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Testing for endocytosis in plants

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Summary. For many years endocytosis has been regarded with great scepsis by plant physiologists. Although now generally accepted, care must still be taken with experiments designed to demonstrate endocytic uptake at the plasma membrane. We have taken a critical look at the various agents which are in use as markers for plant endocytosis, pointing out pitfalls and precautions which should be taken. We also take this opportunity to introduce the tyrphostins – tyrosine kinase inhibitors –, which also seem to prevent endocytosis in plants.

Keywords: Biotinylated marker; FM lipophilic styryl dye; Lucifer Yellow; Tyrphostin; Wortmannin.

Introduction

In 1980 W. J. Cram made the unfortunate statement, "We shall argue firstly, on ultrastructural and physiological grounds, that pinocytosis does not occur and secondly, on theoretical grounds, that it could not occur in a turgid plant cell'' (Cram 1980: p. 1). 25 years on and a good dozen reviews later it can safely be said that endocytosis is not restricted to mammalian cells but is also exhibited by walled organisms, including plants. Nevertheless, it has to be admitted that as far as plants are concerned, the unequivocal demonstration of the internalization of a receptor-ligand complex through a clathrin-coated pit and the recycling of the receptor to the plasma membrane (PM) still remains to be published. Despite the fact that, from the structural view point, it has been known for almost 20 years that plant cells possess endocytic organelles (clathrin-coated pits and vesicles, multivesicular bodies), the commonly held belief among physiologists that turgor pressure in plants would make endocytosis difficult, if not impossible, has delayed the appreciation of this process by the plant community. The lack of obvious molecules for endocytic cargo has not helped this situation. Turgor no longer plays an impeding role for endocytosis in plants (Saxton and Breidenbach 1988, Meckel et al. 2004), and a number of good candidates for internalizable receptors at the PM are now known (Holstein 2002, Geldner 2004).

One of the major problems in studies on endocytosis in plants arises from trying to determine whether a molecule has entered cells by a fluid-phase or a receptor-mediated pathway. Another problem lies in interpreting the results of the application of inhibitors, whose specificities may be wide and whose targets may differ throughout the endocytic pathway. A widely used indicator to discriminate between fluid-phase endocytosis (FPE) and receptor-mediated endocytosis (RME) is the kinetics of uptake. FPE shows linear uptake kinetics with respect to increasing substrate concentration (an inevitable consequence of the nonselective uptake of external fluid) and shows no evidence of saturation over a large external solute range. In contrast, RME saturates with ligand concentration (a consequence of the limited numbers of receptor molecules at the PM). Uptake of labelled solute by FPE is not reduced in the presence of unlabeled solute, while unlabelled free ligand competes for the uptake of labelled ligand during RME. Finally, the presence of a PM receptor allows ligand binding to the PM at either 4 °C or 37 °C, while binding to the cell surface of a solute internalized by FPE is not detectable at either 4 °C or 37 °C.

In this article we present a critical overview of the agents which have been used to demonstrate the process of endocytosis in plants, including markers and inhibitors.

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Markers

Lucifer Yellow: a reliable marker for fluid-phase endocytosis?

The extent to which plant cells can undergo FPE, as well as its physiological significance, is still a matter of debate. It also appears that the ability to undergo FPE may be restricted to some cell types (for a review, see Šamaj et al. 2004). Classical fluid-phase markers, such as colloidal gold- or fluorescently labelled globular proteins (e.g., bovine serum albumin [BSA]), or the enzyme horseradish peroxidase, penetrate extremely slowly through plant cell walls, making it difficult to evaluate the process. The ideal marker for FPE would therefore be a compound (1) whose passage to the PM is not impeded by the cell wall, (2) that does not enter cells by any carrier-mediated process, and (3) that has a low intrinsic membrane passive permeation. Lucifer Yellow (LY) would appear to fulfill these criteria. A disulfonated 4-aminonaphthalimide, LY is a membrane-impermeative fluorescent marker and has been extensively used as a fluid-phase marker in a range of eukaryotic cells (Swanson et al. 1985, Riezman 1985, Basrai et al. 1990, Wiederkehr et al. 2001). LY has also been widely used as a marker for FPE in plant cells (Oparka et al. 1988; Wright and Oparka 1989; Hillmer et al. 1989, 1990; Roszak and Rambour 1997; Cholewa and Peterson 2001; Baluška et al. 2004; Yano et al. 2004).

