ORIGINAL

A histochemical and immunohistochemical investigation of guanase and nedasin in rat and human tissues

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Abstract : Human guanase is known as a specific enzyme in the liver, kidney, and brain. However, its functional significance remains poorly understood. In addition, interestingly, a different organ distribution between humans and rats was suggested. Here, we performed immunohistochemical staining with anti-human nedasin(neuronal and endocrine discs large/SAP102 associated protein), whose sequence was identical to that of guanase, antibody and histochemical staining for guanase in normal tissues of rat and human liver, kidney, and small intestine, and compared the results. Guanase activity was observed uniformly in the rat hepatocytes, biliary epithelium and vascular endothelium cells, while it was localized to the hepatocytes and biliary epithelium in the human liver. When the histochemical staining for guanase and the immunohistochemical staining for nedasin were compared, the stained regions were different in the rat liver but were almost consistent in all human tissues. Totally consistent staining results were also obtained between rats and humans in the other organization except the liver. Based upon the research reports to date, the experiments on guanase and nedasin in rat organs performed in this study are considered to have important implications in the investigation of their physiological significance. J. Med. Invest. 53 : 246-254, August, 2006

Keywords **:** *guanase, nedasin, rat, human*

INTRODUCTION

Guanase is an enzyme discovered in domestic rabbit liver homogenate by Schmidt in 1932(1), and is a deaminase involved in the conversion of guanine to xanthine. Xanthine is further degraded into uric acid by xanthine oxidase. In 1963, Passanenti(2) discovered that serum guanase activity was increased in patients with liver disease. Subsequently many investigators(3-7) studied the clinical significance of measuring serum guanase activity and indicated its usefulness in liver function tests.

Human guanase is abundant in the liver, brain, and kidney, but is scarce in the skeletal muscle, heart muscle, pancreas, and others, in which aspartate aminotransferase(AST) and alanine aminotransferase (ALT)are relatively abundant ; thus, an increase in serum guanase activity is considered specific to liver diseases. Ito *et al* .(8-11) reported the localized distribution of guanase to the cytoplasm of hepatocytes around the human hepatic portal vein, human renal proximal tubule, and mucosal epithelium of the human small intestine by histochemical and immunohistochemical staining, but its functional significance remains poorly understood.

Recent advances in molecular biology have also

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revealed the sequence of the guanase gene and are launched new studies. Yuan *et al*.(12) cloned the guanase gene and reported its DNA sequence. Kuwahara *et al*.(13) reported that an unknown protein that binds to NE-dlg (neuronal and endocrine discs large), one of the MAGUK (membrane -associated guanylate kinase) family proteins that are considered to play various important roles in the adhesion of epithelial cells and synaptic connection between neurons, was cloned and named nedasin (NE-dlg/SAP102 associated protein), whose sequence was identical to that of guanase. However, there is no report concerning the comparison between images of immunohistochemical staining using anti-nedasin antibody and images of histochemical staining for guanase. Thus, comparison between these staining images is considered useful to reveal the physiological significance of guanase and its functional analysis.

Kubo *et al*.(14) performed histochemical staining for guanase and immunohistochemical staining with anti-human nedasin antibody in normal tissues of the human liver, kidney, and small intestine, and reported differences in staining properties in the kidney. In addition, Zoref-Shani E *et al*. (15) examined the guanase activity of various rat tissue extracts and reported the activity in the heart and spleen ; interestingly, a different organ distribution between humans and rats was suggested. Palezki(16)reported high similarities of guanase sequences, as high as 91% between rat and human guanase, so that we thought it possible to perform immunohistochemical staining with anti-human nedasin antibody in rat tissues. Here, we performed histochemical staining for guanase and immunohistochemical staining for nedasin in normal tissues of rat and human liver, kidney, small intestine used as positive control and skeletal muscle used as negative control, and compared the results.

MATERIALS AND METHODS

Reagents

(1) The histochemical staining method

Guanine, N-bis (2-hydroxyethyl) glycine (bicine), xanthine oxidase (XOD) (grade III, 19.8 U/mL), and nitro blue tatrazolium (NBT) were purchased from Sigma Chemical Co. (USA). Other reagents of the highest available purity were obtained from standard sources.

0.1M Bicine buffer, NBT solution, substrate stock solution, substrate solution, and substrate mixture were prepared as described previously(8).

