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Acidic clusters target transmembrane proteins to the contractile vacuole in *Dictyostelium* cells

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Summary

The mechanisms responsible for the targeting of transmembrane integral proteins to the contractile vacuole (CV) network in *Dictyostelium discoideum* are unknown. Here we show that the transfer of the cytoplasmic domain of a CV-resident protein (Rh50) to a reporter transmembrane protein (CsA) is sufficient to address the chimera (CsA-Rh50) to the CV. We identified two clusters of acidic residues responsible for this targeting, and these motifs interacted with the γ -adaptin AP-1 subunit in a yeast protein-protein interaction assay. For the first time we report the existence of an indirect transport pathway

from the plasma membrane to the CV via endosomes. Upon internalization, the small fraction of CsA-Rh50 present at the cell surface was first concentrated in endosomes distinct from early and late p80-positive endosomes and then slowly transported to the CV. Together our results suggest the existence of an AP-1-dependent selective transport to the contractile vacuole in *Dictyostelium*.

Key words: Contractile vacuole, Endosomes, Sorting signals, Clathrin-adaptor, *Dictyostelium discoideum*

Introduction

To survive their natural environment, amoebae such as *Dictyostelium discoideum*, have developed a specialized organelle, the contractile vacuole (CV), which enables them to adapt to osmotic shocks. Morphological and ultrastructural studies have revealed that the CV is composed of a network of ducts connected to giant vacuoles, the bladders or cisternae, which can fuse with the plasma membrane and expel water out of the cell (Gingell et al., 1982; Heuser et al., 1993; Nolte and Steck, 1994; Gerisch et al., 2002). The structure, function and biochemical composition of the CV are reminiscent of the tubulo-vesicular structure seen in acid-secreting gastric parietal cells (reviewed by Yao and Forte, 2003).

Not much is known about the biogenesis of the CV in *Dictyostelium*. CV-resident proteins enter the secretory pathway and are transported through the Golgi apparatus before arriving at the CV. Indeed O-glycosylated proteins, a post-translational modification acquired in the Golgi apparatus, have been identified in CV proteins (Gabriel et al., 1999). Further insights into the traffic of proteins to the CV came from the study of cells deficient for coat proteins involved in vesicular transport. One of the best-characterized vesicular coat proteins is clathrin, which, in association with adaptor-protein (AP) complexes, participates in multiple transport steps (Hirst and Robinson, 1998; Smith and Pearse, 1999). In mammalian cells four different AP complexes have been identified (AP-1 to AP-4), which share a similar composition (Boehm et al., 2001). In addition to clathrin recruitment, AP complexes are also implicated in the sorting of cargo proteins transported by clathrin-coated vesicles. The AP-1 complex contains β 1- and γ -adaptin large chains (80 and 130 kDa

respectively), a μ 1A or μ 1B medium chain (50 kDa) and a σ 1A or σ 1B small chain (20 kDa) (Scales et al., 2000). It ensures transport between the trans-Golgi network (TGN) and late endosomes/lysosomes, but it has also been implicated in several other steps of intracellular transport in the endocytic pathway, in particular in the retrograde transport from endosomes to the TGN of mannose 6-phosphate receptors in mammalian cells, chitin synthase III and syntaxin Tlg1p in yeast cells (Zizioli et al., 1999; Meyer et al., 2000; Valdivia et al., 2002), the transport of the transferrin receptor from apical to basolateral membranes in epithelial cells (Futter et al., 1998), the recycling of the low-density lipoprotein receptor and the transferrin receptor to the basolateral membrane (Gan et al., 2002), and the transport from the TGN to the basolateral membrane of many membrane proteins (Folsch et al., 1999; Folsch et al., 2001).

Clathrin-deficient *Dictyostelium* cells show dramatic morphological and functional defects of the CV (O'Halloran and Anderson, 1992; Wang et al., 2003). Deletion of the *apm1* gene encoding the medium subunit of the clathrin adaptor-protein AP-1 also results in the absence of a characteristic CV (Lefkir et al., 2003). Together these results suggest that clathrin-coated vesicles, and more precisely AP-1 clathrin-coated vesicles, are involved in the biogenesis of the CV. In *Dictyostelium* cells, endosomes and the CV share common proteins such as the proton ATPase (Fok et al., 1993; Heuser et al., 1993; Adessi et al., 1995). However endocytosed fluid phase does not get access to the CV (Gabriel et al., 1999), and no exchange of material between these two compartments has yet been documented. Moreover, despite the close contact between the CV bladder and the plasma membrane during

water expulsion, no direct protein exchange occurs between the CV and the plasma membrane either (Gabriel et al., 1999).

Only a few *Dictyostelium* proteins specifically localized in the CV have been identified, including drainin (Becker et al., 1999), calmodulin (Zhu and Clarke, 1992), rab 11 (Harris et al., 2001) and Rh50 (Benghezal et al., 2001). Rh50 is the *Dictyostelium* homolog of the mammalian rhesus protein. It is an integral membrane protein only found in the CV. There must exist a precise sorting mechanism to ensure the sorting and transport of such proteins to the CV, but this question has not been addressed so far. The targeting to the CV has only been studied for drainin, a cytosolic protein associated with the CV membrane (Becker et al., 1999). The specific localization of this cytosolic protein to the vacuolar membrane is dependent on the presence of a stretch of eight hydrophobic amino acids in its C-terminal half. No transport or CV retention signals have been identified so far for integral membrane proteins.

In this study, we characterized for the first time a signal responsible for the transport of Rh50 to the CV. Making use of chimeric proteins, we showed that acidic-based signals present in the last C-terminal domain of Rh50 are necessary and sufficient for efficient transport to the CV. A fraction of the chimeric proteins can be transported to the cell surface and it is then redirected to the CV through endosomal compartments. We thus provide for the first time evidence for a connection between the plasma membrane, endosomal compartments and the CV network. In addition, we demonstrated a direct interaction between CV-specific acidic signals and the γ subunit of the AP-1 clathrin-adaptor complex. Our results suggest that AP-1 is directly implicated in the transport of Rh50 chimera from intracellular compartments to the CV.