The properties that make LY a useful fluid-phase marker are (1) a high quantum yield that does not vary between pH 1–10; (2) resistance to bleaching; (3) membrane impermeation; (4) highly dissociated (negatively charged) status at pH values >2 (because of a low acidic dissociation constant); (5) lack of toxicity to cells at concentrations normally employed to measure FPE; and (6) lack of cellular metabolism. However, the physicochemical properties of this compound (molecular mass of 443 Da, relatively hydrophobic, with two sulfonic acid groups and a net negative charge) would also appear to make it a virtually ideal substrate for organic anion transport systems that are expressed at high levels in liver and other transporting epithelia (Boyer et al. 1992, Meier et al. 1997, Muller et al. 1997, Masereeuw et al. 1999). These systems exhibit a broad substrate specificity, but prefer negatively charged amphiphilic compounds with a molecular mass of >250 Da. In this respect, LY uptake in skate and rat hepatocytes was temperature-sensitive and partially sodium-dependent, exhibited saturation kinetics, and was inhibited by other organic anions and by competitive inhibitors of organic anion transport (e.g., probenecid, *p*-[dipropylsulfamoyl]-benzoic acid). These data strongly suggest that LY is transported across the plasma membrane of these cells largely via specific carrier-mediated mechanisms, rather than via FPE (Ballatori et al. 1999).

Accumulation of exogenously applied LY in the lysosome of mammalian cells, which represents the final compartment in the endocytic pathway, may also not necessarily be the result of vesicle-mediated FPE. Thus, LY has been shown to be sequestered from the cytosol into the lysosomal system after ATP^{4-} permeabilization of the PM in mammalian cells (Steinberg et al. 1987a, b, 1988). Since probenecid has been shown to inhibit this sequestration (Steinberg et al. 1987b, 1988), one must assume that there is an additional transport system localized at the lysosomal membrane which recognizes LY.

Vacuolar LY accumulation in protoplasts or intact plant cells may depend on the species, tissue, and experimental conditions chosen. Thus, whereas Wright and Oparka (1989) reported LY uptake into the vacuole of mesophyll protoplasts from a variety of dicotyledonous plants, especially from the genus *Nicotiana*, Klein et al. (1997) could not detect LY uptake into the vacuole or the cytosol of barley mesophyll protoplasts, even after 18 h of incubation. Again, whereas vacuolar accumulation of LY has been reported for intact barley roots (Oparka et al. 1988), and for mesophyll cells of *Commelina communis* (Hillmer et al. 1990), in the inner cortex cells of maize root apices, LY collected within tubulovesicular invaginations of the PM and within vesicles, tubules, and small peripheral vacuoles, but never accumulated within the central vacuole (Baluška et al. 2004). A similar ultrastructural membranous localization of LY has been reported for nutrient-absorptive trichomes of a carnivorous bromeliad (Owen et al. 1991).

Despite these contradictory results, the observation that LY when exposed to different plant tissues, cells, or protoplasts, generally does not remain confined to the extracellular space but readily enters the vacuole has led to the idea that the dye accumulates via vesicle-mediated FPE (Oparka 1991, Oparka and Hawes 1992). However, being a disulfonated molecule, LY may represent an analogue of naturally occurring sulfonated compounds in plant cells, e.g., flavonoids. Several directly energized transport systems are known to be located at the vacuolar membrane of plant cells for the uptake of such compounds (Coleman et al. 1997), and indeed a mechanism of this type has been shown to mediate vacuolar uptake of LY in rye (Klein et al. 1997). Moreover, the cytosolic accumulation of LY as a result of probenecid treatment has been reported for suspension-cultured cells of carrot (Cole et al. 1991) and for onion epidermal cells (Oparka et al. 1991). Anion transporters may also be responsible for uptake at the PM.

A case in point is suspension-cultured cells of *Morinda citrifolia* which do not exhibit vacuolar sequestration of LY, the dye instead collecting in the cytosol. However, treatment with probenecid prevented LY from entering the cells (O'Driscoll et al. 1991). Therefore, the demonstration of LY transport to the vacuole is not automatically unequivocal proof of a FPE uptake mechanism.

In summary, LY can indeed be an excellent marker for FPE, but the occurrence of a carrier-mediated process should be tested for in each experimental system, by looking at saturability of uptake (characteristic of carrier-mediated uptake or RME, but not of FPE), sodium-dependence (which is characteristic of some carrier-mediated pathways) and sensitivity to probenecid (or other related inhibitors which interfere with organic acid transport).

