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Co-secretion of soluble caveolin-1 and pepsinogen in the oesophagus of the red-legged frog *Rana aurora aurora*

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Summary

Caveolin-1, an integral membrane protein, is the principal component of caveolae, which are specialised vesicular microdomains of the plasma membrane. Caveolae are found in most cell types, but they are most abundant in adipocytes, endothelial cells, fibroblasts, and muscle cells. Functionally, they have been implicated in endothelial transcytosis, potocytosis, and signal transduction. Recently, caveolin-1 has been found unexpectedly in the cytoplasm, mitochondria and elements of the secretory pathways of exocrine secretory cells. We have co-localised caveolin-1 and pepsinogen immunohistochemically in serous cells of oesophageal glands of the red-legged frog, *Rana aurora aurora*. Thus, according to its intracellular localisation pattern, caveolin-1 may be either a soluble protein, located in secretory droplets, or a protein that is inserted in caveolar membranes. Soluble caveolin-1, which is probably embedded in a lipid particle surrounded by a phospholipid shell, may be involved in intracellular and extracellular lipid transport. In the gut, caveolin-1-rich lipid particles can act as donor particles to facilitate (protein-mediated) intestinal uptake of cholesterol and phospholipids. Our findings strengthen the hypothesis that caveolin-1 has a physiological autocrine/paracrine function and demonstrate that secretion of this protein also occurs in vertebrates other than mammals, such as amphibians, which may be a useful alternative animal model to study caveolin-1.

Introduction

Caveolin-1 is an integral membrane protein (Kurzchalia et al., 1992), with both the N- and C-terminal domains being cytoplasmic, and is the principal component of caveolar membranes. Caveolae are specialised vesicular microdomains of the plasma membrane that are formed by localised

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accumulation of cholesterol, glycosphingolipids, and caveolin. Caveolae can be found individually or in clusters and are found in most cell types, but they are most abundant in adipocytes, endothelial cells, fibroblasts, and muscle cells. Functionally, caveolae have been implicated in endothelial transcytosis, potocytosis, and signal transduction. Caveolin-1 can play a role in intracellular vesicular and cholesterol trafficking (Smart et al., 1996; Anderson, 1998; Okamoto et al., 1998; Uittenbogaard and Smart, 2000).

Recently, caveolin-1 has been found unexpectedly in the cytoplasm, mitochondria and elements of the secretory pathways of exocrine secretory cells. These data suggest that caveolin-1 may also exist in a soluble form as a cytosolic protein or associated with secretory products (Liu et al., 1999; Li et al., 2001). However, the function of secreted caveolin-1 is still unknown, but a role in extracellular lipid transport has been hypothesised (Liu et al., 1999; Li et al., 2001).

To better understand this role of caveolin-1, we have immunohistochemically localised caveolin-1 in secretory cells of the gastro-intestinal tract of an amphibian, the red-legged frog *Rana aurora aurora*. We have previously described the gastro-oesophageal mucosa of this anuran (Ferri et al., 2001). In the red-legged frog, as in other Ranidae, such as *Rana esculenta* (Bani et al., 1992), pepsinogen in the gastric juice is for the larger part synthesised by oesophageal glandular cells, whereas hydrochloric acid is produced by oxyntic cells in fundic glands. Thus, there is a gradient in the production of proteolytic enzymes and hydrochloric acid along the oral–aboral axis of the gastro-oesophageal tract, as has been reported for other non-mammalian vertebrates (Ferri et al., 1999; Liquori et al., 2000). Since peptic glands are particularly voluminous and secrete abundant amounts of pepsinogen, the oesophageal mucosa of this frog seems to represent an appropriate model to investigate the presence and functions of caveolin-1 in the secretory pathway.

Material and methods

Animals

Specimens of *R. a. aurora* from western North America were obtained from Minervini (Bari, Italy).