Results

The C-terminal domain of Rh50 causes localization in the CV

The Rh50 protein is an integral membrane protein analogous to the mammalian rhesus protein (Benghezal et al., 2001). In contrast to the proton ATPase, which is found both in the CV and in endosomes (Fok et al., 1993; Heuser et al., 1993; Adessi et al., 1995), Rh50 is strictly restricted to the CV compartment. This suggests the existence of a highly specific intracellular sorting machinery involved in targeting to the CV, and of targeting signals responsible for the localization of Rh50 in the CV. To determine which domain is responsible for the localization of Rh50 on CV, we expressed a fusion protein composed of the contact site A (CsA) extracellular domain, the transmembrane domain of the integral membrane protein P29F8 (Barth et al., 1994) and the 91 residues forming the Rh50 C-terminal cytoplasmic domain (Fig. 1A). Similar constructs were previously used to study the role of other cytoplasmic sequences in intracellular targeting in *Dictyostelium* (Gabriel et al., 1999; Ravanel et al., 2001).

The reporter protein CsA-Stop harboring only a short cytosolic domain was mostly localized to the plasma membrane, and absent from the CV (Fig. 1B) (Ravanel et al., 2001). By contrast, the addition of the last 91 residues of Rh50 was sufficient to ensure the localization of CsA-Rh50 to the CV as demonstrated by the colocalization of the chimeric protein with endogenous Rh50 or with calmodulin, another CV resident protein (Zhu and Clarke, 1992) (Fig. 1B). In addition, as observed for endogenous Rh50 (Benghezal et al., 2001),

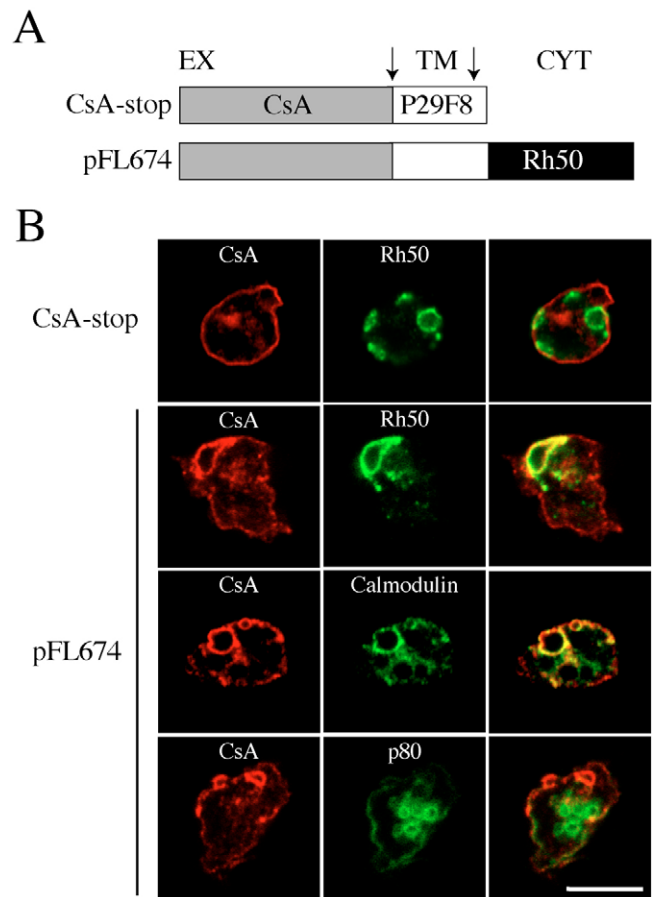


Fig. 1. The last 91 residues of Rh50 are sufficient for membrane protein localization on the CV. (A) Schematic representation of the CsA-Rh50 chimeric protein used in this study. The fusion protein is composed of the contact site A external domain (EX), P29F8 transmembrane domain (TM) and a short cytoplasmic domain (CYT) either followed by a stop codon (CsA-Stop) or the 91 C-terminal residues of Rh50 (pFL674). (B) Confocal microscopy study of cells expressing CsA-Stop or CsA-Rh50 (pFL674) constructs. Cells were double-labeled with the anti-CsA antibody (41-71-21) and polyclonal antibodies to either calmodulin or Rh50 (RH153) or the monoclonal antibody to p80 (H161) directly coupled to Alexa Fluor 488. Merged images are shown in the right-hand column. For calmodulin staining, cells were not fixed in paraformaldehyde but directly incubated in -20°C methanol for 10 minutes. CsA-Rh50 colocalized with endogenous Rh50 or with calmodulin, two CV-resident proteins, but was excluded from early and late p80-positive endosomes. Bar, 10 μm .

CsA-Rh50 was excluded from early and late endosomal vacuoles characterized by the presence of the p80 endosomal marker (Ravanel et al., 2001). Note that, whereas the majority of CsA-Rh50 was found in the CV, a small amount was targeted to the plasma membrane (Fig. 1B).

The C-terminal domain of Rh50 exhibits potential sorting signals

The fact that the Rh50 C-terminal cytoplasmic domain was sufficient for CV targeting suggested that this domain displays sorting signals. Indeed, sequence analysis revealed the presence of three putative sorting signals, a diaromatic motif

A

Rh50	E <u>Y</u> <u>Y</u> Q D S A <u>F</u> <u>W</u> I L P ⁴⁵²
CD-MPR	E Q <u>F</u> P H L A <u>F</u> <u>W</u> Q D L ²³²
h-MR	G A <u>F</u> E N T L <u>Y</u> <u>F</u> N S Q ¹⁴³
HIV-env	<u>Y</u> L <u>W</u> N L L L <u>Y</u> <u>W</u> G R E ⁸⁰⁶

B

Rh50a	A G G <u>D</u> <u>D</u> <u>E</u> <u>E</u> <u>E</u> G V G K <u>E</u> H ⁴⁸⁸
Rh50b	L P S <u>D</u> <u>D</u> <u>E</u> <u>E</u> <u>E</u> <u>D</u> <u>E</u> F K Q <u>E</u> ⁵²⁵
Furin	C P S <u>D</u> <u>S</u> <u>E</u> <u>E</u> <u>D</u> <u>E</u> G R G <u>E</u> R ⁷⁸³
HIV-Nef	W L <u>E</u> A Q <u>E</u> <u>E</u> <u>E</u> <u>E</u> V G F P V ⁷⁰
Ent3a	<u>D</u> <u>E</u> <u>E</u> <u>E</u> <u>D</u> <u>D</u> <u>D</u> <u>D</u> <u>E</u> F S <u>E</u> F Q ²⁷⁶

Fig. 2. Ph50 has three putative sorting signals already described in other proteins. (A) Alignment of the Rh50 protein sequence with diaromatic signals characterized in the cation-dependant mannose 6P receptor (CD-MPR), the human mannose receptor (MR) and the envelope glycoprotein of the human immunodeficiency virus type 1 (HIV-env). Aromatic residues are underlined. (B) Alignment of the two acidic clusters found in Rh50 (Rh50a and Rh50b) with known acidic signals found in furin, HIV-1 Nef and yeast Ent3p. Acidic residues are underlined and the conserved F residue adjacent to acidic clusters is in bold.

