

# Cryptides: Functional Cryptic Peptides Hidden in Protein Structures and Their Novel Signaling Mechanisms

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## Introduction

Neutrophils are a group of leukocytes involved in the innate defense system by the destruction and removal of infected organisms and toxic cell debris produced from inflammatory sites [1]. They quickly infiltrate into injury sites, produce superoxide and digestive enzymes, and phagocytose toxic debris and infected microorganisms. CXC chemokines such as interleukin 8 (IL-8) and complement factors such as C5a produced from inflammatory sites are known to induce migration and activation of neutrophils [2]. Transmigration of neutrophils induced by the stimulation of various inflammations such as reperfusion injury is often observed immediately after tissue injury. Therefore, unknown factors other than chemokines and complement factors to induce immediate transmigration and activation of neutrophils should be present because CXC chemokines including IL-8 are indicated to be translated and then constitutively secreted by the inflammatory stimulation, and complement factors such as C5a are produced by the infectious stimulation. We hypothesized that necrotic or apoptotic cells in tissue injury sites directly produce substances that cause transmigration and activation of neutrophils to respond immediately in acute inflammation and these factors may be present in healthy organs. In the previous studies, therefore, we tried to purify such neutrophil migrating- and activating-factors from porcine hearts and identified a novel class of neutrophil-activating peptides, COSP-1 and fCyt b [3,4]. Surprisingly, these peptides are fragments of mitochondrial proteins. We named such functional cryptic peptides *cryptides* and specifically designated cryptides derived from mitochondrial proteins *mitocryptides* because they are hidden in protein sequences and have different functions from their parent proteins [3-5]. Accordingly, COSP-1 and fCyt b was then renamed mitocryptide-1 and -2, respectively. We also pointed out the presence of many other mitocryptides [5].

**MCT- 1: Val-Thr-Phe-Leu-Leu-Pro-Ala-Gly-Trp-Ile-Leu-Ser  
(COSP-1) -His-Leu-Glu-Thr-Tyr-Arg-Arg-Pro-Glu**

**MCT- 2: formyl-Met-Thr-Pro-Met-Arg-Iys-Ile-Asn-Pro-Leu  
(fCyt b) -Met-Lys-Leu-Ile-Asn**

Fig. 1. Primary structures of human mitocryptides (MCTs) that activate neutrophils.

Because the experimental approaches used to identify these two peptides are laborious, it is necessary to develop more efficient methodology to identify many other functional peptides. Here, we communicate the identification of mitocryptides that activate neutrophils utilizing bioinformatic techniques. We also tried to identify their receptors and discuss novel signaling mechanisms in which many of mitocryptides are involved.

## Results and Discussion

Since mitocryptide (MCT)-1 had a common physicochemical features in the distribution of positively charged and hydrophobic amino acid residues with mastoparan that activates G<sub>i</sub>-type of G proteins directly [6], we predicted neutrophil-activating peptides derived from mitochondrial proteins that activate G proteins utilizing protein data bank SWISS-Prot based on those features. Namely, we extracted human mitochondrial proteins from protein database Swiss-Prot (ver. 50, May, 2006) to make a subset of human mitochondrial proteins at first. Fragment peptides presumably cleaved by various processing peptidases were then collected according to the information of the site of cleavages by those peptidases in each database entry. Sequences of fragment peptides were also created from the proteins' sequences assuming cleavages by caspase, trypsin, and chymotrypsin based on their known/presumed specificities. From the resultant peptide entries, a subset of the peptide database was constructed that contain fragments of 12-36 amino acid residues.

Neutrophil-activating peptides were searched for in the peptide database considering that the target peptides activate G<sub>i</sub>-type G proteins thus have common physicochemical properties with mastoparan as well as MCT-1. Peptides having more than two net positive charges were extracted first. Then, peptides that can form amphiphilic structure having hydrophobic core composed of aromatic or aliphatic residues were chosen. Finally, peptides were selected whose positively charged side chains are oriented on the same side when the peptide is in an  $\alpha$ -helical conformation. Those predicted functional peptides were then chemically synthesized, and their activities to induce  $\beta$ -hexosaminidase release from HL-60 cells differentiated into neutrophilic/granulocytic cells were evaluated.

More than forty peptides including MCT-3, -4, -5, and -6 whose primary structures were not related each other were predicted and confirmed to activate not only G protein directly but also differentiated HL-60 cells. The activities of some of those peptides were shown in Table 1. Among them, MCT-3 and -5 had the most potent abilities

to induce  $\beta$ -hexosaminidase release at less than 100 nM in the differentiated HL-60 cells.

