



# Co-localization hypothesis: A mechanism for the intrapancreatic activation of digestive enzymes during the early phases of acute pancreatitis

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## Abstract

Acute pancreatitis is generally believed to be a disease in which the pancreas is injured by digestive enzymes that it normally produces. Most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens which are normally activated only upon entry into the duodenum but, during the early stages of acute pancreatitis, those zymogens become prematurely activated within the pancreas and, presumably, that activation occurs within pancreatic acinar cells. The mechanisms responsible for intracellular activation of digestive enzyme zymogens have not been elucidated with certainty but, according to one widely recognized theory (the "co-localization hypothesis"), digestive enzyme zymogens are activated by lysosomal hydrolases when the two types of enzymes become co-localized within the same intracellular compartment. This review focuses on the evidence supporting the validity of the co-localization hypothesis as an explanation for digestive enzyme activation during the early stages of pancreatitis. The findings, summarized in this review, support the conclusion that co-localization of lysosomal hydrolases with digestive enzyme zymogens plays a critical role in permitting the intracellular activation of digestive enzymes that leads to acinar cell injury and pancreatitis.

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**Key words:** Acute pancreatitis; Digestive enzymes; Pancreatic acinar cells

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## INTRODUCTION

Acute pancreatitis is an inflammatory disease of the pancreas which, in its severe forms, leads to varying degrees of pancreatic necrosis. Passage of a biliary tract stone into or through the terminal biliopancreatic ductal system is the most frequent inciting cause for clinical acute pancreatitis and studies performed using an opossum model of biliary acute pancreatitis have suggested that the offending stone triggers pancreatitis by obstructing drainage from the pancreatic duct<sup>[1]</sup>. Other studies performed using that same model have indicated that the earliest changes of pancreatitis occur within the acinar cells of the pancreas and that periductal as well as perilobular changes, including cell injury and inflammation, occur at later times<sup>[2]</sup>.

The morphological changes of acute pancreatitis suggest that an autodigestive process has occurred and, indeed, activated digestive enzymes can be detected within the pancreas during clinical acute pancreatitis. Activated digestive enzymes can also be detected within the pancreas during the very early stages of most experimental pancreatitis models. Since the pancreas normally synthesizes and secretes many potentially harmful digestive enzymes, it is generally believed that it is the premature, intrapancreatic activation of those pancreas-derived enzymes which, ultimately, leads to cell injury and acute pancreatitis. However, for the most part, the potentially harmful digestive enzymes that are synthesized and secreted by the pancreas are present, within the gland, as inactive pro-enzymes or zymogens and activation of those zymogens normally occurs only when they reach the duodenum. How, then, might these zymogens be prematurely activated within the pancreas during the early stages of acute pancreatitis and how might that activation lead to acinar cell injury/necrosis?

Not surprisingly, these questions have prompted the performance of many studies, using various experimental models of acute pancreatitis, over the past several decades and several hypotheses have been advanced which address these questions. One of those hypotheses is the so called "co-localization hypothesis" which suggests that, during the early stages of acute pancreatitis, pancreas derived

digestive zymogens become co-localized with lysosomal hydrolases in acinar cell cytoplasmic vacuoles and that, as a result of this co-localization, lysosomal hydrolases such as cathepsin B, activate trypsinogen. According to this hypothesis, trypsin then activates the other digestive enzyme zymogens and the activated digestive enzymes gain access to the cell interior leading to the cell injury/necrosis which typify acute pancreatitis<sup>[3]</sup>.

This review will summarize current evidence that supports the co-localization hypothesis. To place this hypothesis in its proper context, we will begin by briefly summarizing current concepts regarding the physiological synthesis, intracellular trafficking, and secretion of certain types of proteins by the exocrine pancreas. This will be a highly selective review which will primarily draw upon work performed by many collaborators in the authors' laboratory over the past 25 years and the contributions of those individuals are gratefully acknowledged.