(F⁴⁴⁸W⁴⁴⁹) and two acidic motifs (F⁴⁷⁸DDEEE⁴⁸², ⁵¹⁶DDEEED⁵²¹) (Fig. 2).

Diaromatic motifs have been shown to influence endosomal sorting of several mammalian and viral membrane proteins (reviewed by Bonifacino and Traub, 2003), including the cation-dependent mannose 6P receptor (CD-MPR) (Schweizer et al., 1997), the human mannose receptor (MR) (Schweizer et al., 2000), and envelope glycoprotein (Env) of the human immunodeficiency virus type 1 (HIV-1) (Blot et al., 2003). Interestingly, the Rh50 C-terminal domain presents an additional Y located five residues from the diaromatic motif (F⁴⁴⁸W⁴⁴⁹) that could be part of a putative internalization signal (YxxxxF, Fig. 2A) as reported for both CD-MPR and MR (Johnson et al., 1990; Schweizer et al., 2000).

The two Rh50 acidic motifs (designated here Rh50a and Rh50b) belong to another family of sorting signals characterized by clusters of acidic residues often presenting sites for phosphorylation by casein kinase 2 (CKII) (reviewed by Bonifacino and Traub, 2003). In contrast to the membrane-proximal acidic signal of Rh50 (Rh50a), the Rh50b acidic motif shows a consensus CKII phosphorylation site (SxxE, Fig. 2B), although it is not known whether Rh50 is phosphorylated. In addition, an F residue is located directly downstream of the Rh50b motif. This sequence is reminiscent of the acidic-phenylalanine motifs (DE)_{2,3}FxxΦ (where Φ is a hydrophobic residue) recently identified in two yeast epsin-like proteins, Ent3p and Ent5p, and shown to interact with γ-ear domains of GGA2 and AP-1 (Duncan et al., 2003).

Acidic signals are necessary and sufficient for CV targeting

To determine which signal was required for CV targeting, several mutants of the CsA-Rh50 chimera were constructed (Fig. 3). Constructs were stably expressed in *Dictyostelium* cells and the localization of the chimeras was analyzed by confocal microscopy after labeling with anti-CsA (green) and



Fig. 3. Mutations introduced into the cytoplasmic domain of the CsA-Rh50 fusion protein. Positions of the mutations are indicated on the black line representing the cytoplasmic domain of the constructs. Each construct was given a name as indicated to the left of the figure. For pFL905, the sequence in bold corresponds to the last C-terminal acidic signal of Rh50.

anti-calmodulin (red) antibodies (Fig. 4). A domain containing the diaromatic signal (pFL760) alone did not allow the transport of the CsA-Rh50 mutant to the CV. By contrast, chimeras harboring one (pFL759 and pFL786) or two acidic motifs (pFL761) were targeted to the CV, as demonstrated by the colocalization of the chimera with calmodulin.

These results demonstrated that the presence of at least one acidic motif in the cytosolic domain of a chimeric transmembrane protein was sufficient to target it to the CV. To further establish the role of acidic signals in Rh50 targeting, acidic residues of the first acidic signal of Rh50 were mutated to alanine (pFL778) in the context of a construct truncated to the last acidic signal (pFL759). The resulting mutant was not transported to the CV but instead localized at the plasma membrane and in small vesicular structures distinct from the CV (Fig. 4). Mutations of the FW motif to alanine (pFL775) did not prevent transport of the CsA-Rh50 mutant to the CV. Together these results indicate that each acidic motif is necessary and sufficient for the targeting of the CsA-Rh50 chimera to the CV.

Finally, to exclude the possibility that other residues in the Rh50 cytoplasmic tail participate in CV targeting in addition to the acidic signals, the sequence SDDEEEFKQ (corresponding to the last C-terminal acidic signal) was fused to the CsA reporter protein with a spacer sequence of nine amino acids (composed of SG repeats). The presence of the acidic signal (pFL905) allowed transport of the chimera to the CV (Fig. 4). This result indicates that acidic motifs are sufficient for CV targeting, in the absence of any other motif. However the distance of the acidic domain from the transmembrane domain seems important because the direct fusion of the motif to the CsA reporter protein without a spacer sequence prevents CV targeting (data not shown).

Proteins destined to the CV are sorted in intracellular compartments

As mentioned above, targeting of CsA-Rh50 to the CV is very efficient, but a small portion of the chimeric protein is present at the cell surface. This offered us the possibility of following more precisely the intracellular transport of the CsA-Rh50 chimera. For this, cells were first incubated with the anti-CsA antibody for 5 minutes at 4°C, washed and then warmed up for

different periods of time to allow intracellular transport to occur. To monitor the amount of CsA-Rh50 left on the cell surface after internalization and to identify the plasma membrane unambiguously, cells were then transferred back at 4°C and incubated prior to fixation or permeabilization with an anti-mouse secondary antibody coupled to Alexa Fluor 647 (blue). Cells were then fixed, permeabilized to gain access to internalized chimeras and incubated with an anti-mouse secondary antibody coupled to Alexa Fluor 568 (red) to detect the presence of internalized CsA-Rh50 proteins. In addition, to characterize the CV, cells were colabeled with anti-Rh50

(green). Note that this antiserum only recognized the endogenous Rh50 protein but not the CsA-Rh50 chimera (pFL759) (data not shown).