Light microscopy

Four adult specimens of *R. a. aurora* were sacrificed after ether anaesthesia and their digestive tracts were quickly removed and processed for embedding in Technovit 8100 (Heraeus-Kulzer, Wehrheim, Germany). Small samples were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 4 h at 4°C. After several rinses in PBS, the samples were incubated overnight at 4°C in PBS containing 6.8% sucrose. They were then dehydrated at 4°C in a graded series of acetone. Specimens were incubated in the Technovit 8100 monomer solution for 6 h at 4°C under gentle stirring. Polymerisation was carried out on ice for 3 h. Semithin sections (3 μm thick) were cut with glass knives using an Ultratome (LKB, Uppsala, Sweden). Before histochemical and immunohistochemical staining, sections were mounted on microscope slides coated with polylysin and were incubated for 5 min at 37°C in 0.01% trypsin and 0.1% $CaCl_2$, in PBS, pH 7.8, for antigen retrieval.

Histochemistry

Zymogen granules were identified with a modified Bowie's staining method according to Bonucci (1981), or with a combined PAS-Bowie staining method to demonstrate both mucus and zymogen granules simultaneously (Ferri et al., 2001). All sections were counterstained with haemallum.

Some sections were stained using the Masson–Fontana silver method to detect argentaffin endocrine cells (Pearse, 1972).

Immunohistochemistry

Caveolin-1 and pepsinogen were localised using the peroxidase–antiperoxidase (PAP) method. Endogenous peroxidase was blocked by incubating sections in PBS containing 1% H_2O_2 for 10 min at room temperature. After several rinses in PBS, sections were incubated for 2 h at 37°C in a humid Petri dish with the primary antibodies (anti-caveolin-1: ABR, Golden CO, USA; anti-pepsinogen-1: DPC Biermann, Bad Nauheim, Germany) diluted 1:100 in PBS containing 1% normal goat serum (blocking buffer). Sections were washed in blocking buffer and incubated for 1 h at 37°C with goat anti-rabbit IgG (Sigma, St. Louis MO, USA) diluted 1:100 in blocking buffer. After several washes in blocking buffer, sections were incubated with horseradish PAP complex (Sigma) at a dilution of 1:100 for 1 h at 37°C. Finally, immunolabelling was visualised by

incubation with 3-3′-diaminobenzidine–H_2O_2 medium (Graham and Karnowsky, 1966) for 10 min at room temperature.

Controls were performed by using antibodies pre-adsorbed with the respective antigens or by omitting the primary antibodies.

Images were captured using an E600 photomicroscope equipped with a digital camera DMX1200 (Nikon, Kawasaki, Japan).

Electron microscopy

Small samples of gastro-oesophageal mucosa obtained from four adult specimens of *R. a. aurora* were fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 M PBS, pH 7.4, for 4 h at 4°C, and then postfixed in 1% OsO_4 in PBS for 30 min at 4°C. Fixed specimens were washed in several changes of PBS and dehydrated in a graded series of ethanol. Finally, samples were embedded in Epon (Taab, Redding, UK). Sections were mounted on formvar-coated nickel grids and stained routinely with uranyl acetate and lead citrate. Finally, grids were observed with an EM 109 electron microscope (Zeiss, Oberkochen, Germany).

Immunoelectron microscopy

For immunoelectron microscopy, ultrathin non-osmicated sections, mounted on formvar-coated gold grids, were treated with 0.05 M glycine in PBS buffer for 15 min at room temperature. Grids were incubated for 30 min at room temperature with 1% BSA in PBS containing 0.2% gelatin (PBG) and then placed on a drop of anti-caveolin-1, diluted 1:100 in PBG overnight at 4°C. The grids were rinsed with PBG, and then incubated in a dilution of 1:10 of 10-nm gold-conjugated anti-rabbit IgG (Sigma) in PBG for 1 h at room temperature. After several rinses in PBG and distilled water, the grids were lightly stained with uranyl acetate and lead citrate.

Immunolabelling controls were performed by using antibodies pre-adsorbed with antigens or by omitting the primary antibodies.

Results

The oesophagus of the frog *R. a. aurora* was found to be lined with a pseudostratified ciliated epithelium containing abundant amounts of mucous goblet cells. Two different types of goblet cells were found in the epithelium (Fig. 1A). Type-I goblet cells were characterised by supranuclear cytoplasm containing large PAS-positive secretory vesicles that were often confluent. Type-II goblet cells were thinner with ovoid basal nuclei and numerous small secretory granules in the supranuclear cytoplasm. The granules, which were PAS positive, often showed weak immunostaining of pepsinogen.

