Protein palmitoylation in membrane trafficking

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Introduction
Protein palmitoylation was first described more than 20 years ago for the myelin proteolipid protein, but the functional significance of this post-translational modification remains obscure. Progress has been hampered by the lack of specific inhibitors for acylation and because of the difficulty of developing an assay to isolate the palmitoylating enzyme(s). Palmitoylation generally occurs through labile thioester bonds and it is the only lipid modification that is readily reversible. Palmitoylation may determine the extent to which a protein is associated with membranes, but evidence from a number of groups is beginning to suggest that the role of covalent fatty acids is more complex than simply serving as a hydrophobic membrane anchor and suggest instead that it may function to assemble protein–protein complexes at or within the membrane. These interactions could then be modulated by cycles of acylation–deacylation. Although not as common as other reversible modifications of proteins, such as phosphorylation, reversible fatty acylation might be expected to have equally important regulatory functions.

Glick and Rothman [1] first suggested a role for fatty acylation in membrane trafficking by demonstrating that vesicular transport between the cis and medial stacks of the Golgi apparatus is stimulated by palmitoyl-CoA in a cell-free transport assay. Further studies showed that fatty-acyl-CoA is required both for the budding [2,3] and fusion [4] of transport vesicles. The budding of Golgi-coated vesicles was recently reconstituted by using partly purified membranes and these results suggest that palmitoyl-CoA is required for fission of the vesicle from the membrane [3]. To identify an acylated protein that might act as a palmitate donor or acceptor during vesicular transport, we examined protein acylation under conditions where transport is inhibited. It is well recognized that vesicular transport ceases as cells undergo mitosis [5]. We showed that a protein with an apparent molecular mass of 62 kDa was hyperacylated only in mitotic cells, where vesicular transport is inhibited [6]. Further evidence linking p62 acylation and transport was obtained by using other compounds that inhibit vesicular transport. The most effective at stimulating acylation were the fungal metabolite brefeldin A (BFA), and respiratory inhibitors, such as carbonyl cyanide m-chlorophenylhydrasone or sodium azide, which deplete the intracellular levels of ATP. These results suggest that fatty acylation is an important and necessary step for membrane transport and that p62 is a likely candidate for this acylation.

Purification and cloning of p62
Morphological analysis of the endoplasmic reticulum (ER)/Golgi region of the cell has identified a poorly defined subcellular region known as the intermediate compartment (IC) [7,8]. Newly synthesized proteins leave the ER and migrate to the Golgi apparatus through this tubulovesicular compartment. Whether the IC is a specialized domain of the ER or exists as a distinct compartment is still unclear. In our initial studies we showed that p62 co-fractionated on sucrose gradients with a marker for the IC, p58 [9]. By isolating IC membranes it was possible to obtain highly enriched starting material for the purification of p62. By using a method described by Schweizer et al. [10], crude post-nuclear supernatants (PNS) from CHO cells were fractionated on a Percoll gradient. The fractions containing p62 (detected by radiolabel) were then pooled and re-centrifuged on a metrizamide gradient. p62 was efficiently separated from most proteins in the preparation (Figure 1 a–d) and these fractions were also enriched at least 30-fold in the intermediate compartment marker protein p58 (results not shown). Palmitoylated p62 appeared to be associated with a single band on the gel. p62 was further purified by taking advantage of its insolubility in detergents. As shown in Figure 1(a,b) p62 is partly insoluble in Triton X-100 and completely insoluble in the zwitterionic detergent 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS). The bulk of the proteins found in the IC membrane fraction were solubilized by these detergents, providing a significant purification step. The CHAPS-insoluble pellet was then

Abbreviations used: ARF, ADP ribosylation factor; BFA, brefeldin A; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ER, endoplasmic reticulum; IC, intermediate compartment; PNS, post-nuclear supernatants; PVDF, polyvinylidene difluoride.
Mechanisms, Biochemical Regulation and Functional Consequence of Protein Acylation