(2) The immunohistochemical staining method

Anti-nedasin polyclonal antibody (provided by Prof. Hideyuki Saya, Department of Tumor Genetics and Biology, Kumamoto University School of Medicine) was used as a primary antibody in immunohistochemical staining for nedasin ; The Dako CSA (catalyzed signal amplification) system (Dakocytomation) was used as a staining kit ; The Streptavidin/biotin blocking kit(Vector Laboratories) was used to block endogenous biotin. Other reagents of the highest available purity were obtained from standard sources.

Tissues

Rat Livers, kidneys, small intestines, and skeletal musculus collected from three 3-4-week-old male Wistar rats were used. Three healthy human tissues collected from surgically removed liver (metastatic liver cancer), kidney (renal cell carcinoma), and small intestine(the jejunum of a patient with resected gastric cancer) were used. Rat small intestine with guanase mRNA expression(17) and human small intestine whose positive reaction had already been confirmed(14) were used as positive controls ; human and rat skeletal muscle without activity(10) were used as negative controls. Our study plan was approved by the medical ethics committee of The University of Tokushima Graduate School .

Methods

(1) Histochemical staining method for guanase (activity staining)

Each tissue was snap frozen in -80°C isopentan immediately after collection, and 10 μm sections were prepared using a cryostat and put onto glass slides. These sections were fixed with 2.5% glutaraldehyde solution in cacodylate buffer (0.2 M, pH 7.4) for 1 hour at room temperature, and were washed with 0.1 M bicine buffer.

After washing, the sections were immersed in XOD solution (0.1 mL/10 mL buffer) for 15 min to degrade endogenous xanthine and hypoxanthine. Subsequently, after being washed with bicine buffer and immersed in substrate mixture for 1-2 hours, the sections were dehydrated with alcohol and immersed in xylene to be embedded. Each control section was stained simultaneously to evaluate its staining

properties. The staining properties compared with the coloring of the positive control were described as follows : strong coloring (++) ; weak coloring (+); no coloring (−). In addition, hematoxyline and eosin(H&E)staining was performed on each adjacent section.

(2) Immunohistochemical staining method for nedasin

Immunohistochemical staining was performed on the adjacent sections with anti-nedasin antibody as a primary antibody using a CSA staining kit.

After treatment with fixing solution (formalin concentrate solution : methanol=1 : 4) for 20 sec, the sections were immersed in methanol concentrate solution containing 0.3% hydrogen peroxide solution for 30 min to block endogenous peroxidase, and were each washed 3 times with Tris Buffered Saline containing Tween 20(TBST) for 5 min. To block endogenous biotin, streptavidin solution was dripped onto the sections for 15 min, and after washing with TBST for 5 min 3 times, biotin solution was dripped onto the sections for 15 min, followed by washing 3 times with TBST for 5 min. To block non-specific proteins, casein-containing non-specific reaction blocking reagent was dripped onto the sections for 5 min, and after 1 : 1,000 diluted anti-nedasin antibody was dripped for 15 min without washing, the sections were each washed 3 times with TBST for 5 min. For the reaction with a biotin-labeled secondary antibody, a biotin-labeled anti-rabbit immunoglobulin antibody was dripped onto the sections for 15 min, and the sections were each washed 3 times with TBST for 5 min. In addition, streptavidin-biotin complex was dripped onto the sections for 15 min, and the sections were each washed 3 times with TBST for 5 min. Then biotin-labeled tyramide was dripped onto the sections as an amplification reagent for 15 min, and after washing 3 times with TBST for 5 min, peroxidase-labeled streptavidin was dripped for 15 min, followed by washing 3 times with TBST for 5 min.

The coloring reaction was stopped at the point when strong coloring of the positive control sections was confirmed by observing the brownish coloring of 3,3'-diaminobenzidine tetrahydrochloride (DAB) with the naked eye and under a microscope while dripping DAB substrate solution onto the sections, and after the coloring was stopped by immersing the sections in distilled water, the sections were dehydrated with alcohol and immersed in xylene to be embedded. Each control section was stained simultaneously to evaluate its staining properties.

RESULTS

1. Histochemical staining for guanase and immunohistochemical staining for nedasin in each tissue. Histochemical staining for guanase and immunohistochemical staining for nedasin using anti-human nedasin antibody were conducted on fresh frozen sections of rat liver, kidney, and small intestine to compare the staining results, then these results were compared with the data of human tissues.