The mucosa was folded, and contained numerous oesophageal glands. The glands were of the simple or tubular–alveolar type and consisted of PAS-positive mucous cells and serous cells. The latter cell type was filled with large granules that stained intensely using Bowie's staining method (Fig. 1A) and were intensely positive for both pepsinogen and caveolin-1 (Fig. 1B and C).

The stomach of *R. a. aurora* is subdivided into a wide corpus, or fundus, and a short pars pylorica, that are characterised by fundic and pyloric glands, respectively. It was found to be lined with a thick mucosa with few longitudinal folds. A single layer of PAS-positive mucus-secreting cells lined the luminal surface of the stomach (Fig. 1D).

Fundic glands were of the simple or ramified tubular type and consisted mainly of oxyntic cells and mucous neck cells. Scattered argentaffin cells were also present (data not shown).

Oxyntic cells and endocrine cells were PAS negative and did not stain with Bowie's method (Fig. 1D). Immunostaining of pepsinogen or caveolin-1 was not observed (Fig. 1E and F).

Pyloric glands consisted of PAS-positive mucous cells and enteroendocrine cells which did not stain for either pepsinogen or caveolin-1.

At the oesophagogastric junction, mucosal epithelium gradually changes from oesophageal ciliated pseudostratified to a simple gastric epithelium. Towards the stomach, goblet cells showed an increasing number of secretory granules stained with Bowie's method. In this area, oesophageal glands were reduced in volume, and contained only a few pepsinogen granules, whereas the first gastric glands appeared. These glands were mostly alveolar and consisted mainly of mucus-secreting cells, with few oxyntic cells and contained only few pepsinogen-positive granules. Mucous glandular cells were moderately PAS-positive but did not stain for pepsinogen or caveolin-1.

At the EM level, serous cells of oesophageal glands contained an extensive rough endoplasmic reticulum in a vesicular form, and numerous secretory granules (Fig. 2A). These granules were globular and homogeneously electron dense.

Immunogold staining with anti-caveolin-1 resulted in numerous gold particles in secretory granules of serous cells in oesophageal glands that were not associated with vesicular membranes

