGase-F, presumably resulting in removal of all N-linked oligosaccharide residues (because longer incubations with more PNGase-F did not further reduce the size of the resultant, sharp, protein bands), caused a shift of the major 80-kDa band to a core protein of approximately 54 kDa (Fig. 5, lane 3), a size indistinguishable with the observed core molecular mass of both the ecto-

ATPases of chicken (17) and rabbit (27), as well as the human ecto-apyrase, CD39 (31). The sharp faint bands at 35 kDa in lanes 3 and 4 in Fig. 5 were due to the exogenously added PNGase-F enzyme, as shown by the PNGase-F blank sample in lane 4 of Fig. 5 (the migration position of the PNGase-F band is indicated by an asterisk in Fig. 5). The identity of the band having a core protein molecular mass of 43 kDa (Fig. 2, lane 3), which presumably was derived from the diffuse glycoprotein band seen at ∼60 kDa in lane 2 of Fig. 5, is unknown.

N-terminal Sequencing of the Stomach Ecto-ATPase—Mem-Sep fractions with the highest activity (fractions 6–8) were pooled, acetone-precipitated, run on a 5–15% gradient gel, and electroblotted onto PVDF membrane for N-terminal sequencing. The following sequence was obtained (with the corresponding picomoles of amino acid for each cycle shown in parentheses): M(2)-E(2)-Y(31)-K(23)-G(10)-K(22)-V(19)-V(26)-A(17)-G(10)-L(17)-L(21)-T(12)-A(17)-T(11)-W(14)-V(14). The first two amino acids are at lower levels because they were obtained from a second sequencing experiment, which was made necessary due to a sequencer malfunction in the first experiment, resulting in loss of data for the first two amino acid residues. Nevertheless, the sequencing of a second, independ-
Enzymes capable of hydrolyzing extracellular ATP and ADP have been known for a long time, but have only recently been described on a molecular level (15, 16). Many functions have been proposed for these enzymes, including termination of purinergic stimulation of muscle (2, 8), parasite adaptations to evade host defenses (9), and involvement in vesicular transport processes mediated by caveolae (34), as well as regulators of thrombotic processes and in maintaining hemostasis in the circulatory system (7, 10). The lymphoid cell activation antigen, CD39, is found on vascular endothelial cells and has recently been identified as an ecto-apyrase (11), as well as being one of the three important endothelial thromboregulatory systems (along with NO (endothelium-dependent relaxation factor) and the eicosanoids; Refs. 7 and 10) that inhibit platelet aggregation in response to agonists. Soluble forms of apyrases hold promise as being useful clinically as anti tissue graft rejection agents (12, 13), as well as antithrombotic agents acting independently of the aspirin-sensitive pathway (7). The ecto-ATPases may also have a role in cardiovascular well-being, because one putative function of these enzymes is to regulate blood pressure by hydrolyzing ATP used to activate purinergic receptors and modulate vascular smooth muscle tone.

An initial step in defining the functions of the ecto-ATPases and ecto-apyrases is the purification and immunolocalization of both enzymes. In this study, we show the immunolocalization of the ecto-apyrase and the ecto-ATPase in the same tissue (stomach), and demonstrate that these two enzymes are compartmentalized to the glandular cells and the smooth muscle cells, respectively (see Fig. 4, A and B). These results are
consistent with a function of the ecto-apyrase in secretory processes, and a function of the ecto-ATPase in termination of purinergic stimulation of smooth muscle. Whatever the functions, it is clear that the two enzymes are important in different processes in the stomach, due to their very different localizations.

In the chicken gizzard and stomach, the ecto-ATPase is distributed in discrete clusters throughout the smooth muscle cell membrane (see Figs. 3A and 4B), suggesting that the protein is not randomly inserted into the cell membrane, and indicating that the ecto-ATPase is not polarized to any one region of the cell, as is seen in the stomach glandular cells (see Fig. 4A). Clustering of proteins suggests that the ecto-ATPase might form aggregates and/or be associated with other proteins, such as purinergic receptors.

Immunoblots of gizzard membranes with MC 18, an anti-ecto-apyrase monoclonal antibody, produced an 80-kDa immunoreactive band characteristic of the ecto-apyrase (see Fig. 2, lane 3). However, in immunolabeling experiments, MC 18 did not produce visible staining in gizzard (see Fig. 3B). The lack of immunostaining in the gizzard with the anti-apyrase antibody most likely means that the protein is expressed in low levels in the gizzard and/or it is so diffusely distributed that it is below the detection limit of the immunofluorescence technique.