FM dyes: reliable membrane probes for endocytosis?

The amphiphilic styryl dyes FM 4-64, FM 1-43 and FM 2-10, developed by Betz and colleagues for studies on membrane trafficking in the synapse (Betz et al. 1996), have become very popular tools for studies on endocytosis in a range of eukaryotic cell types, including plants. These dyes only fluoresce in a hydrophobic environment and are believed to insert into the outer lipid layer of the PM when applied to cells exogenously (Bolte et al. 2004a). Of the three dyes mentioned above, FM 4-64 appears to be the most reliable for endocytosis studies, since the others, which are less hydrophobic, can be removed from the PM by repeated washing (Meckel et al. 2004). When presented to cells held at low temperatures, FM dyes remain in the PM but become internalized upon returning the cells to normal growth temperature (e.g., Vida and Emr 1995, Fischer-Parton et al. 2000) (Fig. 1a). In time, the dyes are transported from the PM to the tonoplast, where the majority of the dye molecules remain (e.g., Kutsuna et al. 2003, Yano et al. 2004) (Fig. 1c). This movement is generally considered to occur via vesicles (Bolte et al. 2004a), although nonvesicular modes are possible but remain to be demonstrated (Read and Kalkman 2003).

As originally demonstrated for yeast cells (Vida and Emr 1995), FM dyes label small punctate structures on their way to the tonoplast (Fig. 1c). The organelles labelled do not belong to the early secretory pathway, although eventually the Golgi apparatus can receive label (Bolte et al. 2004a). The principal intermediates appear to lie on the endocytic pathway, since a colabelling with the endo-

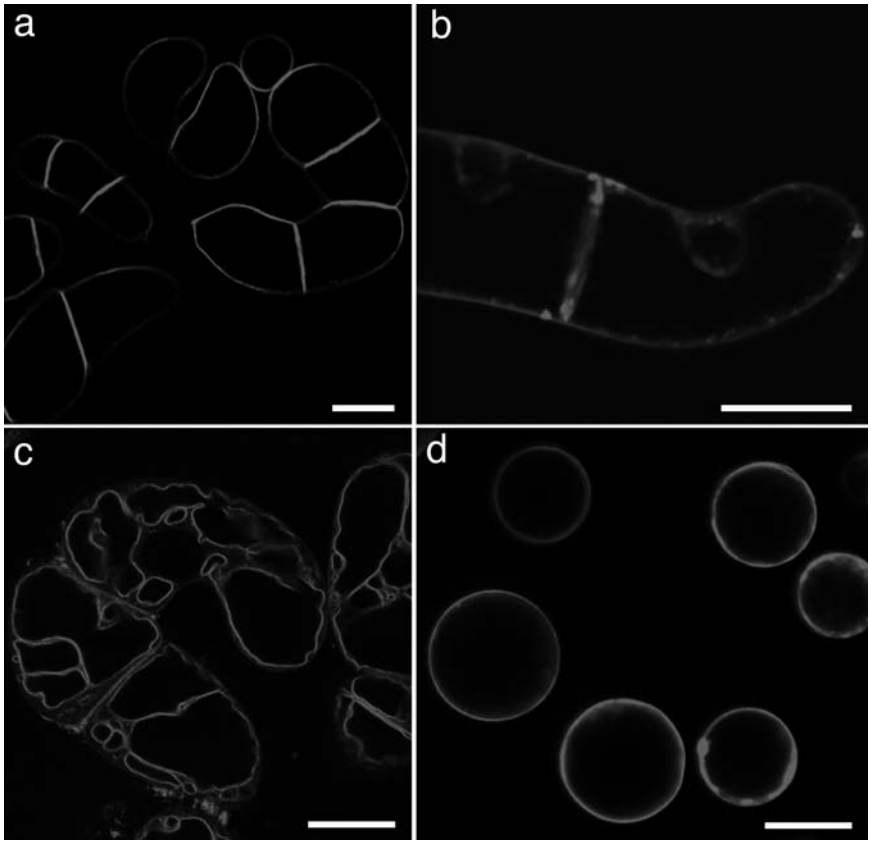

Fig. 1a–d. FM 4-64 labelling of plant cells and protoplasts. **a–c** Tobacco BY2 cells showing initial labelling of the PM (**a**), then labelling of putative endosomal compartments several minutes after incubation in the dye at room temperature (**b**), and finally 24 h after exposure when the dye is now exclusively in the tonoplast (**c**) (micrographs courtesy of Dr. Yuji Moriyasu). **d** *Arabidopsis thaliana* mesophyll protoplasts (micrograph courtesy Dr. Inhwan Hwang). The protoplasts, which are known to be secretion competent, have been incubated at room temperature for 24 h, but no internalization of the FM dye can be seen. Bars: 10 μm