Figure 1

p62 is located in the intermediate compartment and is insoluble in detergents

p62 was purified on a metrizamide density gradient as described by Hauri and Schweizer [8]. Acylated p62 is recovered in three fractions (9–11) in this gradient (fluorogram (c)). Under these conditions p62 is efficiently separated from the bulk of the other proteins (Coomassie-blue-stained gel, (d)). The arrow shows that the acylated band corresponds to the higher of the two light bands that are found in this region of the gel. IC membranes were then solubilized in the zwitterionic detergent CHAPS or in the non-ionic detergent Triton X-100 and pelleted at 100,000 g for 1 h. The major proteins found in this membrane fraction were solubilized by these detergents but p62 remained insoluble: (a) fluorogram showing the palmitate-labelled p62 band; (b) the Coomassie-blue-stained gel. The black dot in (b) indicates the Coomassie-blue band that is palmitoylated. Proteins were separated by 5–20% polyacrylamide gel. The positions of molecular mass markers are indicated, in kilodaltons.

(a) kDa

(b)

(c) kDa

(d)

Proteins were separated by SDS/PAGE on a very long (32 cm) 8% gel and transferred to polyvinylidene difluoride (PVDF) membrane. This long-format gel was used to obtain maximal separation between the p62 band and any other contaminants. p62 appears as a significant Coomassie-labelled band in this gel (Figure 2). The acylated p62 band from three identical lanes was cut out and sequenced on an Applied Biosystems 477A protein sequencer. The amino terminus of p62 was not blocked and 20 amino acids were obtained:

PSAKQRGSKGGHSA

(underline)

Using this new sequence information we isolated a cDNA clone encoding p62.

To confirm that the cDNA clone we obtained was correct, we subcloned the luminal domain into the Ndel/XhoI site of the bacterial expression vector pET-15b (Novagen). This expression vector has been modified to include a polyhistidine tag that can be used to affinity-purify the expressed protein by metal chelation chromatography. Efficient expression of the recombinant protein was obtained when the cells were induced at 25°C, and a band with the predicted molecular mass appeared in the crude extract of the bacterial cells. The polyhistidine-tagged protein was purified in a single step by binding to an affinity column charged with Ni²⁺ and eluted with imidazole. This material was further purified by SDS PAGE and used as an antigen to immunize rabbits. The polyclonal antiserum obtained recognized a protein with an apparent molecular mass of 62 kDa in CHO, NRK, HeLa, PC12 and M17 cells by immunoblotting. This

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The supernatant and pellet fractions of CHAPS-solubilized IC membranes were separated by SDS PAGE on a very long (32 cm) 8% gel followed by transfer to a PVDF membrane. p62 appears as a significant Coomassie labelled band. Only the region between the 66 kDa marker and the 43 kDa marker is shown. (a) Coomassie blue stain; (b) fluorogram.

Figure 2
The p62 band isolated for peptide sequencing

An acylated 62 kDa protein is immunoprecipitated with polyclonal antiserum against a recombinant protein produced from the cDNA encoding p62

CHO cells were radiolabelled with [3H]palmitate in the presence or absence of BFA and extracted in immunoprecipitation buffer. The extracts were then incubated with pre-immune serum (pre) or with serum (Ab) containing antibodies against the recombinant p62 protein and immune complexes collected on staph A cells. The immunoprecipitated 62 kDa protein was hyperacylated in the presence of BFA.

Secondly, the dissociation of coatomer can be prevented by treatment with either GTP-γ-S or aluminium fluoride [15,20,21]. I have developed an assay that measures the acylation in vitro of p62 in perforated cells (D. I. Mundy, unpublished work). Acylation was time dependent, did not occur at 4°C and required ATP and cytosol. In addition, both GTP-γ-S and aluminium fluoride blocked the acylation of p62 in this assay, suggesting that when coatomer is locked on to membranes p62 is not acylated. I have also demonstrated that the acylation of p62 is blocked by mastoparan, a basic peptide that mimics part of the cytoplasmic domain of receptors that couple to heterotrimeric G proteins. Kristakis et al. [22] showed that mastoparan also stimulates the binding of coatomer to membranes. This is particularly interesting because several studies have suggested that heterotrimeric G-proteins participate in or regulate coatomer binding [23, and references therein].