The CsA-Rh50 chimera present at the cell surface was rapidly internalized and accumulated in punctuate intracellular structures as early as five minutes after warming up the cells (Fig. 5). After 60 minutes of internalization, in addition to these initial endosomal structures, CsA-Rh50 localized on the CV network as observed by colabeling with anti-Rh50 (Fig. 5) and anti-calmodulin antibodies (data not shown). Only very minor amounts of the CsA-Rh50 chimera colocalized with p80-positive endosomal vacuoles even after 2 hours of internalization (Fig. 6A). Note that the CV compartment was not observed upon staining with the anti-CsA antibody (Fig. 6A) because p80 endosomal vacuoles and the CV are not usually in the same cellular region (Ravel et al., 2001). In Fig. 6A, we specifically selected pictures appropriate for the observation of p80 endosomal vacuoles but usually not for the detection of the CV network. Thus, the punctuate structures containing internalized CsA-Rh50 are distinct from p80-positive endosomal vacuoles and represent distinct endosomal structures. Using the same experimental procedure, no endocytosis was detected when cells expressed a transmembrane CsA protein with a short cytoplasmic domain (data not shown) (Ravel et al., 2001).

To establish that endocytosed CsA-Rh50 proteins are initially present in endosomal structures distinct from the Golgi apparatus, cells expressing CsA-Rh50 were transfected with a green fluorescent protein (GFP)-tagged Golgi marker. Golgesin is a protein associated with membranes of the Golgi apparatus and post-Golgi vesicles in *Dictyostelium* cells. GFP-tagging of golgesin at the C-terminal extremity [golgesin (C)-GFP] results in the specific localization of the protein in the Golgi (Schneider et al., 2000). After 5 minutes of internalization CsA-Rh50 did not colocalize with golgesin (C)-GFP (Fig. 6B). Even after longer internalization times, CsA-Rh50 was still excluded from the Golgi apparatus (data not shown).

Together these results indicate that CsA-Rh50 mislocalized at the cell surface is constantly retrieved from the surface back to the CV. This transport involves transit through an endosomal compartment distinct from p80 endosomal vacuoles and from the Golgi complex. However, confocal microscopy studies with antibodies to known lysosomal proteins did not convincingly allow us to further establish the nature of this compartment.

To gain further insights concerning the site where sorting to the CV occurs, we analyzed the transport of CsA-Rh50 proteins where sorting motifs have been mutated. CsA-Rh50 (pFL759) carries a putative diaromatic signal and an acidic signal, and internalization experiments were repeated with cells that express constructs exhibiting only one potential sorting motif (see Fig. 3). Mutation of the FW signal to alanine residues (pFL775) did not affect transport of the chimera to the CV (Fig. 7A) indicating that the acidic cluster is sufficient for the sorting of Rh50 to the CV. By contrast, mutation of the acidic cluster to alanine (pFL778) resulted in a loss of CV targeting (Fig. 7B). However it is noticeable that this mutated chimeric protein still gained access to a punctuate endosomal compartment with an internalization rate comparable to pFL759 and pFL775 (data not shown). Apparently, transport of pFL778 to the endosomes still proceeded, but a further step

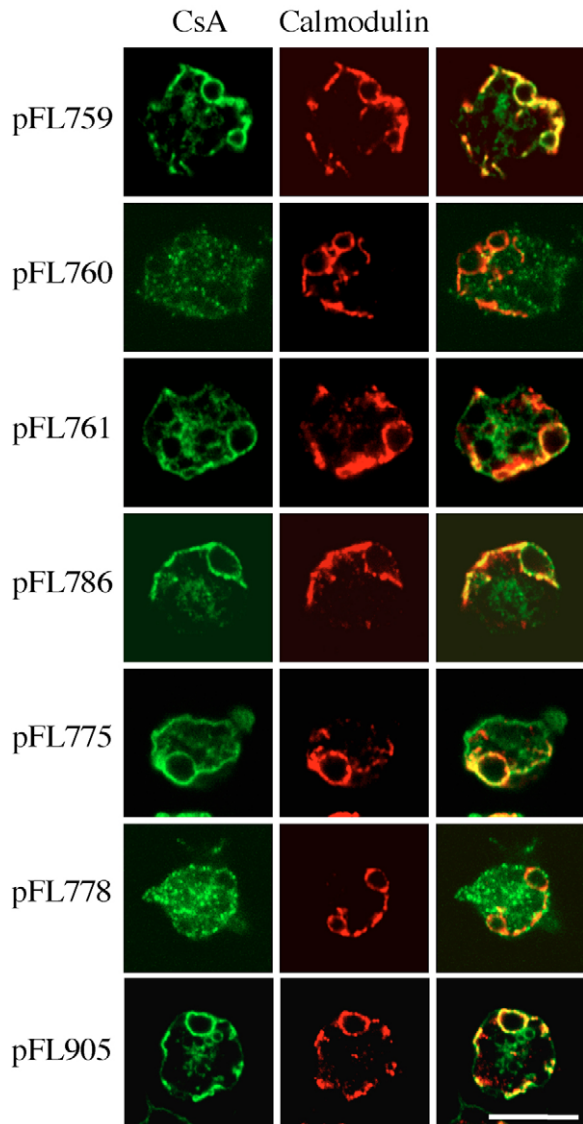


Fig. 4. Rh50 acidic clusters are sufficient for CV targeting. *Dictyostelium* cells expressing the indicated construct were double-labeled with the anti-CsA antibody (41-71-21) and the polyclonal antibody to calmodulin and analyzed by confocal microscopy. Although a domain containing only the diaromatic signal (pFL760) did not allow the transport of the CsA-Rh50 mutant to the CV, domains containing one (constructs pFL759 and pFL786) or two acidic signals (pFL761) were sufficient for targeting to the CV. Mutation of the acidic residues to alanine (pFL778) prevents the expression of CsA-Rh50 to the CV. Bar, 10 μ m.

of intracellular sorting was abrogated in this mutant. This suggests that the acidic motif is recognized in an intracellular compartment, and further supports the notion that transport from the cell surface to the CV occurs through at least one endosomal compartment and not by a direct exchange between the plasma membrane and the CV.

