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Research paper

The endocytic pathways of a secretory granule membrane protein in HEK293 cells: PAM and EGF traverse a dynamic multivesicular body network together

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ABSTRACT

Peptidylglycine α-amidating monooxygenase (PAM) is highly expressed in neurons and endocrine cells, where it catalyzes one of the final steps in the biosynthesis of bioactive peptides. PAM is also expressed in unicellular organisms such as *Chlamydomonas reinhardtii*, which do not store peptides in secretory granules. As for other granule membrane proteins, PAM is retrieved from the cell surface and returned to the trans-Golgi network. This pathway involves regulated entry of PAM into multivesicular body intralumenal vesicles (ILVs). The aim of this study was defining the endocytic pathways utilized by PAM in cells that do not store secretory products in granules. Using stably transfected HEK293 cells, endocytic trafficking of PAM was compared to that of the mannose 6-phosphate (MPR) and EGF (EGFR) receptors, established markers for the endosome to trans-Golgi network and degradative pathways, respectively. As in neuroendocrine cells, PAM internalized by HEK293 cells accumulated in the trans-Golgi network. Based on surface biotinylation, >70% of the PAM on the cell surface was recovered intact after a 4 h chase and soluble, bifunctional PAM was produced. Endosomes containing PAM generally contained both EGFR and MPR and ultrastructural analysis confirmed that all three cargos accumulated in ILVs. PAM containing multivesicular bodies made frequent dynamic tubular contacts with younger and older multivesicular bodies. Frequent dynamic contacts were observed between lysosomes and PAM containing early endosomes and multivesicular bodies. The ancient ability of PAM to localize to ciliary membranes, which release bioactive ectosomes, may be related to its ability to accumulate in ILVs and exosomes.
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1. Introduction

Peptidylglycine α-amidating monooxygenase (PAM), a cuproenzyme, is best known for its essential role in the biosynthesis of the many α-amidated peptides stored in secretory granules and released by neurons and endocrine cells in response to specific signals. A bifunctional integral membrane enzyme, intact PAM protein is deposited on the cell surface during the process of granule exocytosis. Governed by trafficking determinants in its cytosolic domain, PAM is retrieved from the cell surface by clathrin-mediated endocytosis and can be returned to the trans-Golgi network or degraded. Endoproteolytic cleavages in the linker regions connecting the catalytic cores of PAM can occur in secretory granules, generating catalytically active soluble fragments that are released along with the product peptides (reviewed in Kumar et al., 2016a).

It is now clear that expression of PAM is not limited to neurons and endocrine cells. Expression of various preprohormones and monoclonal antibodies in cell lines that do not form secretory granules consistently demonstrates that the low levels of PAM activity detected in these cells are sufficient to amidate peptidylglycine substrates as they pass through the secretory pathway. In addition, it is now known that PAM is expressed in *Chlamydomonas reinhardtii*, a unicellular green alga. The localization of CrPAM (*Chlamydomonas*

Abbreviations: CSFM, complete serum-free medium; EGF, epidermal growth factor; HEK, human embryonic kidney; HSG, HEPES-buffered saline with glucose; ILV, intralumenal vesicle; MPR, mannose-6-phosphate receptor; PAM, peptidylglycine α-amidating monooxygenase; PAL, peptidyl-α-hydroxyglycine α-amidating lyase; PHM, peptidylglycine α-hydroxylating monooxygenase; CrPAM, *Chlamydomonas reinhardtii* PAM.

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reinhardtii PAM) to the Golgi complex and to the ciliary membrane led to the identification of PAM in both motile and primary cilia in mammals (Kumar et al., 2016b).

Protein trafficking through the endocytic pathway in HEK293 cells, which engage in constitutive secretion but do not produce secretory granules, has been studied in great detail. Stable expression of rat PAM-1 in HEK293 cells demonstrated production of active enzyme that cycled onto and off of the plasma membrane (Tausk et al., 1992; Milgram et al., 1993). These well characterized cells provide an ideal system for focusing on the endocytic trafficking of PAM.

In AtT-20 cells, PAM protein internalized from the plasma membrane rapidly appears in the intralumenal vesicles (ILVs) of multivesicular bodies (Bäck et al., 2010). Protein entry into ILVs generally leads to lysosomal degradation, as best shown for EGF and the EGF receptor (Gruenberg and Stenmark, 2004; Tomas et al., 2014). The mechanism for delivery of ILVs to lysosomes remains a question of debate (Bright et al., 2005; Gan et al., 2009; Pryor and Luzio, 2009). PAM routed to ILVs largely escapes lysosomal degradation (Bäck et al., 2010), as has been observed for tetraspanins (Gruenberg and Stenmark, 2004), MHC class II proteins (Kleijmeer et al., 2001) and the cation independent mannose-6-phosphate receptor (Kobayashi et al., 1998).

The aim of this study was to determine the endocytic pathway taken by PAM-1 internalized from the surface of stably transfected HEK293 cells and to compare PAM trafficking to the routes taken by EGF/EGF receptor complexes and by cation independent mannose-6-phosphate receptors, markers for degradative and endosome to trans-Golgi network trafficking, respectively.

2. Materials and methods

2.1. Cells

HEK293 cells stably transfected with vector encoding rat PAM-1 (Tausk et al., 1992; Milgram et al., 1993) were maintained in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum and 0.5 mg/ml G418 and passed weekly. Complete serum-free medium (CSFM) is the same medium without serum but with insulin-transferrin-selenium (Life Sciences).