Thirdly, although BFA blocks the cycling of coatomer onto membranes, it also causes the dissociation of several other peripheral membrane proteins, including γ-adaptin and an unidentified p200 protein [24,25]. PtK1 cells are of interest because they are resistant to the effects of BFA on coatomer, but not to its effects on these other peripheral membrane proteins [26]. There is no acyla-
tion of p62 in PtK1 cells treated with BFA [27], which is further evidence that the acylation of p62 is related to the disruption of coatamer binding and not to any other effects that BFA may have.

p62, coatamer and membrane traffic

Although coatamer was first identified in intra-Golgi transport, evidence obtained from yeast genetics and the use of transport assays in vitro and in vivo suggests that coatamer is also involved in ER–Golgi transport [28–31]. This is supported by immunoelectron microscopy, which has demonstrated that coatamer is found predominantly in the IC and on the cis face of the Golgi rather than in the stacks [32–34]. The precise role of coatamer in ER–Golgi transport has not been established [35], making it difficult to predict how p62 and coatamer function are related. The simplest interpretation, that it is involved in vesicle budding as suggested by the Golgi studies, is complicated by the identification of a second coat complex (COP II), that is required for budding from the ER in yeast, which does not include coatamer [36]. It may be that there are two classes of transport vesicles that bud from the ER or that there are two separate budding steps, one to the IC and one from the IC to the Golgi complex. However, recent results strongly suggest that coatamer is involved in retrograde transport from the Golgi to the ER [37]. The fact that disruption of coatamer function also blocks anterograde transport may suggest that some crucial component must constantly be recycled or, alternatively, that a functional acceptor compartment is necessary for forward transport, and emphasizes the dynamic relationship between these two organelles.

If the original findings are correct, and budding of coated vesicles (retrograde or anterograde) requires fatty acylation, then one possibility is that p62 is an acylated protein required for budding. The accumulation of palmitate on p62 when transport is inhibited may therefore reflect the trapping of a fission protein in its acylated state. Using the antibodies we developed against p62, we have examined the subcellular localization of p62 by immunofluorescence microscopy. We found that p62 defines a very extensive compartment, much more extensive than is seen with other markers of the IC. It remains to be established whether this reflects the true extent of the IC or a subdomain of the ER. However, it raises the possibility that p62 is a fission protein required for anterograde transport from the ER. It may or may not also function as the retrograde fission protein. Another interesting possibility, suggested by both the predicted structure of p62 and its extensive distribution, is that it may function to recognize cargo, which in turn triggers the assembly of coat structures and budding. p62 is predicted to be a type II membrane protein with a large luminal domain and approximately 100 amino acids on the cytoplasmic side of the membrane. This structure is reminiscent of a transmembrane signalling molecule where the binding of cargo to the luminal domain would trigger a change on the cytoplasmic face. Particularly intriguing is the finding that activators of heterotrimeric G-proteins affect the acylation of p62 in perforated cells (D. I. Mundy, unpublished work). The classical function of heterotrimeric G-proteins is to transduce extracellular (luminal) signals to intracellular effectors by coupling with transmembrane receptors. Recent studies have implicated this class of GTP-binding proteins in membrane trafficking or sorting, and at the trans-Golgi network the evidence suggests that they control vesicle budding [23,38]. As already discussed, heterotrimerics have been implicated in the binding of coatamer to membranes. Interestingly, trimeric G-proteins were recently shown to be palmitoylated [39,40], and Degtyarev et al. [41] have demonstrated that the acylation of the α subunit is stimulated when G, is activated by its receptor. There have also been reports that the activity of heterotrimeric G-proteins can be regulated by the acylation of an interacting protein [42,43]. p62 may activate a heterotrimeric G-protein either directly, by serving as an acyl donor, or indirectly, because reversible palmitoylation of p62 itself controls its interaction with the heterotrimeric G-protein. Although these possibilities are interesting they are highly speculative. They do, however, provide a framework to begin to unravel the function of p62 and to determine whether its function is regulated by palmitoylation.