somal markers GNOM (Geldner et al. 2003) and Rab5 homologues (Ueda et al. 2001, Bolte et al. 2004b), as well with prevacuolar compartment markers (Bolte et al. 2004b, Tse et al. 2004), has been observed.

Despite their attractiveness for endocytosis studies, one should be aware of the toxicity of the FM dyes (Bolte et al. 2004a, Meckel et al. 2004), and of inconsistencies in the results obtained with the different dyes. Although structurally similar, the FM dyes differ in charge and different labelling patterns have been recorded both in fungal (Fischer-Parton et al. 2000) and plant cells (Meckel et al. 2004). In contrast to FM 4-64, FM 1-43 and FM 2-10 label mitochondria in addition to putative endosomal intermediates (Meckel et al. 2004). According to the latter authors, mitochondrial staining with FM 1-43 and FM 2-10 can only occur if these less hydrophobic dye forms escape from the membranes of early endocytic compartments and enter the cytosol, from where they are taken up by the mitochondria. Finally, there are curious cases where FM 4-64 is taken up by the PM but does not internalize, although the cells in question are known to be secretion competent (Fig. 1d).

Biotinylated markers: markers for receptor-mediated endocytosis?

Biotinylated proteins have been found to enter plant cells by a process with the characteristics of RME. Originally tested in cultured soybean cells (*Glycine max* Merr. cv. Kent) (Horn et al. 1990, 1992; Low et al. 1993; Low and Chandra 1994), they have been also successfully used as markers for RME in cultured rice cells (*Oryza sativa* cv. Taipei) (Bahaji et al. 2001, 2003) and also in protoplasts isolated from either rice or *Arabidopsis thaliana* or cultured BY2 cells (Elena Ortiz-Zapater and Fernando Aniento, unpubl. observations), indicating their suitability as markers for RME in a wide range of suspension-cultured cell types. Horn et al. (1990) showed temperature-dependent uptake of fluorescein isothiocyanate-labelled biotinylated insulin and hemoglobin in cultured soybean cells by immunofluorescence, while nonbiotinylated controls did not bind to the cells nor were internalized to any measurable extent. ^{125}I-labeled biotinylated RNase A was used to measure kinetics of uptake. It was found that the uptake of biotinylated proteins was 50-fold higher than that of nonbiotinylated controls but reached saturation with time, possibly due to the internalization or down-regulation of available receptors. The same saturation kinetics has been obtained when looking at the uptake of biotinylated BSA in cultured rice cells (Bahaji et al. 2001). Free biotin can compete with biotinylated proteins in the uptake process (Horn et al. 1990, Bahaji et al. 2001), and the uptake of biotinylated BSA has been shown to saturate with the concentration of the marker, both in soybean (Low et al. 1993) and in rice cells (Bahaji et al. 2001). Biotinylated proteins have also been found to be specifically internalized in isolated protoplasts (either from rice or *Arabidopsis thaliana* or BY2 suspension cultures), in a time- and temperature-dependent process. Therefore, the uptake of biotinylated proteins in plant cell suspensions has all the characteristics of RME. There is, however, a discrepancy concerning the final destination of the internalized markers. While internalized biotinylated horseradish peroxidase in cultured soybean cells has been proposed to be delivered predominantly into the cytoplasm (Low et al. 1993), biotinylated BSA internalized in cultured rice cells was undetectable in the cytosolic fraction and found predominantly in intracellular membranes (Bahaji et al. 2001).

Experiments with isolated protoplasts have also shown the utility of biotin-fluorescein as a marker for RME. In contrast to fluorescein, biotin-fluorescein has been observed to enter *Arabidopsis thaliana* protoplasts in a temperature-dependent manner, to be found in cytosolic vesicular structures (possibly endosomal compartments), and to end up in the central vacuole. These observations now open up the possibility of following the transit of endocytosed markers through endosomal compartments by fluorescence microscopy (Elena Ortiz-Zapater and Fernando Aniento, unpubl. observations).