Figure 1 shows tissue images of rat liver. In the image of histochemical staining for guanase (Figure 1A), a moderate coloring (+) of guanase activity was almost uniformly observed in the hepatocytes and portal components such as the biliary epithelium, vascular endothelium cells. In the image of immunohistochemical staining for nedasin (Figure 1B), a brown discoloration due to DAB oxidation was observed in the hepatocytes but not in portal and central venous components. Different results were obtained by comparing histochemical staining for guanase and immunohistochemical staining for nedasin.

Figure 2 shows tissue images of human liver. Histochemical staining and immunohistochemical staining in the hepatocytes showed strong (++) and moderate (+) coloring, respectively, while histochemical staining and immunohitochemical staining in the biliary epithelium showed moderate (+) and strong (++) coloring, respectively.

Figure 3 shows tissue images of rat kidney. As for the guanase activity in rat kidney tissue, strong (++) and weak coloring were observed in the proximal and distal renal tubules, respectively (Figure 3A), and as for the immunohistochemical staining for nedasin, strong (++) and moderate (+) brown discoloration due to DAB oxidation were observed in the proximal and distal renal tubules, respectively (Figure 3B) ; thus, slightly different results were obtained between the histochemical staining for guanase and the immunohitochemical staining for nedasin.

In the human kidney shown in Figure 4, strong (++) coloring of guanase activity was observed in the proximal renal tubule (Figure 4A), and as for immunohistochemical for nedasin, strong (++) and moderate (+) brown discoloration due to DAB oxidation were observed in the proximal and distal

renal tubules, respectively.

Figure 5 shows tissue images of rat small intestine. In the histochemical staining for guanase, guanase activity was localized to the mucosal epithelium, but was not observed in other regions (Figure 5A). In the immunohistochemical for nedasin, a brown discoloration due to DAB oxidation was localized to the mucosal epithelium, but was not observed in other regions (Figure 5B); thus, consistent staining results were obtained between the histochemical staining for guanase and the immunohistochemical for nedasin. These are the same results as those for human small intestine tissue shown in Figure 6.

In the histochemical staining (activity staining) of the frozen sections of rat and human skeletal muscle that were used as negative controls, no guanase activity was observed. In addition, immunohistochemical staining for nedasin was conducted, and no brown discoloration due to DAB oxidation was observed.

Figures 5 and 6 show the guanase staining images of the positive control sections. Strong coloring was observed in the positive controls, rat (Figure 5A) and human (Figure 6A) mucosae of the small intestine, and staining properties at this level were described as (++).

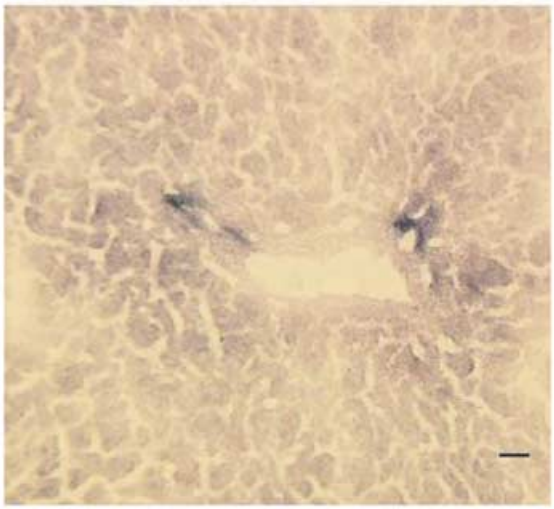
2. Comparison between activity staining for guanase and immunostaining for nedasin in each tissue

Similar results were obtained by staining 15 times under the same conditions. The results of the histochemical staining for guanase and the immunohistochemical staining for nedasin of rat and human liver, kidney, and small intestine are shown in the table.

In the livers, guanase activity was observed uniformly in the rat hepatocytes, biliary epithelium and vascular endothelium cells, while it was localized to the hepatocytes and biliary epithelium in the human tissues. When the histochemical staining for guanase and the immunohistochemical staining for nedasin were compared, the stained regions were different in rats but were almost consistent in humans.

Totally consistent staining results were obtained between rats and humans in the kidney, small intestine used as positive control and skeletal muscle used as negative control, whereas differences were observed in the liver.