Rh50 acidic clusters interact with the ear domain of γ AP-1

The sequence homology between Rh50b and Ent3p acidic clusters (Fig. 2) suggested that Rh50 acidic motifs interacts with the ear domain of the γ -subunit of the AP-1 clathrin adaptor complex as previously reported for Ent3p (Duncan et al., 2003). To test this hypothesis, we performed yeast two-hybrid interaction assays between the γ -ear domain of AP-1 and Ent3p or Rh50 C-terminal domain (Fig. 8). Both Ent3 and Rh50 proteins interacted with the γ -ear domain of AP-1 in this assay whereas no interaction was detected with another subunit of AP-1, μ 1, involved in the recognition of tyrosine-based sorting signals. A mutation of one acidic cluster to alanine residues led to a reduction of the interaction between the γ -ear domain and Rh50 suggesting that the two acidic clusters of Rh50 acted synergistically in the optimal recruitment of γ . Recently the crystal structure of the human γ 1-ear domain has revealed a cluster of basic residues required for the recruitment of accessory proteins, γ -synergin and Rabaptin 5 (Nogi et al., 2002). To test whether this cluster was also involved in the binding of Rh50 C-terminal domain, residues R⁸⁷¹ and K⁸⁷³ in *Dictyostelium* γ -ear (equivalent of human γ 1-ear residues R⁷⁹⁵ and K⁷⁹⁷) were mutated to glutamine. As observed in Fig. 8, both mutations inhibited the interaction with both Rh50 and Ent3p proteins in this yeast two-hybrid assay. Note that the interaction between GGA (another γ -ear-containing protein) and Rh50 was not tested here because BLAST searches failed to detect any GGA proteins in *Dictyostelium* (data not shown). Thus it seems likely that AP-1 coated vesicles are implicated in the transport of Rh50 to the CV, by virtue of a direct interaction between the Rh50 cytosolic acidic motifs and the basic residues of the γ -AP1 ear domain.

Discussion

Characterization of a signal for targeting a membrane protein to the CV

In this study, we report for the first time the identification of a sorting signal responsible for the specific targeting of a transmembrane protein to the contractile vacuole in the model organism *Dictyostelium discoideum*. Here, we made use of a chimeric approach to determine the domain of Rh50 required for proper CV targeting. Rh50 is a polytopic protein with a C-terminal domain exposed to cytoplasm of the cell. We demonstrated that the fusion of the last 91 amino acid of Rh50 was sufficient to direct a CsA/P29F8 chimera,

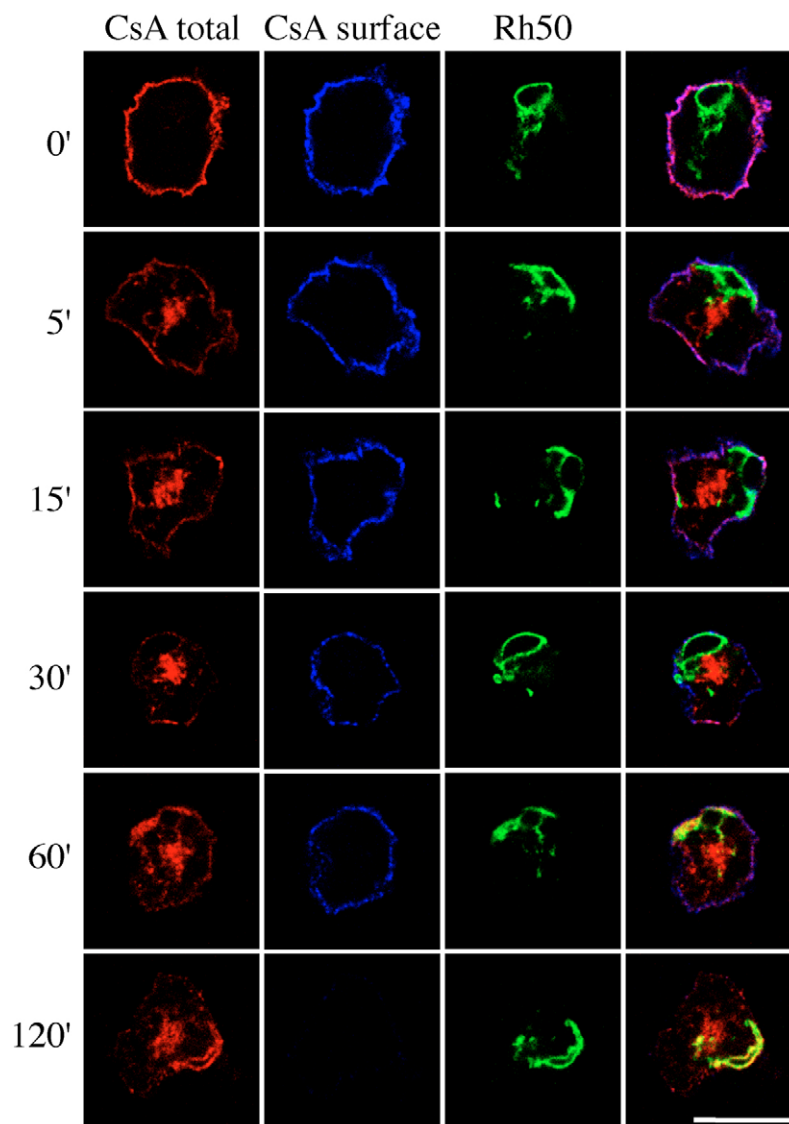


Fig. 5. Transport of CsA-Rh50 from the plasma membrane to the CV. Cells expressing pFL759 were incubated with the anti-CsA antibody for 5 minutes at 4°C, washed and then warmed up for the times indicated on the left. The amount of CsA-Rh50 left on the cell surface after internalization was monitored by incubating cells at 4°C for 30 minutes with an anti-mouse secondary antibody coupled to Alexa Fluor 647 (CsA Surface, blue staining). Then, cells were fixed, methanol-permeabilized and incubated with an anti-mouse secondary antibody coupled to Alexa Fluor 568 to detect internalized CsA-Rh50 as well as any remaining construct on the cell surface (CsA Total, red staining). The CV compartment was decorated with a polyclonal antibody to Rh50 (RH153, green staining). Cells were observed by confocal microscopy. CsA-Rh50 was rapidly internalized, accumulated in vesicular structures and after 60 minutes of internalization localized on the CV network. Bar, 10 μ m.

normally expressed at the cell surface, to the CV. Further mutagenesis experiments established that two clusters of acid residues were responsible for the targeting of the chimera to the CV. This is the first evidence for a specific sorting signal in an integral membrane protein resident of the CV and thus this study provides the first clues on the molecular machinery responsible for the integrity and the biogenesis of the CV in *Dictyostelium* cells. The characterization of other