## PANCREATIC ACINAR CELL BIOLOGY

### *Protein assembly*

On a "gram of protein-per-cell" basis, pancreatic acinar cells synthesize more protein than any other cell in the body. Digestive enzymes, which are secreted from the pancreas and designed to act within the gastrointestinal tract, account for most of the protein mass synthesized by acinar cells but acinar cells also synthesize many other types of proteins, including the hydrolytic enzymes that are designed to digest un-needed material within intracellular lysosomes. Both types of enzymes, secretory digestive enzymes and lysosomal hydrolases, are assembled on ribosomes attached to the rough endoplasmic reticulum (ER). As they are assembled, they elongate within the cisternae of the rough ER until a signal peptide is added and, at that point, they are cleaved freeing them to assume their tertiary structure within the ER. Many secretory digestive enzymes are synthesized as pro-enzymes or zymogens which are inactive until they are proteolytically processed following secretion. Many of the lysosomal hydrolases are also synthesized as inactive pro-enzymes but their post-translational processing and activation is accomplished within the cell.

### *Intracellular trafficking of secretory proteins<sup>[4]</sup>*

Following their assembly within the ER, the enzymes destined for secretion are carried within small transport vesicles to the Golgi stacks where they are sorted and some are post-translationally modified. Those that are destined to be secreted in a constitutive manner are then transported to the cell surface where they exit the cell. Most of the secreted digestive enzymes and their zymogens, however, are subject to regulated discharge - i.e. they are stored within the cell until their secretion is accelerated in response to extracellular stimuli. Following their assembly and transport to the Golgi, these proteins traverse the Golgi stacks and they are then packaged within condensing vacuoles which evolve into zymogen granules as they mature and migrate towards the luminal surface of the cell. There, by mechanisms which are regulated

by neurohormonal secretagogue stimuli, fusion/fission of the zymogen granule membrane with the luminal plasmalemma occurs and this leads to the formation of a fusion pore which permits the granule contents to be discharged into the extracellular (i.e. ductal) space. In an overall sense, this regulated pathway allows for the massive and rapid discharge of intracellularly stored proteins which, in the case of pancreatic acinar cells, consists primarily of digestive enzymes<sup>[5]</sup>.

### *Intracellular trafficking of lysosomal hydrolases<sup>[6]</sup>*

The lysosomal hydrolases are a group of more than 50 dissimilar acid hydrolases which function to degrade un-needed cellular material as well as material taken up by cells via either endocytosis or phagocytosis. As noted for other proteins, lysosomal hydrolases are assembled within the cisternae of the rough ER and, in common with other newly synthesized proteins, the nascent lysosomal hydrolases are transported to the Golgi complex which they traverse from its *cis* to its *trans* surface, passing through the *medial* and *trans*-Golgi subcompartments. In contrast to proteins destined for either secretion or transport to other intracellular sites, the lysosomal hydrolases are sorted from other newly synthesized proteins as they traverse the Golgi stacks by a complex mechanism in which N-acetylglucosamine phosphates are added to mannose residues and then the N-acetylglucosamine groups are removed, leaving the remaining mannose residues phosphorylated at the 6-position. Those mannose-6-phosphorylated lysosomal hydrolases are then bound to receptors in the *trans*-Golgi that specifically recognize mannose-6-phosphate residues. The mannose-6-phosphate receptors along with their associated lysosomal hydrolases are then shuttled to the pre-lysosomal compartment where, as a result of the acidic pH of this compartment, dissociation of the hydrolase/receptor complexes occurs, thereby liberating the hydrolases within the pre-lysosomal compartment and allowing the now unoccupied receptors to return to the Golgi where they are free to bind and transport additional mannose-6-phosphorylated lysosomal hydrolases. Of potential significance however, is the fact that, even under physiological conditions, sorting of lysosomal hydrolases from secretory proteins in the Golgi is incomplete and, as a result, a fraction of the newly synthesized lysosomal hydrolases enters the secretory pathway<sup>[7-9]</sup>. This fraction of the lysosomal hydrolases is subject to regulated secretion from the cell along with other secretory proteins<sup>[10]</sup>. Another fraction of lysosomal hydrolases may also be secreted from the cell by being enclosed within so-called secretory lysosomes<sup>[11]</sup>. Lysosomal hydrolases may also be constitutively secreted from the cell without ever being packaged within lysosomes.

Many of the lysosomal hydrolases are initially synthesized as inactive or only partially activated pro-enzymes. Their complete activation is achieved through post-translational processing of the pro-peptide as it undergoes intracellular transport to pre-lysosomes<sup>[12]</sup>. Together, pre-lysosomes, lysosomes, endosomes, phagosomes, and autophagosomes function as an

interconnected network of organelles which contain a wide variety of acid hydrolases capable of degrading nucleic acids, proteins, carbohydrates, and lipids.