DISCUSSION



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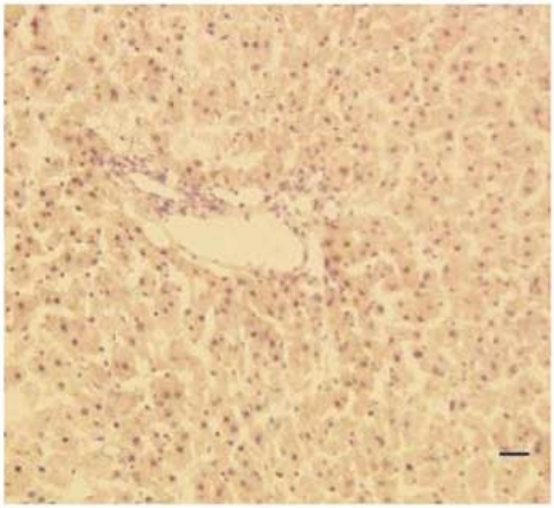

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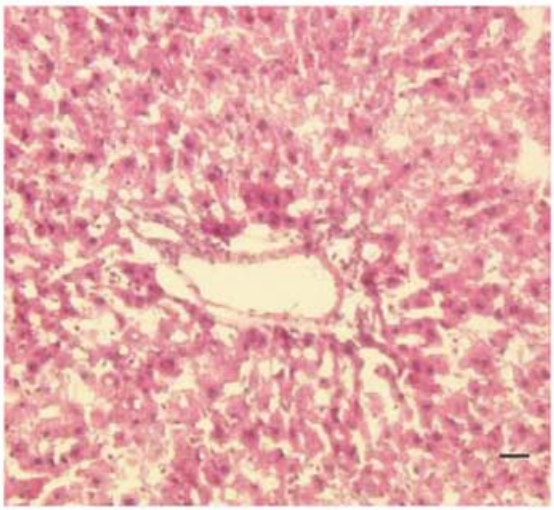

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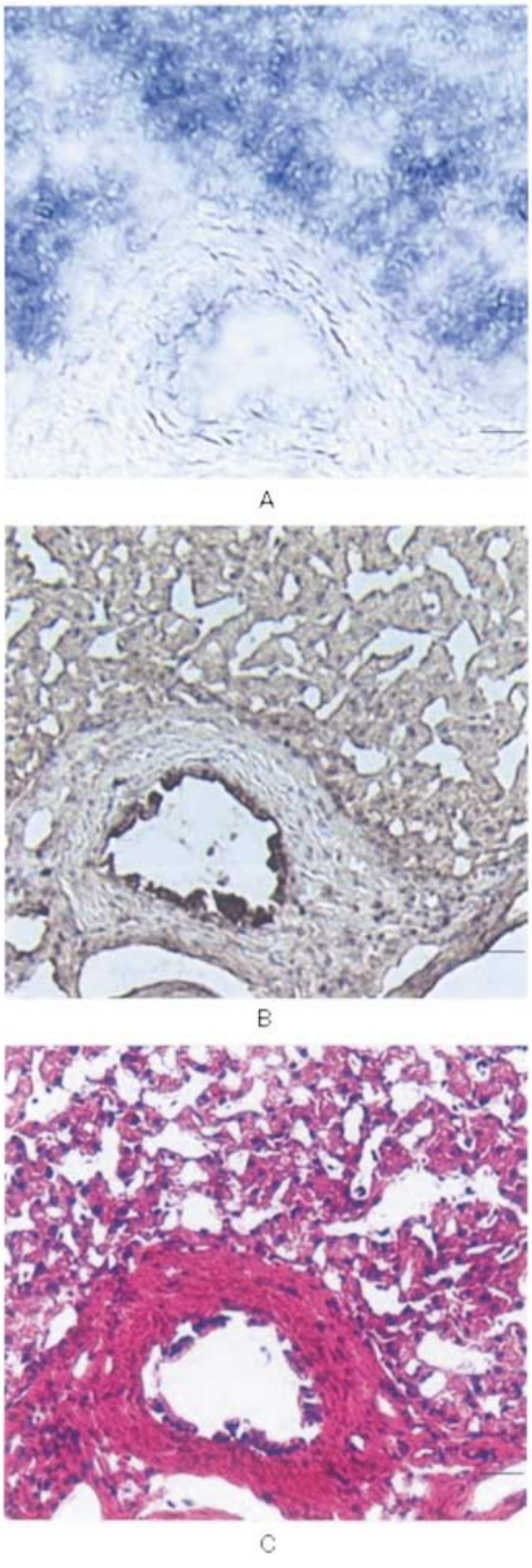
Figure 1(A) Images of histochemical staining for guanase on the frozen sections of the rat normal liver : Guanase activity was uniformly observed, in the portal components and the hepatocytes.
Figure 1(B) Images of immunohistochemical staining for nedasin on the same sections : a brown discoloration due to DAB oxidation was observed moderately in the hepatocytes, but not in the portal components.
Figure 1(C) Images of H&E staining on the same sections are shown.