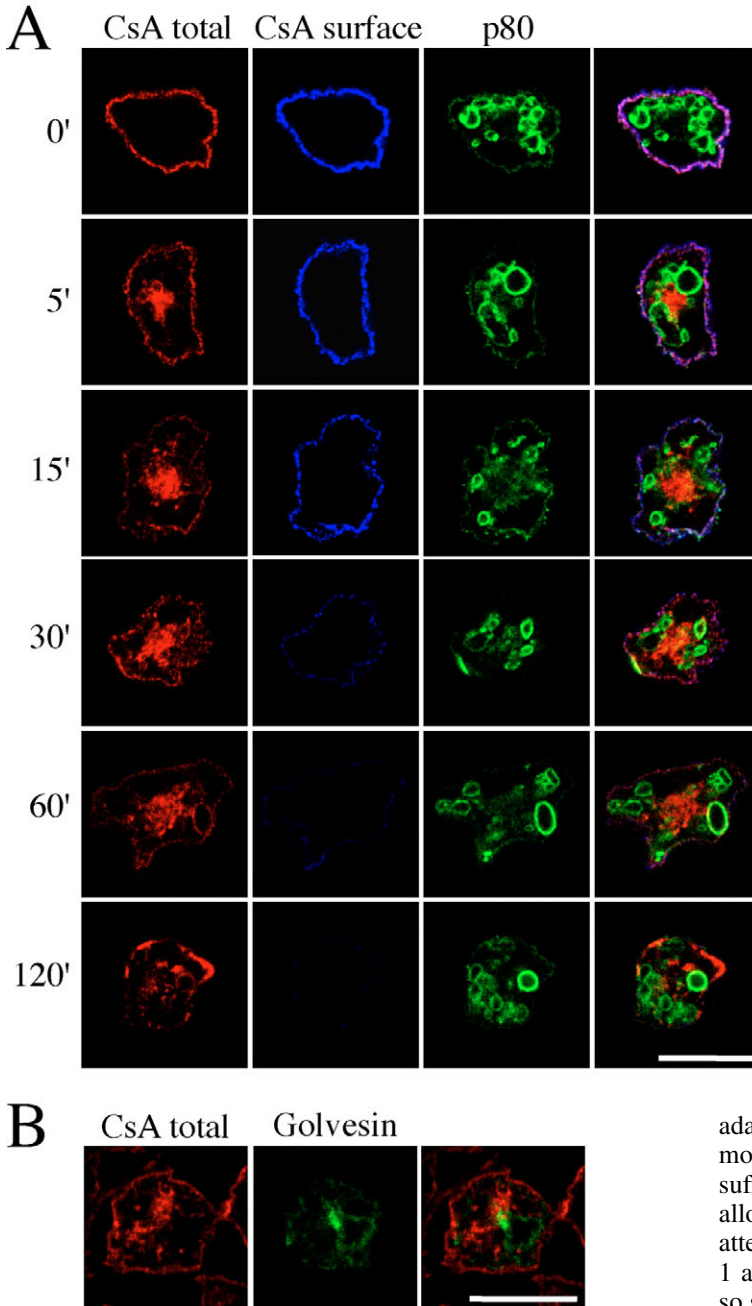


Fig. 6. Internalized CsA-Rh50 is excluded from p80-containing endosomes and from the Golgi apparatus. (A) Cells expressing pFL759 were treated as described in Fig. 5. At the end of the procedure, early and late endosomal vacuoles were labeled with the monoclonal antibody to p80 (H161) directly coupled to Alexa Fluor 488 (p80, green staining). CsA-Rh50 barely colocalized with p80 endosomes even after 2 hours of internalization. Note that the CV compartment was not detected here upon staining with the anti-CsA antibody because p80 endosomes and the CV localize in different areas of the cell and that we selected by confocal microscopy a plane appropriate for the observation of p80 endosomes but excluding the CV network. (B) The Golgi marker golgesin (C)-GFP was expressed in cells expressing pFL759. Cells were treated as described in Fig. 5. After 5 minutes of internalization, CsA-Rh50 did not colocalize in golgesin (C)-GFP positive structures. Bar, 10 μ m.

direct proteins to different intracellular compartments. The amino acid sequence flanking the acidic clusters could be an important factor. For instance, the retrieval of the prohormone-processing enzyme furin from endosomes to the TGN depends on the phosphorylation of the CKII sites close to the acidic signal (Jones et al., 1995). Furthermore these signals and their flanking regions can be recognized by different sets of molecules. Some acidic signals specifically interact with a protein termed PACS-1 (Wan et al., 1998), which is essential for the endosome to TGN transport pathway, whereas the yeast Ent3p acidic-phenylalanine signal interacts with γ -ear containing proteins, GGA and γ -adaptin (Duncan et al., 2003).

Here we demonstrate that both acidic clusters of Rh50 can weakly interact with γ -adaptin as revealed by a yeast two-hybrid protein-protein interaction assay. The requirement for the presence of two acidic clusters to detect a significant interaction with γ -adaptin presumably reflects the weak affinity of each individual motif for γ -adaptin in vitro. This weak affinity could be sufficient in vivo because the presence of only one signal allows the transport of CsA-Rh50 to the CV. Despite several attempts, we failed to detect a direct interaction between AP-1 and the acidic signals in vivo (data not shown). This is not so surprising because the interaction between targeting signals and adaptor proteins is very transient and involves only a very small fraction of the protein at any given time. Such interactions have always proved difficult to detect even for the well-characterized tyrosine-based signals (Bonifacino and Traub, 2003). To our knowledge, Rh50 is the first cargo protein whose transport may be dependent upon the direct interaction of the AP-1 γ -ear domain and acidic signals. However we cannot exclude the fact that individual acidic signals are interacting with another protein such as PACS-1 (though *Dictyostelium* presents no proteins homologous to PACS-1), which would mediate the interaction with AP-1 and participate in the targeting of Rh50 to the CV.

The interaction between γ -adaptin and Rh50 is consistent with our previous study that demonstrated the role of AP-1 coat in the biogenesis of the CV (Lefkir et al., 2003). AP-1-coated vesicles could play an essential role in intracellular transport

transmembrane CV proteins should reveal whether these acidic motifs are generic signals for CV targeting or if other signals also function in this transport pathway. The fact that the cytoplasmic tail of Rh50 contains CV targeting signals does not preclude the fact that other domains of the protein also participate in the transport of native Rh50 to the CV because redundant sorting signals are common in membrane proteins.

Rh50 acidic clusters and the AP-1 vesicular coat

Acidic clusters have been previously shown to function as sorting signals in different transport pathways, namely in endosomes to TGN, endosomes to multivesicular bodies, and plasma membrane to endosomes (reviewed by Bonifacino and Traub, 2003). It is not clear how one family of signals can

of membrane proteins to the CV, accounting for the fact that the loss of AP-1 results in the complete loss of a functional CV. In addition to AP-1, other factors might participate to the transport of Rh50 to the CV network. Further experiments will be required to fully address the role of AP-1 in the delivery of Rh50 to the CV network.

