

The p47 co-factor regulates the ATPase activity of the membrane fusion protein, p97

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Abstract The highly conserved ATPase p97, a member of the AAA-ATPases, is found in a complex with its co-factor p47 in rat liver cytosol. Previously it had been shown that p97-mediated reassembly of Golgi cisternae from mitotic Golgi fragments requires p47 which mediates the binding of p97 to a Golgi t-SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment factor receptor), syntaxin 5. Here we show that it also suppresses the ATPase activity of p97 by up to 85% in a dose-dependent and saturable manner suggesting that it has other roles in the membrane fusion cycle.

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Key words: p97; p47; AAA-ATPase; Membrane fusion; SNARE; *N*-Ethylmaleimide-sensitive factor; Soluble *N*-ethylmaleimide-sensitive factor attachment protein

1. Introduction

Cytoplasmic membrane fusion events require members of the AAA family of ATPases (ATPases associated with different cellular activities). The best characterised is NSF (*N*-ethylmaleimide-sensitive factor, and the yeast homologue, Sec18p), first identified as a necessary component of intra-Golgi [1] and then shown to be involved in other vesicle-mediated transport steps [2,3]. Another is p97 (and the yeast homologue, Cdc48p), which was originally shown to be involved in the fusion of ER membranes as part of the process of karyogamy [4] and then shown to be involved in the reassembly of Golgi cisternae from fragments generated either by treatment with mitotic cytosol [5] or by specific drugs such as ilimaquinone [6]. Others include Pas1 and Pas8, involved in peroxisome biogenesis, and Vps4p, involved in yeast endosomal membrane traffic (for review, see [7]).

The role played by these ATPases is best understood in the case of NSF. The fusion of vesicles with target membranes is mediated by SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) – v-SNAREs on the vesicle interact specifically with t-SNAREs on the target membrane. At the level of the presynaptic membrane, the t-SNARE, syntaxin [8], interacts with the v-SNARE, VAMP/synaptobrevin [9,10] in the docked synaptic vesicles. The formation of SNARE pairs is thought to bring the two membranes together and catalyse the fusion reaction [11]. The SNARE pairs, now in the same membrane, are then thought to be broken by the action of NSF, hydrolysing ATP and preparing each SNARE for another round of membrane fusion [12,13]. The mechanism of action of p97/Cdc48p is

thought to be similar except that the substrate is not a v-t SNARE pair but a t-t SNARE pair [14]. This is most clear in the case of yeast ER-ER membrane fusion which only requires the t-SNARE, Ufe1p, and none of the other ER to Golgi v- or t-SNAREs [15]. Mammalian Golgi-Golgi fusion requires the t-SNARE, syntaxin 5, but not the Golgi v-SNARE, GOS-28 [16].

Neither ATPase binds directly to SNAREs. In the case of NSF, binding is mediated by SNAPs (soluble NSF attachment proteins). α -SNAP is essential whereas γ -SNAP augments the binding [17]. On the other hand, binding of p97 is mediated by p47 [16]. p47 is a trimeric complex that binds to one end of the p97 hexameric barrel [18]. For Golgi reassembly from mitotic Golgi fragments, both ATPases are needed and both compete for syntaxin 5 [16]. This is because p47 competes with α -SNAP and vice versa.

Since α -SNAP and p47 both mediate the binding of their respective ATPases to SNAREs we asked whether they shared other properties. α -SNAP alone will activate the ATPase activity of NSF [19] presumably as part of its function in breaking up SNARE pairs. Here we show that p47 regulates the ATPase activity of p97, but the effect is an inhibition, not an activation.

2. Materials and methods

2.1. Purification of p97 and p47

Rat liver p97 and recombinant His₆-tagged p47 were purified as described previously [18].

2.2. ATPase assay

The assay relied on the release of P_i from [α -³²P]ATP and subsequent separation and measurement of [α -³²P]ADP and [α -³²P]ATP by thin layer chromatography [20]. The assay mixture (40 μ l) comprised 50 mM Tris-HCl (pH 9) containing 150 mM KCl, 2.5 mM MgCl₂, 15% glycerol and 2 mM ATP unless otherwise stated. The assay was performed for 30 min at 37°C. At four time points during the reaction, 1 μ l of the mixture was applied to polyethyleneimine cellulose thin layer plates (Polygram CEL 300PEI). Chromatography was carried out in 0.7 M LiCl, 1 M acetic acid over a distance of 5 cm for about 15 min. ATP and ADP spots were quantitated using a phosphorimager (Molecular Dynamics). The assays were performed with either 0.5 μ g of purified p97 alone or p97 preincubated with varying amounts of p47 for 30 min on ice.