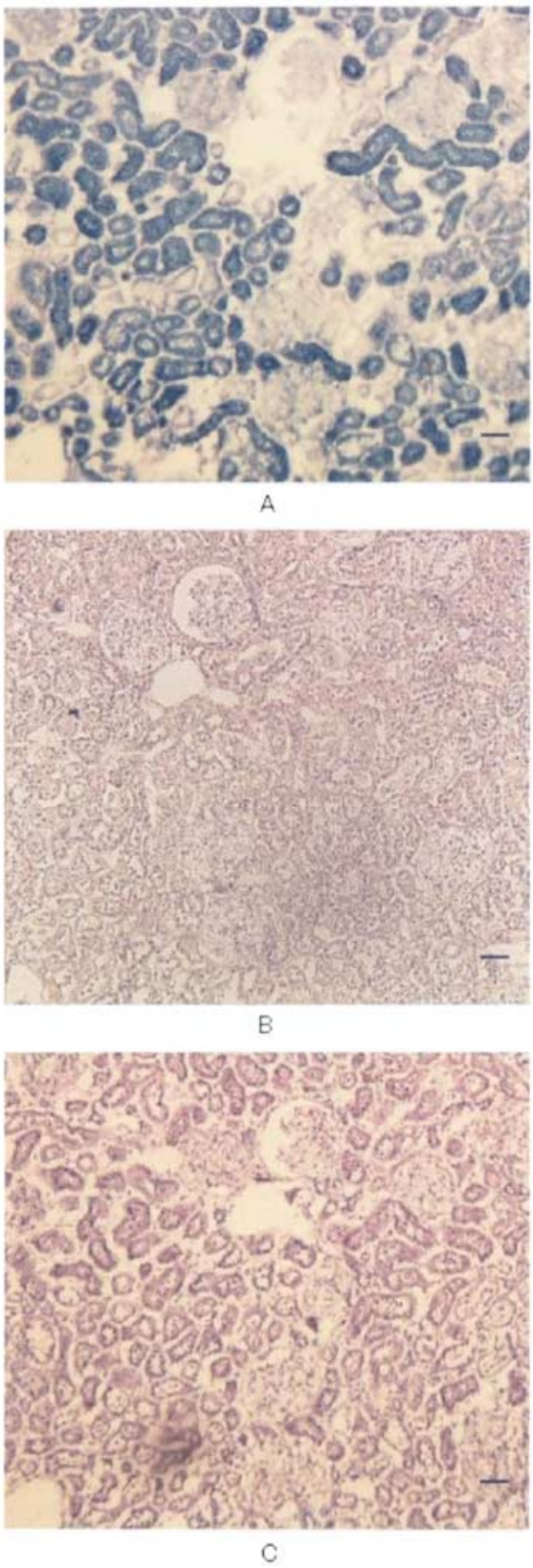
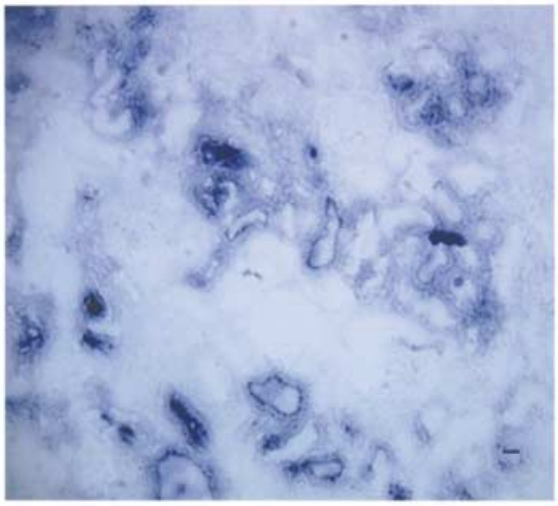


Figure 2(A) Images of histochemical staining for guanase on the frozen sections of the human normal liver : Guanase activity was localized to the hepatocytes and biliary epithelium.
Figure 2(B) Images of immunohistochemical staining for nedasin on the same sections:a brown discoloration due to DAB oxidation was localized to the hepatocytes and biliary epithelium.
Figure 2(C) Images of H&E staining on the same sections are shown.