2.2. Surface biotinylation

HEK293 cells stably expressing rat PAM-1 (Tausk et al., 1992; Milgram et al., 1993) were incubated for 30 min at 37 °C in CSFM. Before labeling with cell impermeant sulfo-NHS-LC-biotin, cells were rinsed with 15 mM HEPES, 120 mM NaCl, 2 mM $CaCl_2$, 4 mM KCl, 25 mM glucose, pH 7.5 (HSG). For assessing steady state plasma membrane localization, surface biotinylation was carried out on ice and all solutions used were pre-chilled. For assessment of endocytic trafficking, surface biotinylation was carried out for 10 min at 37 °C. Sulfo-NHS-LC-biotin (1.25 mM dissolved in HSG) (Pierce) was applied on ice or at 37 °C. The reaction was quenched by replacing the biotin-containing HSG with 2 mg/ml BSA in CSFM (quenching medium); after 5 min, the quenching medium was replaced with CSFM. Cells were either extracted immediately (Pulse) or incubated in CSFM containing 1 mg/ml BSA for up to 4 h (Chase). Cells were extracted into 20 mM Na TES, 10 mM mannitol, 1% TX-100, pH 7.4 (TMT) supplemented with protease inhibitors (30 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 16 μg/ml benzamidine) and particulate material was removed by centrifugation at 14,000 rpm for 20 min. After centrifugation of chase media, protease inhibitors were added. Clarified lysates and media were incubated with neutravidin beads (40 μl slurry) (Pierce) for 1 h at 4 °C. Beads were rinsed twice with TMT containing protease inhibitors and once with buffer lacking detergent before elution into Laemmli sample buffer by heating for 5 min at 95 °C. Eluates and the corresponding inputs were analyzed on 4–15% gradient gels and non-saturated images were quantified using SynGene software.

2.3. Antibodies

The following antibodies were used: rabbit polyclonal JH629 [rPAM-1(394–498), exon A (Yun et al., 1995)], mouse monoclonal anti-syntaxin 6 (ab 610635, BD Transduction Laboratories), mouse monoclonal anti-mannose-6-phosphate receptor (cation independent) (2G11, Abcam), mouse monoclonal anti-EGFR antibody (ab30, Abcam). Alexa Fluor 555 or 488 labeled JH629 antibody was prepared using affinity purified antibody and APEX antibody labeling kits (Molecular Probes) according to the protocol provided by the manufacturer. For uptake studies EGF, biotinylated, complexed to Alexa Fluor 555 or 488 streptavidin (Molecular Probes; E35350, E13345) or dextran, Alexa Fluor 488 or 568, 10,000 MW, final concentration 50 μg/ml (Molecular Probes; D22910, D22912) were used.

2.4. Immunofluorescence and live cell imaging

For uptake studies, cells were kept in DMEM-HEPES containing 1% BSA, incubated with antibody to the Exon 16 region of PAM, JH629 1:2000, antibody to MPR (used at 2 μg/ml) or EGF Alexa Fluor 488 or 555 8 μg/ml at 37 °C for 5 min, then chased in culture medium. The cells were fixed with 4% paraformaldehyde, initially on ice, and permeabilized with 0.2% Triton-X100. Cells were either first incubated with primary antibody (syntaxin 6 antibody) for 1 h, then Alexa Fluor conjugated secondary antibody for 1 h, or only Alexa Fluor conjugated secondary antibodies for 1 h. The cells were photographed with a Leica TCS SP8 confocal microscope with a 65× oil immersion lens.

Confocal live cell imaging was performed on a Leica TCS SP8 attached to a DMI 6000 inverted microscope equipped with a full enclosure temperature controlled environmental chamber was used. The cells were grown in glass-bottom dishes (LabTek, Nunc) incubated in DMEM-HEPES + 1% BSA with directly Alexa 488 or 555 labeled JH629 PAM antibody and Alexa 488 or 555 conjugated EGF 8 μg/ml for 10 min, then chased in DMEM-HEPES + 1% BSA. Images were recorded with a 63× water immersion lens and data acquired with LAS AF 3 (Leica). Time-lapse images were obtained every 735 ms for 66 s (90 frames). Images were deconvoluted with Huygens Professional (SVI) and renderings made with Imaris 7.2 (Bitplane). For analysis of Pearson's and Mander's coefficients, the JACoP plugin of ImageJ (National Institutes of Health) was used. Colocalization analysis on individual endosomes was performed manually on the deconvoluted images. For analysis of contacts, randomly chosen PAM containing endosomes were traced manually frame by frame; the number of contacts during the period the endosome could be traced and the duration of contacts were quantified by recording the number of frames in which each contact was visible.

2.5. Electron microscopy

For colloidal gold labeling at the ultrastructural level, cells were incubated for 20 min at 4 °C with PAM antibody JH 629 (1:500) or MPR antibody (16 μg/ml) or EGFR antibody (8 μg/ml) and Alexa Fluor 555 EGF (0.2 μg/ml) in DMEM-HEPES-0.2% BSA. They were rinsed in DMEM-HEPES-BSA at 4 °C and then incubated for 20 min at 4 °C with 10 nm colloidal gold conjugated goat anti-mouse IgG

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