We have shown previously that the chicken ecto-ATPase has high sequence homology with mouse and human CD39 (35), which are ecto-apyrases. The sequence similarity observed between ecto-apyrase and ecto-ATPase enzymes is also found in other species. In Toxoplasma gondii, the ecto-ATPases and the ecto-apyrases are so closely related that the two proteins apparently differ in only 16 amino acids (36). This is consistent with the immunological cross-reactivity observed on Western blots observed in this work (see Fig. 6), as well as previous studies showing immunological cross-reactivity between ecto-ATPases and ecto-apyrases on Western blots (33, 35). It follows that any study involving antibodies against the ecto-ATPases or ecto-apyrases must show specificity for one enzyme in the presence of the other enzyme to be interpreted correctly, as was done in this study.

The stomach ecto-apyrase was purified without using any antibody affinity methods. Instead, techniques similar to those used earlier in the purification of the chicken gizzard (17) and rabbit skeletal muscle (27) ecto-ATPases were used. The specific ATPase activity of the purified ecto-apyrase (75,000 μmol of ATP/mg of protein/h, Table 1) compares favorably with those reported using antibody affinity chromatography to isolate the chicken oviduct ecto-apyrase (48,000 μmol of ATP/mg of protein/h; Ref. 20). However, the ΔT/Pase/ΔD/Pase ratio of the partially purified ecto-apyrase was somewhat higher than those reported previously (see Table I). In mammalian tissues the ATPase and ADPase activity of purified ecto-apyrases is approximately equivalent (ATPase/ADPase ≈ 1), while CD39 has a slightly higher ATPase/ADPase ratio of 1.6 (11). Strobel et al. reported a ratio of 4 for the ecto-apyrase isolated from chicken liver and oviduct (29). It is possible that the higher ATPase/ADPase ratio of 6.7 reported here for the purified stomach ecto-apyrase is due to a small amount of ecto-ATPase that was co-purified with the ecto-apyrase. It is obvious from the immunolocalization results that the ecto-ATPase is also present in the chicken stomach used to purify the ecto-apyrase, and it is likely that the two enzymes will co-purify in many types of chromatography. From the very high specific activity (Table 1) and the SDS-PAGE gel analysis (see Fig. 5, lane 2) of our purified ecto-apyrase, it is apparent that the preparation is not grossly contaminated. However, due to the high specific activity of the ecto-ATPase, it would take only a small amount of ecto-ATPase to be present in the ecto-apyrase preparation to bias the ATPase/ADPase ratio. Another possible explanation for the differences in ATPase/ADPase ratios is that the differences might be due to isoforms being expressed in the different species and/or organ systems. A final, less interesting, explanation could be that the ATPase/ADPase ratio is very sensitive to slight variations in the assays used by different laboratories.

The immunoreactivity of the purified chicken stomach enzyme with MC18, a monoclonal antibody against the chicken oviduct ecto-apyrase, along with an identical glycosylated size to the oviduct enzyme, as well as a deglycosylated size of 54 kDa (see Fig. 5, lane 3) consistent with the sizes reported for all of the vertebrate ecto-ATPases and ecto-apyrases, suggest that the stomach ecto-apyrase reported in this work is identical to the chicken oviduct enzyme characterized by Strobel et al. (20).

In addition, the enzymatic properties and immunoreactivity of all of these chicken enzymes suggests that they are all closely related to human CD39 ecto-apyrase. Clearly, the function of the stomach apyrase could not be the same as that proposed for human endothelial CD39 (maintenance of hemostasis). This means that either the same enzyme could perform different functions when expressed in different tissues and cell types, or that there is a family of ecto-apyrases (isozymes) that are structurally closely related, but functionally distinct, and expressed in a tissue-specific manner.

One key step in the purification of the stomach ecto-apyrase is the elution from the Cibacron blue column by a gradient of urea. The purified proteins bound the nucleotide-binding dye so tightly that conventional methods of elution such as salt gradients (0–2 M NaCl) or nucleotide competition (5 mM ATP) were ineffective. Why the chicken stomach ecto-apyrase reported here bound so tightly to this matrix while others have reported that other ecto-apyrases could be eluted from Cibacron blue matrices using much less stringent conditions (37) is unclear. It is possible that the detergent (digitonin) or the different buffer conditions used in this work are critical. The fact that the enzymatic activity is stable in high concentrations of urea is also of note, and is another line of evidence indicating the high stability of this class of enzymes to many types of denaturants.

In summary, we have described a system in which the structure-function relationship of the ecto-ATPases and ecto-apyrases can be examined. We have shown that the proteins are compartmentalized in functional regions of the stomach, with the ecto-ATPase distributed in clusters throughout the smooth muscle membranes, while the ecto-apyrase is diffusely localized near the apical membrane of the secretory cells. The punctate localization of the ecto-ATPase in the stomach and gizzard suggests that the protein might be clustered around other proteins or form aggregates. Finally, we have devised an ecto-apyrase purification from a readily available tissue that can be examined. The results of these studies further the development of ecto-apyrase-specific probes.

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