Inhibitors

Wortmannin

The drug wortmannin is known to block protein transport to the vacuole in plant cells (Matsuoka et al. 1995, DiSebastiano et al. 1998). Emans et al. (2002) showed that it severely reduces the uptake of the dye FM 1-43 by tobacco BY2 cells. Wortmannin is a well-known inhibitor of phosphoinositol-3 kinase activity in both animal (Vanhaesebroeck et al. 1997) and plant cells (Matsuoka et al. 1995). In animal cells, it causes multivesicular endosomes to dilate and lose their internal vesicles (Bright et al. 2001, Sachse et al. 2002). Recently, Tse et al. (2004) have been able to demonstrate that the target of wortmannin action in plant cells is also a multivesiculate organelle which lies at the junction of the biosynthetic and endocytic pathways to the vacuole. The morphological response to the drug is similar to that seen in mammalian cells. Thus, wortmannin can be regarded as an endocytosis inhibitor, but very much downstream of the internalization event at the PM.

Brefeldin A

The fungal metabolite brefeldin A (BFA) interferes with vesicle-mediated protein trafficking by binding to the complex formed between the small GTPase ARF and its guanidine nucleotide exchange factor (Jackson and Casanova 2000). There are 9–12 ARF GTPases, and 8 ARF-GEFs in the *Arabidopsis thaliana* database (Jürgens 2004). Some ARF-GEFs are BFA-resistant and BFA sensitivity can be designed by exchanging specific amino acids in the BFA-binding pocket (Geldner et al. 2003). One BFA-sensitive ARF-GEF is GNOM, which is necessary for the ordered localization of PIN1, an auxin efflux carrier located at the PM (Steinmann et al. 1999). After BFA addition, PIN1, as well as other PM proteins, becomes localized in an enlarged endosome-derived compartment termed the "BFA compartment" (Geldner et al. 2001). Although the composition of this compartment is not entirely clear (see Šamaj et al. 2004), it is of significance that it stains positively with AtARF1 antibodies (Baluška et al. 2002, Couchy et al. 2003). Thus, in addition to its more familiar effects on the early secretory pathway (reviewed by Nebenführ et al. 2002), it is now well established that BFA can interfere with the recycling of proteins between endosomal compartments and the PM. As stressed by Geldner (2004: p. 554), "... the action of BFA on the endomembrane system is determined by the presence, localization and relative concentration of resistant and sensitive ARF/ARF-GEF complexes in the cell". BFA is therefore by no means a specific endocytosis inhibitor, and its effects on endocytosis in some plants may even be secondary, but it remains a useful diagnosic tool.

Cytoskeleton inhibitors

There is now a large body of evidence from yeast and mammalian cells implicating the actin cytoskeleton in endocytosis (see review by Engqvist-Goldstein and Drubin 2003). Indeed, it would seem that localized domains of endocytic activity at the PM act as organizing centers for actin assembly (Engqvist-Goldstein et al. 2004). Many, but not all of the molecules known to link the actin cytoskeleton to the clathrin-endocytic machinery (dynamins, profilins, ARP2/3 complex) are present in plants (Holstein 2002, Hussey et al. 2002). However, the current evidence in support of a role of the actin cytoskeleton in plant endocytosis is circumstantial and is mainly based on drug studies (reviewed by Šamaj et al. 2004). The actin-depolymerizing agents cytochalasin D and latrunculin B inhibit the formation of BFA compartments (see below) in which cell wall pectins and PM proteins accumulate (Geldner et al. 2001, Baluška et al. 2002, Yu et al. 2002). Cytochalasin D treatment also perturbs the endocytic uptake of sterols, in a manner similar to that observed in the actin2 mutant of *Arabidopsis thaliana* (Grebe et al. 2003). It has also been recently shown that probenecid-insensitive LY uptake in maize root cells is inhibited by latrunculin B and the myosin inhibitor 2,3-butanedione monoxime (Baluška et al. 2004).