Figure 3(A) Images of histochemical staining for guanase on the frozen sections of the normal rat kidney : Guanase activity was observed in the proximal renal tubule.
Figure 3(B) Images of immunohistochemical staining for nedasin on the same sections : a brown discoloration due to DAB oxidation was observed in the proximal and distal renal tubules.
Figure 3(C) Images of H&E staining on the same sections are shown.



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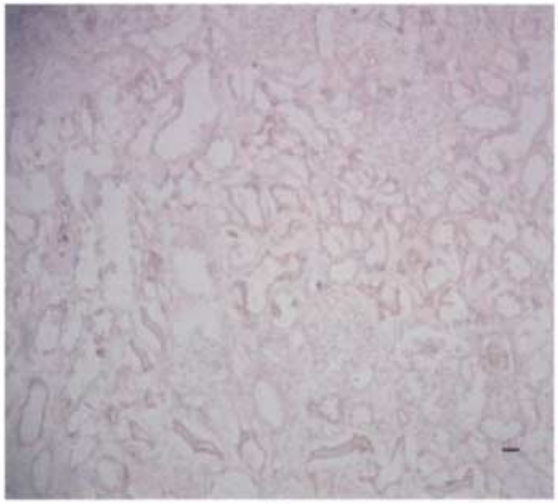

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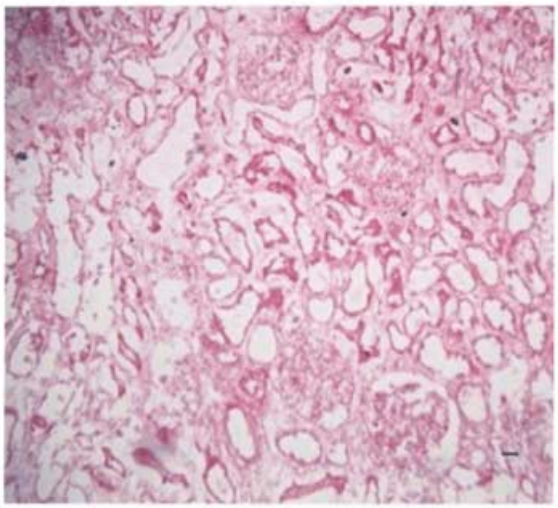

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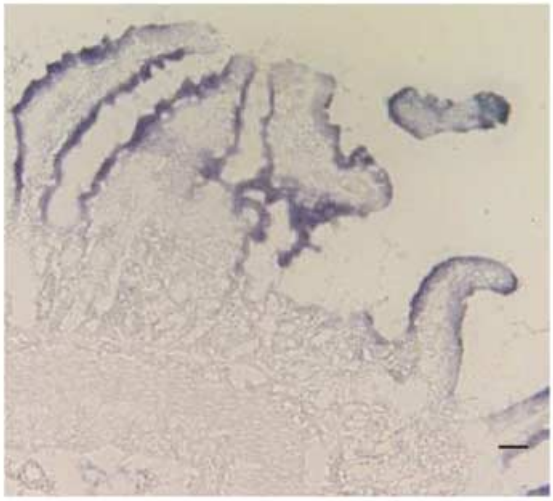
Figure 4(A) Images of histochemical staining for guanase on the frozen sections of the normal human kidney : Guanase activity was observed in the proximal renal tubule.
Figure 4(B) Images of immunohistochemical staining for nedasin on the same sections : a brown discoloration due to DAB oxidation was observed in the proximal and distal renal tubules.
Figure 4(C) Images of H&E staining on the same sections are shown.