### Activation of digestive enzyme zymogens

Some of the digestive enzymes (e.g. amylase, lipase, DNAase, RNAase) are synthesized and secreted from pancreatic acinar cells as active enzymes but others, including most of the potentially harmful digestive enzymes (e.g. trypsin, chymotrypsin, phospholipase, elastase, carboxypeptidase) are synthesized as inactive pro-enzymes or zymogens. Under physiological conditions, activation of these zymogens does not occur until they reach the duodenum where the brush border enzyme enterokinase (enteropeptidase) catalytically activates trypsinogen and trypsin then catalyzes the activation of the other zymogens. In most instances, activation involves cleavage of the zymogen and release of an “activating peptide” which, prior to its release, had maintained the zymogen in its inactive state. Thus, quantitation of free activating peptide levels may provide information regarding the extent of zymogen activation prior to that measurement<sup>[13]</sup>.

### Protective mechanisms

In a general sense, the acinar cells of the pancreas are protected from the injury which might be inflicted by premature, intracellular activation of trypsinogen and other digestive enzyme zymogens by virtue of 3 features. The first, as noted above, is the fact that most of the potentially harmful digestive enzymes are normally present within acinar cells as inactive zymogens. The second is the fact that potent inhibitors of trypsin are synthesized and co-transported through the cell along with trypsinogen and those inhibitors are available to dampen any trypsin activity that might arise as a result of intracellular trypsinogen activation. The third protective feature of pancreatic acinar cells is the fact that, throughout their intracellular trafficking within the cell, digestive enzymes and their zymogens are sequestered from the remainder of the cell by being enclosed within membrane bounded organelles. Very recently, two reports have appeared that suggest that acinar cells may have yet another protective feature. Pancreatic acinar cells express proteinase-activated receptor-2 (PAR2) which is a tethered ligand receptor that is activated by trypsin and, as shown by Namkung *et al*<sup>[14]</sup> and Sharma *et al*<sup>[15]</sup>, activation of pancreatic PAR2 triggers events that protect the pancreas from pancreatitis.

## THE CO-LOCALIZATION HYPOTHESIS

### History

The so-called “co-localization hypothesis” grew out of studies, performed more than two decades ago, that employed the diet-induced and the secretagogue-induced models of acute experimental pancreatitis<sup>[16-20]</sup>. Those studies had shown that digestive enzyme synthesis and intracellular transport continue during the early stages of pancreatitis, but secretion of newly synthesized digestive enzymes from acinar cells is blocked. Those studies had

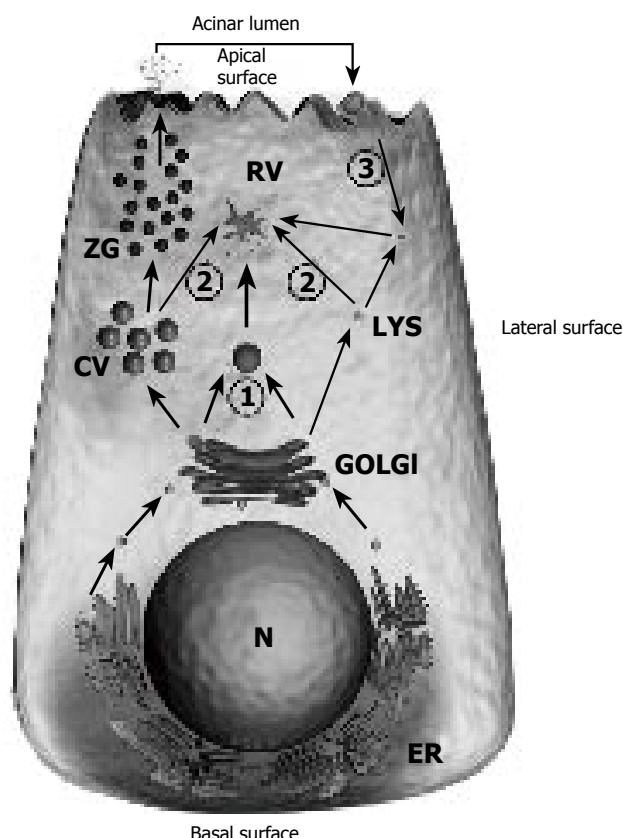
also shown that, prior to the appearance of evidence reflecting acinar cell injury, the normal segregation of digestive enzyme zymogens from lysosomal hydrolases is perturbed and, as a result, both types of enzymes become co-localized within intra-acinar cell cytoplasmic vacuoles. That co-localization phenomenon could be demonstrated using techniques of immunolocalization as well as using techniques of subcellular fractionation. Finally, those studies had shown that digestive enzyme zymogens, including trypsinogen, are activated within acinar cells at very early stages during the evolution of experimental pancreatitis-i.e. prior to the appearance of cell injury. Since the lysosomal hydrolase cathepsin B is known to be capable of activating the digestive zymogen trypsinogen<sup>[21,22]</sup> and, since trypsin can activate the other digestive zymogens, these various observations suggested the following 3-tiered hypothesis: (a) that perturbation of normal intracellular trafficking of digestive zymogens and lysosomal hydrolases, is a very early event during the evolution of pancreatitis and that, as a result of this perturbation, digestive enzyme zymogens become co-localized with lysosomal hydrolases within acinar cell cytoplasmic vacuoles; (b) that, as a result of this co-localization phenomenon, the lysosomal hydrolase cathepsin B (and, most likely, other lysosomal hydrolases) activates trypsinogen and trypsin activates the other zymogens; and (c) that the organelles containing intracellularly activated digestive zymogens become fragile and they release their content of activated enzymes within the cell interior where those enzymes trigger changes leading to cell injury/death<sup>[23]</sup>.