3. Results

p97 was purified to homogeneity from rat liver cytosol. High salt treatment reduced the amount of bound p47 to a non-detectable level (Fig. 1, lane 1). Recombinant His₆-tagged p47 was expressed and purified from *Escherichia coli* lysates (Fig. 1, lane 2) [18].

Pure p97 had an ATPase activity of 18.0 (mean \pm 1.2 S.E.M., n = 3) μ mol ATP/mg p97/h at 37°C at pH 9.0. This

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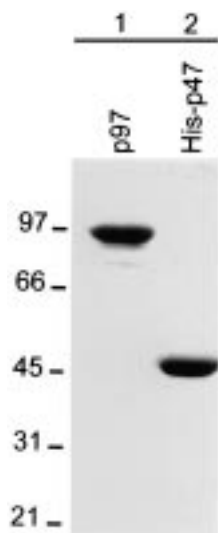


Fig. 1. Purified p97 and p47. p97 and His₆-p47 were purified as described. Samples (p97: 3 μ g; His-p47: 4 μ g) were fractionated by SDS-PAGE and stained with Coomassie blue. Note the absence of p47 in the p97 fraction. Molecular markers are indicated on the left side.

is similar to the previously published value for rat p97 (17 μ mol/mg/h [21]), but lower than that of p97 from *Xenopus laevis* (43.2 μ mol/mg/h [23]) or of yeast cdc48 (93.6 μ mol/mg/h [19]) measured under their respective optimal conditions. Addition of p47 at a molar ratio of 2:1 (p97:p47) to form the stable p97/p47 complex reduced the ATPase activity by 77% to 4.1 (mean \pm 0.1 S.E.M., $n=3$) μ mol/mg p97/h. p47 alone had no detectable ATPase activity.

Fig. 2 shows that the inhibition by p47 was both dose-dependent and saturable. At the molar ratio of p47 to p97 in the stable cytosolic complex of 0.5, the inhibition was 75% of that of p97 alone. A further 10% increase in inhibition was observed when the molar ratio of p47 to p97 was raised to 1.0, equivalent to two trimers of p47/hexamer of p97.

To investigate the mechanism of p47 inhibition, the ATPase activities of both the p97 and the p97/p47 complex were measured at different concentrations of MgATP varying between

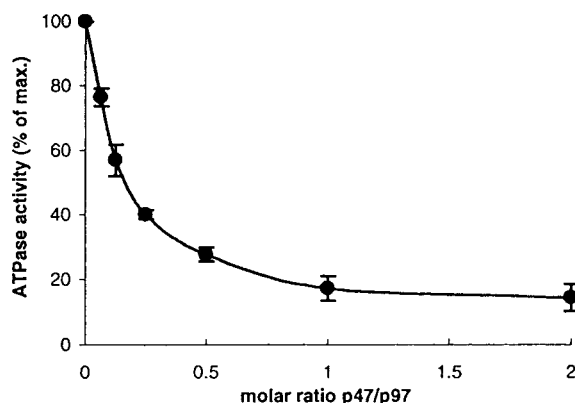


Fig. 2. The inhibition of p97 ATPase by p47 is dose-dependent. Standard ATPase assays were carried out over 30 min using 0.5 μ g p97 after 30 min preincubation with varying amounts of p47 at the molar ratios indicated. Data shown represent the mean \pm S.E.M. ($n=3$). The ATPase activity is shown as a percentage of the activity of p97 without p47 preincubation in each experiment.

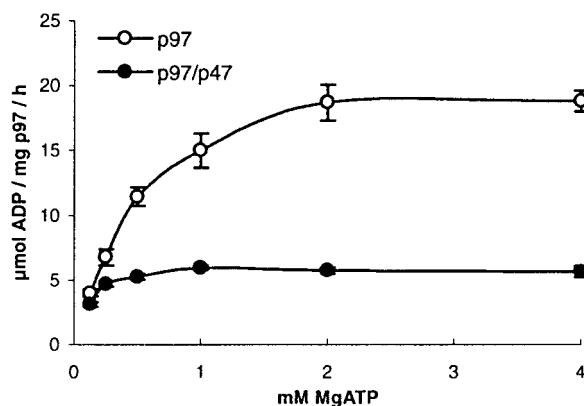


Fig. 3. The inhibition by p47 is non-competitive. ATPase assays were performed under standard conditions: 0.5 μ g p97 preincubated without (\circ) or with (\bullet) 0.25 μ g p47, but in varying MgATP concentrations as indicated. The data represent the mean \pm S.E.M. ($n=3$).