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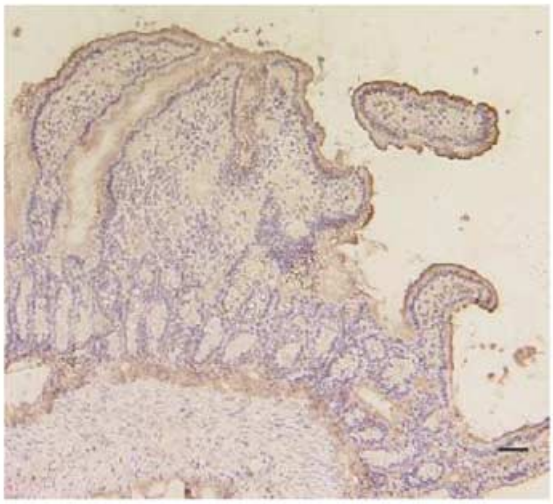

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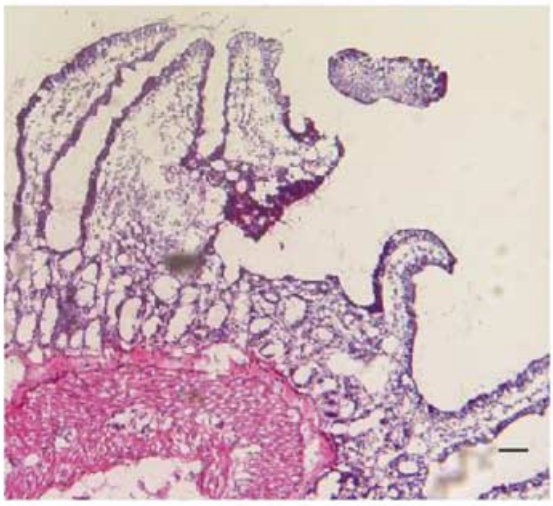
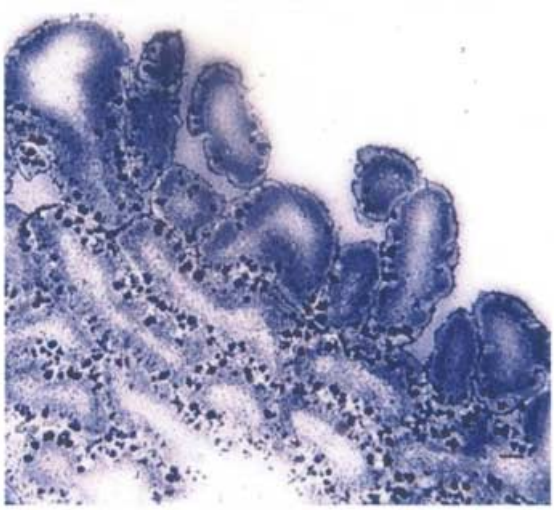


C

Figure 5(A) Images of histochemical staining for guanase on the frozen sections of the normal rat small intestine : Guanase activity was localized to the mucosal epithelium, but was not observed in other regions.
Figure 5(B) Images of immunohistochemical staining for nedasin on the same sections : a brown discoloration due to DAB oxidation was localized to the mucosal epithelium, but was not observed in other regions.
Figure 5(C) Images of H&E staining on the same sections are shown.



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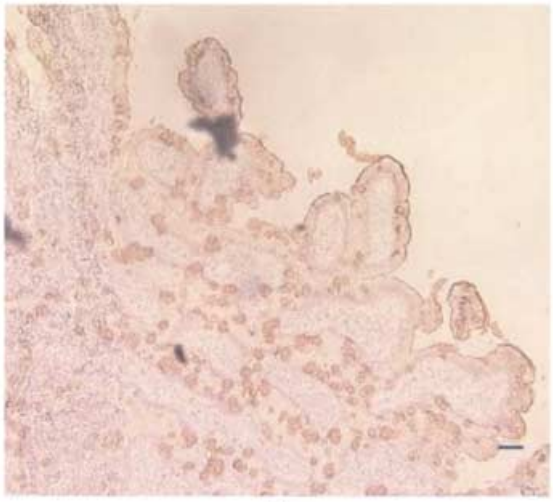