A new transport pathway between the plasma membrane and the CV

Previous studies have ruled out a direct exchange of membrane components between the CV and the plasma membrane during the transient fusion of the bladder with the plasma membrane, which allows water expulsion in hypo-osmotic conditions. Indeed components of the plasma membrane labeled with the fluorescent dye Cy3.5 failed to accumulate in the bladder during its periods of discharge and refilling (Gabriel et al., 1999). This result is in agreement with the observation that several plasma membrane proteins such as p25 and p80 are excluded from the CV (Ravel et al., 2001) (data not shown). An indirect exchange between the plasma membrane and the CV via endosomes has also been excluded using a similar approach. After 10 minutes of internalization of cell surface proteins labeled with Cy3.5 into endosomes, no labeling could be detected in the CV (Gabriel et al., 1999). Although no longer internalization times were tested in these experiments owing to technical limitations, the fact that p80, a marker of early and late endosomes, is excluded from the CV might appear to strengthen the notion that there is no communication between endocytic and CV compartments. However the existence of proteins present in both the CV and endosomes suggests a partial, difficult-to-study link between these two compartments.

We noted that a small amount of CsA-Rh50 is present at the cell surface rather than in the CV. This gave us the opportunity to further explore the possibility of the exchange of membrane constituents between the plasma membrane and the CV. The most striking result is that the surface pool of CsA-Rh50 protein is constitutively internalized and rapidly concentrated in a perinuclear compartment distinct from the Golgi apparatus. This compartment does not contain detectable amounts of p80 protein, a marker of early and late endosomal vacuoles (Ravel et al., 2001). Therefore, in *Dictyostelium* cells newly internalized CsA-Rh50 is rapidly sequestered in endosomes distinct from p80-positive endosomal vacuoles.

What is the fate of proteins localized to this compartment? Confocal microscopy studies revealed that the chimera is detected in the CV only after 1 hour of internalization, the signal getting significantly stronger after 2 hours. Therefore the rapid accumulation of internalized proteins in endosomes strongly suggests that CsA-Rh50 proteins first transit through these endosomes, before reaching the CV. The sorting of CsA-

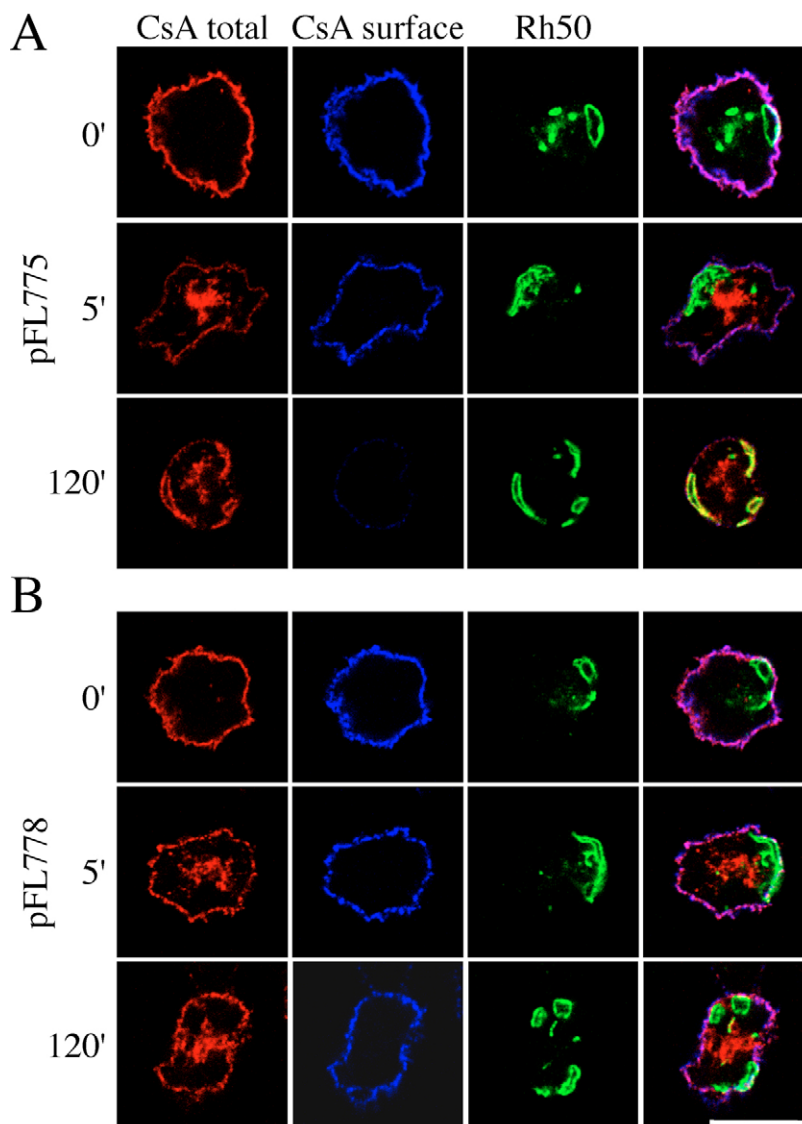


Fig. 7. The acidic cluster is required for transport of CsA-Rh50 from the plasma membrane to the CV. Cells expressing pFL775 (A) or pFL778 (B) were treated as described in Fig. 5. Mutation of the FW signal to alanine residues (pFL775) did not affect the transport of the chimera to the CV whereas mutation of the acidic cluster to alanine (pFL778) inhibited CV targeting. Bar, 10 μ m.