0.125 and 4 mM. The effect of p47 could not be competed by increasing concentrations of MgATP (Fig. 3) clearly showing non-competitive inhibition. Whereas the V_{\max} of p97 alone was estimated at 24 μ mol/mg/h with a K_M of 0.62 mM MgATP, the activity of the p97/p47 complex did not exceed 5 μ mol/mg/h.

The previous experiments were carried out at pH 9.0, the pH at which the enzyme shows maximum ATPase activity. To show that the inhibitory effect of p47 on p97 was also significant at physiological pH, the ATPase activity of p97 and the p97/p47 complex was measured at pH 7.4, pH 8.3 and pH 9.0 (Fig. 4). Consistent with previous work [24], p97 showed a maximum at pH 9.0. The inhibitory effect was significant under all three conditions tested with the highest reduction of about 85% at pH 7.4 compared with 78% when measured at pH 9.0.

4. Discussion

In addition to linking p97 to syntaxin 5 during the process of Golgi reassembly, we now show that p47 markedly inhibits the ATPase activity of this fusion protein. Approximately 75% of the ATPase activity of the p97 was suppressed by the addition of p47 to a molar ratio equivalent to that found in the

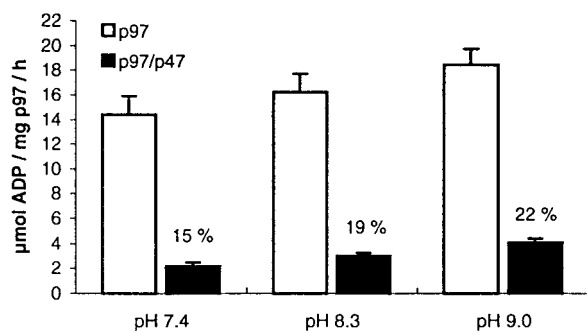


Fig. 4. The inhibition by p47 is significant at physiological pH. ATPase assays were performed under standard conditions: 0.5 μ g p97 preincubated without (\square) or with (\blacksquare) 0.25 μ g p47, but at varying pH as indicated. The data represent the mean \pm S.E.M. ($n=3$). The percentage values indicate the activity of p97/p47 compared to p97 alone at the same pH.

cytosolic p97/p47 complex. Twice this level of p47 increased the inhibition to a maximum of about 85%. It is not clear whether the excess is needed to ensure that all the p97 is in the complexed state or whether more than one p47 trimer can bind to each p97 hexamer.

The role played by this regulation in the functioning of the p97 ATPase is far from clear. p47 carries out an analogous function to α -SNAP which mediates the binding of NSF to SNAREs. α -SNAP also regulates the ATPase activity of NSF but the effect is an activation, not an inhibition [19]. However, the activation is modest (about 2-fold) and it only occurs if the α -SNAP is first immobilised on a plastic surface. It has no effect in free solution. NSF has two ATP binding sites and it has been suggested that α -SNAP activates the site that is thought to be involved in the fusion function of the protein (the other is thought to bind and not hydrolyse ATP and to stabilise the oligomeric structure of NSF [25]). p97 also has two predicted ATP binding sites but further work will be needed to determine which can hydrolyse ATP and which are affected by p47.

The differential effect of α -SNAP and p47 on their respective ATPases could indicate a different mechanism of action. After binding to SNAREs, the appropriate ATPase activity could be stimulated either by the continued presence of α -SNAP or by the loss of p47. This can be examined in more detail once the SNARE cycle has been reconstituted for the p97 ATPase.

An alternative possibility is that the p47-mediated inhibition of the ATPase activity has no direct role in the fusion cycle itself. p97 is an abundant protein, it is at least 5–10-fold more abundant than NSF judged from purification tables [1] and our own unpublished data. Furthermore, p97 is a more active ATPase than NSF. Estimates vary but, in general, it would appear that the ATPase activity of p97 would be about 5-fold higher than that of NSF. Taken together this would mean that the ATPase activity of p97 in the cell is about 50-fold higher than that of NSF. Perhaps the function of p47 is to quench this activity until such time as it is needed for membrane fusion reactions. Consistent with this idea is the fact that p97 is tightly complexed with p47 in the cytosol [18] whereas NSF is free and only complexes with α -SNAP that has bound to SNAREs on the membrane [17].

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