### Challenges

Subsequent to its initial proposal, the co-localization hypothesis was challenged on two grounds. The first was the fact that, while most lysosomal hydrolases have a pH optimum of roughly 5.0, the site of co-localization during the early stages of pancreatitis appears to have a neutral pH. Subsequent studies characterizing the pH optimum for cathepsin B, however, indicated that considerable (although perhaps not optimal) activity was also present at pH 7.0<sup>[24]</sup> and, thus, this concern regarding the co-localization hypothesis appears to be unwarranted. The second challenge to the hypothesis, however, proved to be more substantial because it focused on the issue of causality. Its advocates suggested that the co-localization phenomenon, although real, might be a response to the early injury of pancreatitis. They argued that the co-localization of digestive enzymes with lysosomal hydrolases might simply be a reaction to injury, rather than causing this injury<sup>[25]</sup>. Some even suggested that cell injury-induced co-localization might be a protective response which could allow lysosomal hydrolases to inactivate and degrade those prematurely activated digestive zymogens<sup>[26]</sup>.

## THE EVIDENCE THAT CO-LOCALIZATION LEADS TO ZYMOGEN ACTIVATION IN PANCREATITIS

The remainder of this review will be devoted to an



**Figure 1** Acinar cell demonstrating mechanisms of co-localization. Arrows represent physiological and pathological pathways. Co-localization can occur as a result of (1) missorting of lysosomal hydrolases from digestive zymogens in the Golgi, (2) fusion of zymogen granules with lysosomes and (3) reuptake into the lysosomal compartment of secreted digestive zymogens. Note the ruptured co-localization vacuole (RV) and release of activated digestive enzymes inside the cell. (ER = endoplasmic reticulum, N = nucleus, LYS = lysosomes, CV = condensing vacuoles and ZG = zymogen granules).

examination of the evidence which suggests that the co-localization phenomenon is not simply a reaction to injury or a protective response but that it is, in fact, an early and critical event which leads to zymogen activation and acinar cell injury in pancreatitis. Ideally, studies addressing these issues would be performed using clinically derived material and patients with acute pancreatitis but, unfortunately, those studies are not possible since patients with acute pancreatitis are only identified after the very early phases of the disease have been completed and access to the pancreas of patients with early acute pancreatitis is generally not possible. Thus, by necessity, studies testing the co-localization hypothesis have all been performed using various models of acute pancreatitis in experimental animals.

Validation of the co-localization hypothesis would require fulfillment of the following 6 postulates: (1) The co-localization phenomenon should be observed in all experimental models of acute pancreatitis. (2) Lysosomal hydrolases should be capable of activating digestive enzyme zymogens such as trypsinogen. (3) During the evolution of pancreatitis, the co-localization phenomenon should occur prior to zymogen activation and cell injury. (4) The co-localization phenomenon and digestive zymogen activation should occur within the same intracellular

compartment. (5) Digestive zymogen activation should be dependent upon the presence of lysosomal hydrolase activity. (6) Prevention of co-localization should interfere with zymogen activation but prevention of zymogen activation should not interfere with co-localization.