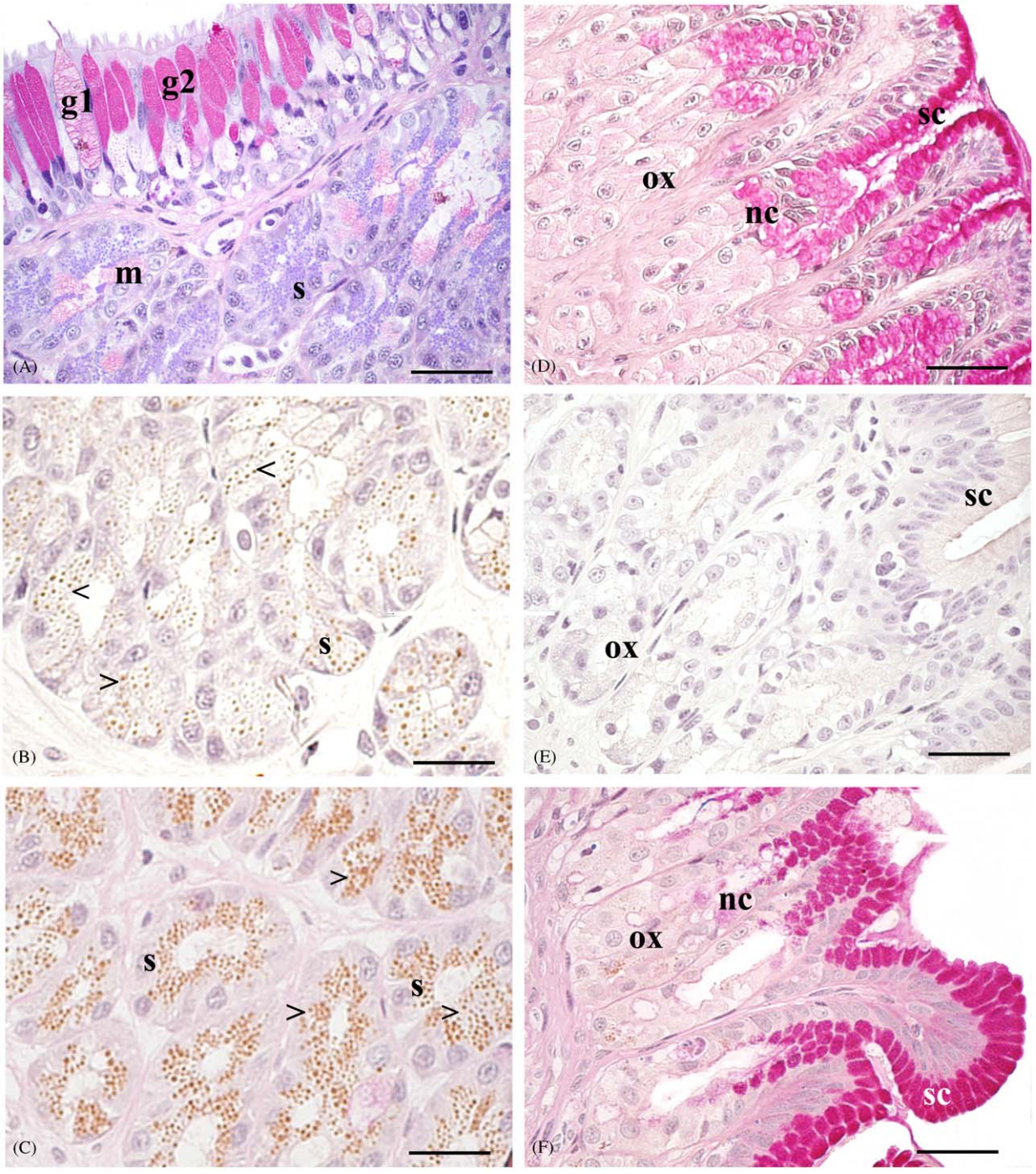

Figure 1. Gastro-oesophageal mucosa of the frog *R. a. aurora* stained with different histochemical methods. (A–C) Oesophageal mucosa. (A) The ciliated epithelium contains two types of goblet cells (g1, and g2). Oesophageal glands consist of serous (peptic) cells (s) and mucous cells (m). PAS-Bowie-haemallum staining. (B) Numerous pepsinogen-positive granules (arrows) are present in serous cells (s) in oesophageal glands. Anti-pepsinogen staining and haemallum. (C) Secretory granules (arrows) are stained for caveolin-1. (s) Serous cells. Anti-caveolin-1 and haemallum staining. (D–F) Gastric mucosa. (D) The mucosa is lined with a single layer of mucus-secreting cells (sc). Gastric glands mainly consist of mucous neck cells (nc) and oxyntic cells (ox). PAS-Bowie-haemallum staining. (E) Oxyntic cells (ox) are not positive after anti-pepsinogen staining. Anti-pepsinogen and haemallum staining. (F) Immunostaining is not present in oxyntic cells (ox) after staining with anti-caveolin-1. Anti-caveolin-1 and haemallum staining. Bars: (A,D–F) 30 μm; (B,C) 25 μm.