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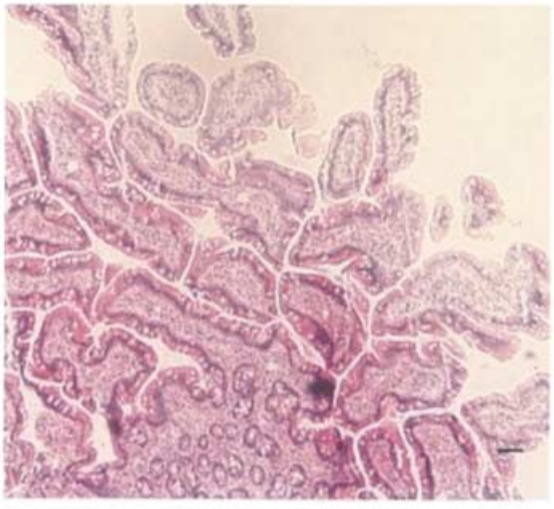

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Figure 6(A). Images of histochemical staining for guanase on the frozen sections of the normal human small intestine : Guanase activity was localized to the mucosal epithelium, but not was observed in other regions.
Figure 6(B). Images of immunohistochemical staining for nedasin on the same sections:a brown discoloration due to DAB oxidation was localized to the mucosal epithelium, but not in other regions.
Figure 6(C). Images of H&E staining on the same sections are shown. *Bar,100 μm

Table. Activity staining for guanase and immunostaining for nedasin in rat and human tissues

		Rat		Human	
		guanase	nedasin	guanase	nedasin
Liver	hepatocytes	+	+	++	+
	portal vein	+	−	−	−
	artery	+	−	−	−
	biliary epithelium	+	−	+	++
Kidney	proximal renal tubules	++	++	++	++
	distal renal tubules	−	+	−	+
	glomerulus	−	−	−	−
Small intense	mucosal epithelium	++	++	++	++
	tela submucosa	−	−	−	−
Muscle		−	−	−	−

Guanase is an enzyme that degrades guanine into xanthine and ammonia, and the resulting xanthine is degraded into uric acid by xanthine oxidase (XOD). As for histochemical studies on guanase, Ito *et al*.(8) reported an investigation of tissue distribution in human normal cases ; Norstrand *et al*. (18) in the human central nervous system ; and Palezki (16) in the mouse and rat central nervous. Kuwahara *et al*. cloned an unknown protein (nedasin), which binds to NE-dlg, one of the MAGUK family proteins that are considered to have various important functions in the adhesion of epithelial cells and synaptic connection between neurons, and reported its identical sequence homology to that of guanase. In addition, Firestein *et al*.(17) and Akum *et al*.(19) reported that the sequence of cypin (cytosolic PSD-95 interactor), a regulator of PSD-95 postsynaptic protein in rat central nervous system, was identical to guanase, and then they separately investigated the functions such as synapse formation and development, morphogenesis of dendrite, and formation of microtubule assembly by separating the linkage between PSD-95 and SAP-102.

When the human organs were stained, the stained regions in the kidney differed between the immunohistochemical staining with anti-human nedasin antibody and histochemical staining for guanase. Kuwahara *et al*.(13) reported that nedasin had 4 variants, including S, V1, V2, and V3 forms, which were organ-specific. The anti-human nedasin antibody that the authors used was against both S and V1 forms, suggesting the possibility that this might have resulted in the differences in the staining properties. Among the rat organs used in this study, different staining properties were also observed in the liver and kidney, and these are likely to have resulted from the different distribution of

the variant forms. Guanase is considered to be one of nedasin 4 variants, or have four variant forms in different activity.

The actual physiological significance of guanase is still poorly understood. Palezki(16) are considering that guanase is involved in the regulation of neurologic functions via the small GTP protein system in the brain ; this idea is consistent with that of Kuwahara *et al*.(13) that nedasin binds to NE-dlg protein to regulate its function. This verifies the hypothesis that the differences in the nerve distribution between humans and rats are reflected in the different localization of nedasin and guanase. In addition, Firestein *et al*.(17) have already reported that there are various types of small GTP proteins and their regulators, and that SSSV, an amino acid sequence specific to the 3' termini of S form nedasin and cypin, is involved in the binding affinities of neural proteins. Variant forms of nedasin and guanase other than the S form may regulate the functions of non-neural small GTP proteins and be involved in the absorption of glucose, amino acids, fat, electrolytes, and others in the kidney and small intestine. However, these are only hypotheses and further studies are needed for a more complete understanding. Based upon the research reports to date, the experiments on guanase and nedasin in rat organs performed in this study are considered to have important implications in the investigation of their physiological significance, including their relevance to neurologic functions and absorption.

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