The evidence supporting fulfillment of these 6 postulates will now be summarized.

### **The co-localization phenomenon occurs in all models of experimental pancreatitis**

As noted above, early studies performed using the diet-induced and the secretagogue (caerulein)-induced models of experimental pancreatitis and employing the techniques of both subcellular fractionation and immunolocalization, indicate that the co-localization phenomenon occurs in both of those models<sup>[16-20,9]</sup>. Subsequent studies using the rabbit model of duct obstruction<sup>[27]</sup>, the closed duodenal loop model<sup>[28]</sup>, and the taurocholate model of retrograde duct injection<sup>[29]</sup>, indicate that the co-localization phenomenon occurs in each of those models as well. In each of these models, co-localization is an early event which can be demonstrated to occur prior to the appearance of cell injury/necrosis.

The mechanism(s) responsible for the co-localization phenomenon in each of these models appears to be different (Figure 1). In the diet-induced model, it reflects fusion of zymogen granules with lysosomes (i.e. crinophagy) while, in the secretagogue-model, it reflects both crinophagy and defective sorting of lysosomal hydrolases away from secretory proteins because of a defect in processing lysosomal hydrolases prior to their passage through the medial Golgi compartment. In the opossum model, co-localization occurs because previously secreted digestive zymogens are taken up by acinar cells via endocytosis and then transported to the endosome/lysosome compartment.

### **Lysosomal hydrolases such as cathepsin B can activate trypsinogen**

Studies independently performed and reported by at least 3 groups of investigators have shown that cathepsin B can activate trypsinogen under in-vitro conditions<sup>[21,22,24]</sup>. The most recent of those studies evaluated the pH profile of this activation process and found that a considerable degree of activation could occur at pH 7.0<sup>[24]</sup>.

### **The co-localization phenomenon occurs prior to acinar cell injury/necrosis**

If the co-localization phenomenon is a reaction to cell injury, it should occur after changes indicative of that injury are already demonstrable while, if it is the cause of cell injury, it should occur prior to the appearance of that injury. Time-dependence studies using the secretagogue-induced model have been performed to evaluate this issue<sup>[30]</sup>. In these studies, pancreatic tissue was homogenized and subcellular fractionated to obtain zymogen granule-enriched and lysosome-enriched fractions at selected times after the start of caerulein administration. Redistribution of cathepsin B from the lysosome-enriched to the zymogen granule-enriched fraction was noted within



15 minutes after the start of caerulein administration and, at roughly this same time, evidence of trypsinogen activation (i.e. increased trypsin activity and increased pancreatic levels of trypsinogen activation peptide) was also first detected. In contrast, hyperamylasemia, pancreatic edema and acinar cell injury/necrosis were only detected at later times. Thus, these studies indicate that the co-localization phenomenon occurs prior to, and not after, cell injury in pancreatitis.

#### **Co-localization and zymogen activation occur within the same intracellular compartment during pancreatitis**

Studies addressing this issue have been performed using antibodies directed against the lysosomal hydrolase cathepsin B and antibodies directed against the peptide released during the process of trypsinogen activation (i.e. trypsinogen activation peptide, TAP). During the early stages of secretagogue-induced pancreatitis, both types of antibodies were found localized in the same cytoplasmic vacuoles<sup>[31,9,20]</sup>. Similar observations have been made in studies using antibodies directed against lysosomal membrane proteins<sup>[32]</sup>. In both cases, the site of co-localization appeared to be in the area of the *trans*-Golgi network. Taken together, these immunolocalization studies support the conclusion that co-localization and activation occur within the same intracellular compartment.