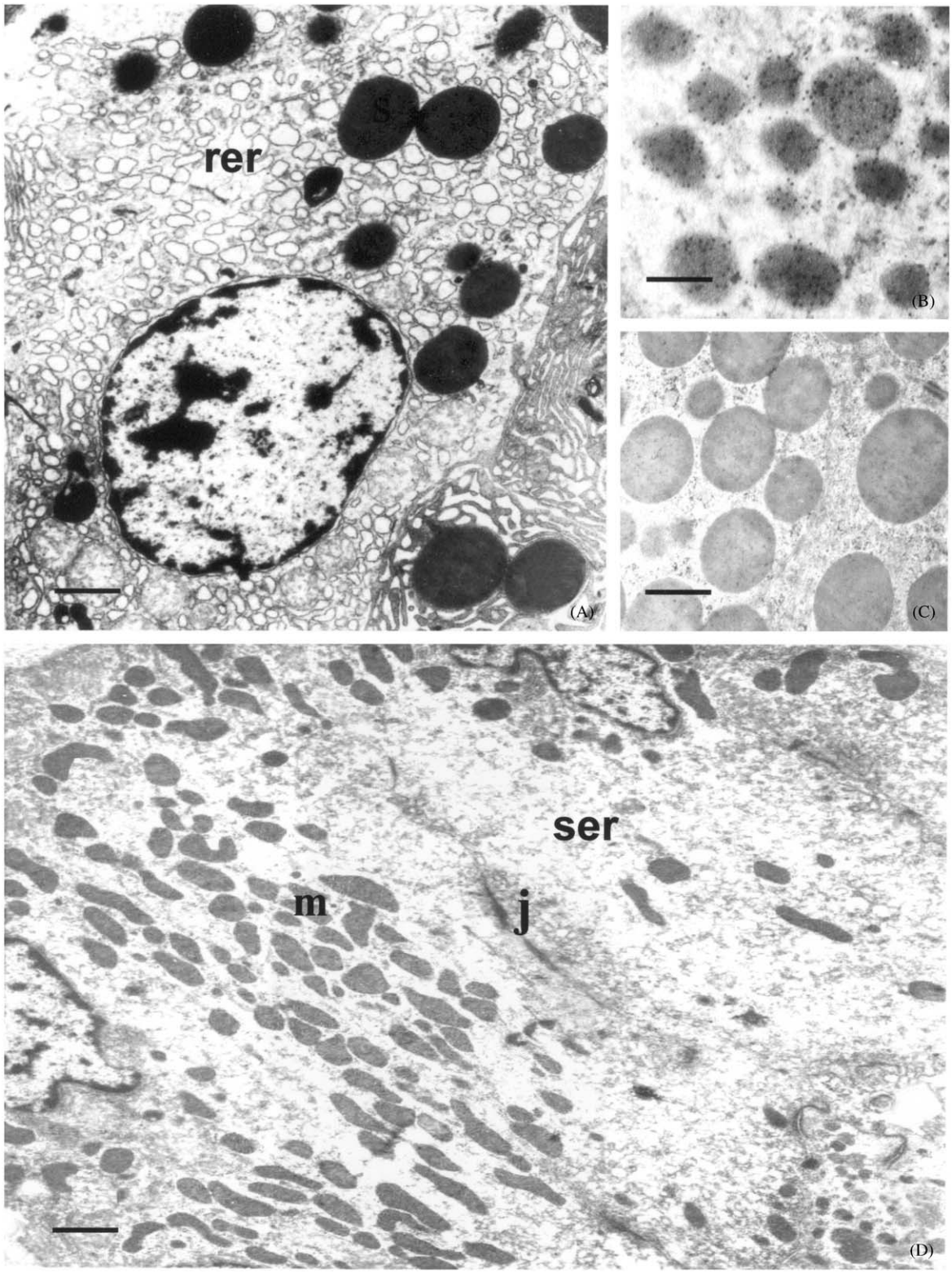

Figure 2. Electron micrographs of secretory cells in gastro-oesophageal mucosa of *R. a. aurora*. (A–C) Oesophageal peptic cells; (D) gastric oxyntic cells. (A) Peptic cells show a well-developed rough endoplasmic reticulum (rer) and numerous electron-dense secretory granules. Uranyl acetate–lead citrate staining. (B) Secretory granules contain numerous gold particles after anti-caveolin-1 staining. (C) After negative control staining, in which the primary antibody was substituted by PBS, the secretory granules did not show gold particles. (D) Oxyntic cells of the gastric glands contain numerous mitochondria (m), an extensive smooth endoplasmic reticulum (ser), whereas they lack secretory granules. (j) Junctional complexes. Uranyl acetate-lead citrate staining. Bars, 1 μm.