Rh50 to the CV is likely to take place in the endosomes, where internalized CsA-Rh50 has a rather long residency time. This sorting process probably depends on the presence of acidic clusters, because mutations of the acidic clusters to alanine residues do not abolish access of CsA-Rh50 to these punctuate endosomal structures. Based on our yeast two-hybrid experiments, we propose that AP-1 could participate to the selection of Rh50 in this compartment. Whether this compartment is only an intermediate compartment between the plasma membrane and the CV or a more ubiquitous endosomal structure where sorting to different organelles could take place is currently under investigation. Interestingly, the CV network resembles the tubulo-vesicular structure seen in acid-secreting gastric parietal cells. In these cells, clathrin and AP-1 are thought to be involved in protein targeting to the tubulo-




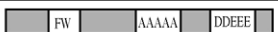
	γ -ear	γ -ear R ⁸⁷¹ Q	γ -ear K ⁸⁷³ Q	μ 1
Ent3p	232.9	42	0.6	2.3
	250.7	2.2	0.7	2.1
	2.3			
	18.7			
	21.4			

Fig. 8. Rh50 acidic clusters interact with the ear domain of γ AP-1. The ability of the Rh50 cytoplasmic domain to interact with the γ -ear domain of AP-1 was tested in a yeast two-hybrid assay. Interaction of proteins was determined by measuring β -galactosidase activity in liquid conditions. Results, expressed in arbitrary units, correspond to the average of three independent experiments. The s.e.m. was less than 5%. Both Ent3p and Rh50 proteins comparably interacted with the γ -ear domain of AP-1 in this assay but not with the μ 1 subunit of the AP complex. Notably, mutation of either acidic cluster to alanine residues led to a dramatic reduction of the interaction between the γ -ear domain and Rh50.

vesicular network (reviewed by Yao and Forte, 2003). Therefore, a role for AP-1 in many vesicular trafficking events seems to be supported in various organisms and is a genuine feature of the AP-1 clathrin-adaptor complex.

Materials and Methods

Cell culture and Antibodies

D. discoideum strain DH1-10 (Cornillon et al., 2000) was grown at 22°C in HL5 medium and subcultured twice a week. Cells were not allowed to reach a density of more than 2×10^6 cells/ml. Antibodies used were a rabbit antiserum to the C-terminal cytosolic tail of Rh50 (controls showed that pFL759 was not recognized by this antiserum) (Benghezal et al., 2001), a rabbit antiserum to calmodulin (a kind gift from T. Soldati, University of Geneva, Switzerland) (Ulbricht and Soldati, 1999) and a mouse monoclonal antibody to CsA (mAb 41-71-21) (Bertholdt et al., 1985). Note that in our cell culture conditions (low density), endogenous CsA was not expressed and therefore this anti-CsA antibody only recognized CsA-Rh50 chimeras. To allow simultaneous labeling with an anti-p80 antibody and another mouse monoclonal antibody, the anti-p80 H161 antibody (Ravel et al., 2001) was directly coupled to Alexa Fluor 488 (Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions.

Plasmids and cell transfection

The cDNA encoding the contact site A (CsA) protein with the transmembrane domain of the integral protein P29F8 (Barth et al., 1994) in the expression vector pDCEV4 was kindly provided by G. Gerisch (Max-Planck-Institut für Biochemie, Martinsried, Germany). The sequence of the cytoplasmic domain of the CsA-Stop construct is KTRVSQNSG. To obtain fusion proteins of this short cytoplasmic domain with Rh50 (CsA-Rh50), a *Bst*XI-*Xho*I fragment was replaced with the DNA encoding the 91 C-terminal residues of Rh50. All mutations and truncations described in Fig. 3 were produced by PCR using pairs of oligonucleotides containing either *Bst*XI or *Xho*I sites at the 5' and 3' regions respectively. PCR fragments were digested by *Bst*XI and *Xho*I, cloned into *Bst*XI/*Xho*I sites of pDCEV4-CsA-Rh50 and sequenced (Genome express, Grenoble, France). Plasmids were linearized by *Sca*I and transfected in *Dictyostelium* by electroporation as described (Cornillon et al., 2000).

The golgesin (C)-GFP expression vector (Schneider et al., 2000) was a kind gift from A. Mueller-Taubenberger (Ludwig Maximilians University Munich, Muenchen, Germany). The neomycin-resistance gene present in this vector was substituted with the blasticidin-S-resistance gene. After electroporation, cells expressing golgesin (C)-GFP were selected with 10 μ g/ml blasticidin S.

Immunofluorescence microscopy

For immunofluorescence analysis, cells were applied on a glass coverslip for 2 hours, then fixed with 4% paraformaldehyde for 30 minutes, washed and permeabilized with methanol at -20°C for 2 minutes. Cells were incubated with the indicated antibodies for 30 minutes, and then stained with the corresponding fluorescent secondary antibodies for 30 minutes. Cells were observed by laser-

scanning confocal microscopy (Zeiss LSM 510). For calmodulin staining, cells were directly fixed by incubation with methanol at -20°C for 10 minutes and treated as described above.

For internalization studies, cells were first incubated with the anti-CsA antibody for 5 minutes at 4°C , washed and then warmed up in culture medium for different periods of time. At each time point, cells were returned to 4°C and incubated with an anti-mouse secondary antibody coupled to Alexa Fluor 647 for 5 minutes. Cells were then fixed with 4% paraformaldehyde for 30 minutes, washed, permeabilized with -20°C methanol for 2 minutes, and incubated with an anti-mouse secondary antibody coupled to Alexa Fluor 568.

Testing protein-protein interaction by two-hybrid assay

Two-hybrid assays were carried out using the Matchmaker LexA two-hybrid system (Clontech Laboratories, Palo Alto, CA). The DNA sequence encoding the AP-1 γ -ear domain (residues 664 to 895) was fused to the DNA binding protein LexA in the expression vector pEG202. Point mutations in γ -ear (R⁸⁷¹Q and K⁸⁷³Q) were made by PCR, sequenced and cloned into pEG202. To test the ability of the Rh50 cytoplasmic domain to interact with the AP-1 γ -ear domain, the DNA sequence encoding the last 91 residues of Rh50 was fused to the B42 activation domain in the vector pJG4-5 containing the inducible GAL1 promoter. For specificity controls, Ent3p (full-length) and p80 (51 amino acid residues of the cytoplasmic loop of p80, ELEK to CLHA) DNA sequences were cloned into pJG4-5 and cotransfected in the reporter yeast with the AP-1 γ -ear-containing construct. Transformed EGY48 yeasts expressing the plasmid p80-LacZ were tested for their ability to grow on selective plates (synthetic complete medium without leucine and containing galactose) and give a blue color on Xgal-supplemented plates. For more accurate results, the β -galactosidase activity was determined in liquid conditions for a fixed number of yeast cells using O-nitrophenyl- β -D galactopyranoside as a substrate. The background activity in cells expressing only the B42 activation domain fusion protein was subtracted.

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