#### **Zymogen activation and pancreatitis severity are dependent upon cathepsin B activity**

This issue has been addressed in two ways. In one, cathepsin B inhibition was achieved using the cell permeant and highly specific cathepsin B inhibitor CA-074me. The effect of this inhibitor in two dissimilar models of pancreatitis (secretagogue-induced and taurocholate-induced) was evaluated<sup>[33]</sup> and, in both models, inhibition of cathepsin B was found to prevent trypsinogen activation. That inhibition was also found to reduce the severity of pancreatitis. The second approach to this issue involved the use of genetically modified mice that did not express cathepsin B and, in those mice, both trypsinogen activation and the severity of pancreatitis were found to be reduced<sup>[34]</sup>. These observations support the conclusion that cathepsin B (either alone or, perhaps with other lysosomal hydrolases) plays a critical role in trypsinogen activation during pancreatitis and that, in pancreatitis, trypsinogen activation is closely related to the severity of the disease.

#### **Prevention of the co-localization phenomenon interferes with zymogen activation but inhibition of zymogen activation does not prevent co-localization**

The co-localization of digestive zymogens with lysosomal hydrolases in both the secretagogue-induced and the taurocholate-induced models of pancreatitis is dependent upon the activity of phosphoinositide-3-kinase (PI3K) and co-localization can be prevented in those models by administration of the PI3K inhibitors wortmannin and LY49002. In a recently reported study, administration of those PI3K inhibitors was found to reduce trypsinogen activation<sup>[35]</sup>. These findings indicate that prevention of co-localization, by administration of PI3K inhibitors, can

reduce zymogen activation in these models of pancreatitis.

As noted above, CA-074me is a potent in-vivo cathepsin B inhibitor and administration of CA-074me to animals can prevent trypsinogen activation during pancreatitis. To further evaluate the possible causative role of co-localization in zymogen activation, mice subjected to secretagogue-induced pancreatitis were given CA-074me and the effect of inhibiting trypsinogen activation (via inhibition of cathepsin B) on the co-localization phenomenon was evaluated (Van Acker *et al.*, submitted for publication). Under these conditions, co-localization of lysosomal hydrolases with digestive enzymes was still observed even though zymogen activation had been prevented. Taken together, these findings are consistent with the conclusion that zymogen activation is dependent upon the co-localization phenomenon and they are inconsistent with the theory that zymogen activation leads to the co-localization phenomenon.

### **SUMMARY**

Acute pancreatitis is generally believed to be a disease in which the pancreas is injured by digestive enzymes that it normally produces. Most of the potentially harmful digestive enzymes produced by pancreatic acinar cells are synthesized and secreted as inactive zymogens which are normally activated only upon entry into the duodenum but, during the early stages of acute pancreatitis, those zymogens become prematurely activated within the pancreas and, presumably, that activation occurs within pancreatic acinar cells. The mechanisms responsible for intracellular activation of digestive enzyme zymogens have not been elucidated with certainty but, according to one widely recognized theory (the "co-localization hypothesis"), digestive enzyme zymogens are activated by lysosomal hydrolases when the two types of enzymes become co-localized within the same intracellular compartment.

This review has been focused on the evidence supporting the validity of the co-localization hypothesis as an explanation for digestive enzyme activation during the early stages of pancreatitis. Indeed, there is considerable evidence that, under appropriate conditions lysosomal hydrolases such as cathepsin B are capable of activating digestive enzyme zymogens such as trypsinogen. Normally, that activation does not occur because, under physiological conditions, newly synthesized lysosomal hydrolases are segregated from digestive enzyme zymogens as the two types of enzymes traffic through acinar cells. However, during pancreatitis, that segregation is perturbed and lysosomal hydrolases become co-localized with digestive enzyme zymogens within cytoplasmic vacuoles. Evidence supporting the co-localization hypothesis as an explanation for intracellular zymogen activation include the following: (a) the co-localization phenomenon is observed in virtually all of the experimental models of pancreatitis that have been studied; (b) lysosomal enzymes such as cathepsin B can activate digestive zymogens such as trypsinogen and trypsin can activate the other zymogens; (c) the co-localization phenomenon occurs prior to the appearance of cell injury/necrosis during pancreatitis; (d) co-localization and zymogen activation occur within the

same intracellular compartment; (e) zymogen activation is dependent upon the presence of lysosomal enzyme (i.e. cathepsin B) activity; and finally, (f) preventing the co-localization phenomenon prevents zymogen activation during pancreatitis but preventing zymogen activation does not, necessarily, prevent the co-localization phenomenon. Taken together, these findings support the conclusion that co-localization of lysosomal hydrolases with digestive enzyme zymogens plays a critical role in permitting the intracellular activation of digestive enzymes that leads to acinar cell injury and pancreatitis.

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