(Fig. 2B). Labelling was not observed in control sections when the primary antibody was substituted by PBS (Fig. 2C), or when antibodies were used that were pre-adsorbed with the respective antigens. Some gold particles were present in oesophageal goblet cells. Immunolabelling was not observed in mucous glandular cells (data not shown). Oxyntic cells of gastric glands were rich in mitochondria but lacked secretory granules (Fig. 2D) and did not show any immunostaining of caveolin-1 (data not shown).

Discussion

Caveolin-1 was originally described as an integral membrane protein at the cytoplasmic surface of caveolar membranes of fibroblasts, endothelial cells, adipocytes and muscle cells. It represents an important structural protein for directing the formation of caveolar membranes and plays a role in transcytosis, potocytosis, and signal transduction. Another crucial function of caveolin-1 is the supply of cholesterol to caveolae (Anderson, 1998).

In *R. a. aurora,* most pepsinogen is produced by oesophageal peptic glands. Nevertheless, a small amount of pepsinogen is produced by goblet cells, as was revealed by a moderate staining with Bowie's method, and the moderate immunostaining of pepsinogen in oesophageal goblet cells. These goblet cells were not positive for caveolin-1. Pepsinogen production by oesophageal epithelial cells has been found in the rock snake (Imai et al., 1991). Oesophageal pepsin has also been shown by biochemical methods in the toad *Bufo marinus* (Taylor and Tyler, 1986; Ruiz et al., 1993). We have co-localised caveolin-1 and pepsinogen immunohistochemically in serous cells of oesophageal glands of the red-legged frog, *R. a. aurora*, whereas pepsinogen or caveolin-1 was not observed in fundic glands.

Caveolin-1 is an unusual membrane protein. Recently, it has been found that it can enter the secretory pathway in mammalian cells, and has an autocrine/paracrine function. Tahir et al. (2001) reported aberrant secretion of caveolin-1 by human prostate cancer cells. This secretion promoted the progression of cancer in vivo. In addition, caveolin-1 was secreted into the medium and suppressed apoptosis in cultured human and mouse prostate cancer cells (Tahir et al., 2001; Wu et al., 2002). Nevertheless, caveolin-1 is not secreted exclusively by cancer cells. In mammals, caveolin-1 is also secreted by normal serous secretory cells of exocrine glands associated with the digestive tract, such as salivary glands, pancreatic acinar glands and chief cells of gastric glands (Liu et al., 1999; Li et al., 2001). In these cells, stimulation with secretagogues caused a significant increase in the amount of caveolin-1 which was co-secreted with enzymes that were specific for each cell type (Liu et al., 1999).

According to its intracellular localisation, caveolin-1 may either be a soluble protein, localised in secretory droplets, or a protein inserted in caveolar membranes (Liu et al., 1999; Li et al., 2001). It has been hypothesised that hydrophobic regions of caveolin-1 in its soluble form are embedded in lipid particles which are surrounded by a phospholipid shell, and constitute a lipid complex with properties of a high-density lipoprotein (HDL) particle (Li et al., 2001). This soluble form may be involved in intracellular and extracellular lipid transport, particularly in the delivery of cholesterol to caveolae, lipid droplets, mitochondria and the rough endoplasmic reticulum.

The function of caveolin-1 that is secreted by peptic cells in association with pepsinogen into the oesophageal lumen in *R. a. aurora* is not clear. The caveolin-rich lipid particles that are secreted are transported from the oesophageal lumen to other regions of the digestive tract, where they may be internalised. Scavenger receptors have been reported in brush border membranes of the intestinal epithelium (Acton et al., 1994; Landschulz et al., 1996; Hauser et al., 1998). In particular, class B type-I scavenger receptors mediate selective uptake of HDL cholesteryl esters (Hauser et al., 1998; Werder et al., 2001). Caveolin-1-rich lipid particles may represent specific ligands of class B type-I scavenger receptors and can act as donor particles to facilitate (protein-mediated) intestinal uptake of cholesterol and phospholipids.

In conclusion, our findings support the hypothesis that caveolin-1 has an autocrine/paracrine function and demonstrate that secretion of this protein also occurs in vertebrates other than mammals, such as amphibians. Further studies are needed to explain how caveolin-1 enters the secretory pathway and how the secreted protein functions. Frogs may be a useful alternative animal model to study these issues.

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