Table 1. The activities of identified mitocryptides to induce  $\beta$ -hexosaminidase release in HL-60 cells differentiated into neutrophilic/granulocytic cells

Peptide	EC <sub>50</sub> ( $\mu$ M)	n
mitocryptide -1	3.5	6
-2	0.03	6
-3	0.23	8
-4	0.3	7
-5	0.38	6
-6	1.1	6

To purify receptor proteins for MCTs, biotinylated MCT-3 extended Cys residue at N- or C- terminus were chemically synthesized and proteins bound to these MCT-3 derived peptides were purified as shown in Fig. 2. Namely, MCT-3 derivatives were incubated with intact HL-60 cells differentiated neutrophilic/granulocytic cells and protein molecules that bound to biotinylated MCT-3 derivatives were purified using streptavidin beads subsequently after the solubilization of the cells with the detergent-containing buffer.

It was demonstrated that biotinylated MCT-3 derivatives bound to a protein whose molecular weight was about 83 kDa and competed with its unlabeled peptide for the binding to this protein. These results indicated that MCT-3 bound to the 83 kDa protein specifically. Moreover, MCT-3 derivatives competed with other MCTs such as MCT-5 and -6 for the binding to the 83 kDa protein. These findings suggest that MCTs bind to the identical 83 kDa protein specifically and propose that this 83 kDa protein is the receptor molecule for MCTs. Taken together with the facts that MCTs involving MCT-3, -5 and -6 have common features such as the distributions of charged and hydrophobic amino acid residues, whereas homologies in their primary structures were not apparent, it was proposed that the 83 kDa proteins are the common receptors for many MCTs.

Since the presence of common receptor molecules for MCTs were suggested, we investigated the effects of the mixture of MCT peptides at the concentrations that did not cause the stimulation by each peptide. The peptide mixture markedly promoted  $\beta$ -hexosaminidase release in the differentiated HL-60 cells (data not shown), indicating the presence of novel accumulative signaling mechanisms by those MCTs whose primary structures are not homologous. These findings suggest that many of MCTs may have important physiological functions that have not been identified so far.

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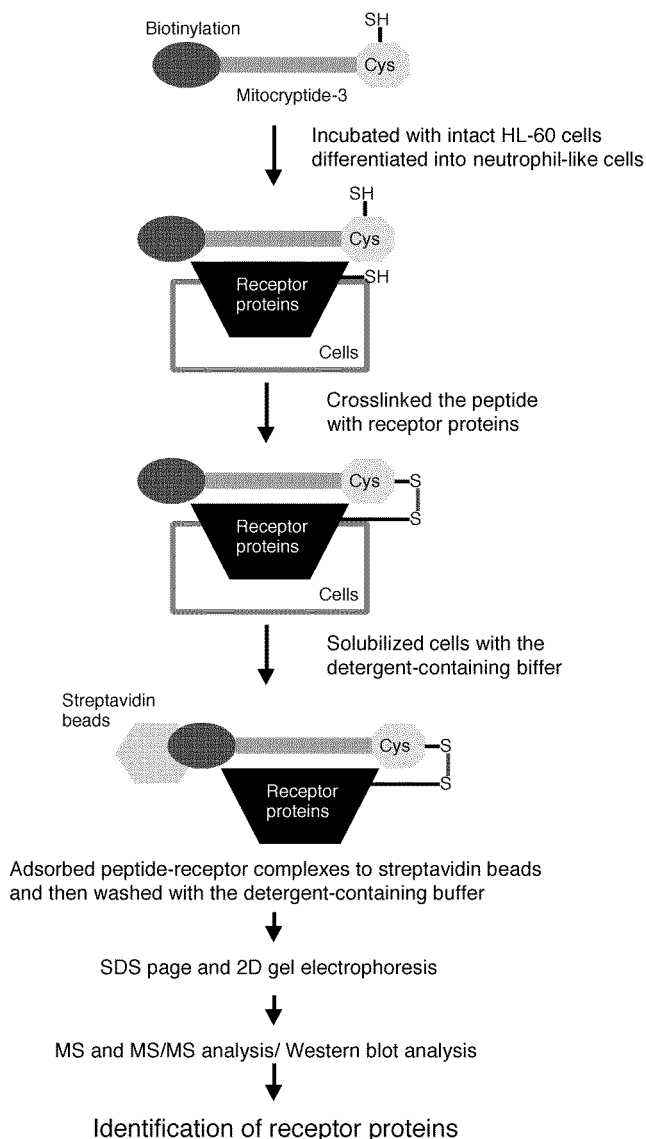


Fig. 2. Purification and identification procedures of receptor proteins for mitocryptides.

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