Tyrphostins

Tyrphostins are structural analogs of tyrosine (Fig. 2). They were initially developed as substrate-competitive inhibitors of the epidermal growth factor receptor tyrosine kinase (Yaish et al. 1988, Lyall et al. 1989, Gazit et al. 1989). Tyrphostins have subsequently been used to investigate the physiological role of many different tyrosine kinases. Some tyrphostins have also been reported to inhibit endocytosis and autophagy (Holen et al. 1995) and vesicle formation from the trans-Golgi network (Austin and Shields 1996), thus implying a possible role for tyrosine kinases in these processes. Because of the increasing interest in receptor-kinases at the plant PM, and their possible internalization via clathrin-coated vesicles, tyrphostins may well prove to be a useful diagnostic tool for those wanting to test for endocytosis in plants. Since they are not well known in plant circles, we present here a more detailed overview of the tyrphostins.

The internalization of many membrane proteins depends on sorting motifs within their cytosolic domains. One such motif contains a critical tyrosine residue within the sequence YXXϕ, where ϕ represents a bulky hydrophobic

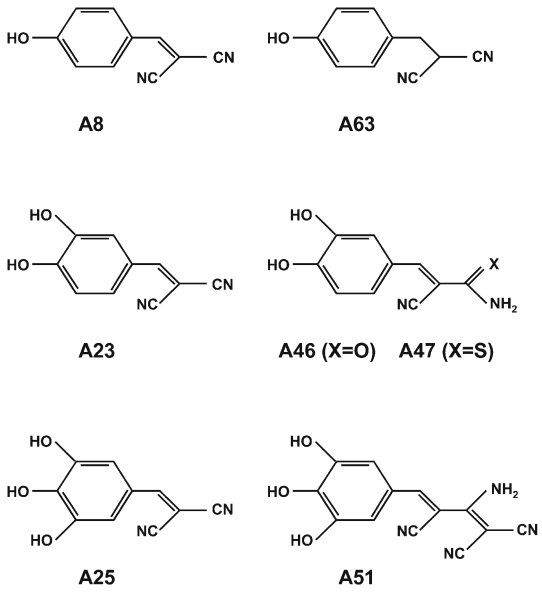

Fig. 2. Structures of tyrphostins used to interfere with RME

residue (Trowbridge et al. 1993, Marks et al. 1997). Examples of these motifs are the YTRF endocytosis signal of the human transferrin receptor or the YQRL motif of TGN38. Tyrosine-based motifs conforming to this consensus sequence can interact directly with the medium (μ) chain subunits of heterotetrameric adaptor complexes (AP) involved in several intracellular trafficking pathways (for a review, see Kirchhausen 1999). The AP-2 adaptor complex facilitates incorporation of transmembrane proteins containing YXXϕ motifs into clathrin-coated vesicles at the PM (Kirchhausen 1999 and references therein). The μ subunit from all four adaptor complexes has been shown to interact with the YXXϕ motif, with the precise sequence and context of this motif determining the specificity of the interaction (Dell'Angelica et al. 1997, Stephens and Banting 1998). These interactions are critically dependent on the tyrosine residue (Ohno et al. 1995, Boll et al. 1996, Stephens et al. 1997). Structural studies on peptides containing these motifs have shown that the critical tyrosine residue projects away from the peptide backbone, residing in either a tight turn or an α-helix structure (Collawn et al. 1989, Eberle et al. 1991, Wilde et al. 1994, Owen and Evans 1998).

The YXXϕ internalization motif is remarkably similar to sequences in which the tyrosine residue can be phosphorylated and, once phosphorylated, bind to Src homology 2 (SH2) domains (Zhou et al. 1993). It is now clear that although both tyrosine kinases and medium subunits of adaptor complexes recognize essentially the same motif, the two can be discriminated in that very few tyrosine-based motifs which have been shown to interact with μ chains can also act as substrates for tyrosine kinases (Stephens and Banting 1997, Shiratori et al. 1997, Chuang et al. 1997). Furthermore, although both μ chains and tyrosine kinases accommodate the tyrosine side chains as part of their interaction with the YXXϕ motif, there is no great similarity between the YXXϕ binding sites on μ chains and those on tyrosine kinases (Owen and Evans 1998).

Molecular modelling of tyrphostins into the μ2 tyrosine-binding pocket has revealed that the phenyl rings of tyrphostins A8, A23, A46, and A47 are accommodated within the tyrosine-binding cleft in μ2, whereas those of tyrphostins A25 and A51 are not (Banbury et al. 2003). Tyrphostins A8 and A63 are monohydroxylated at position 4 of the phenyl ring in their structure (i.e., the same as tyrosine side chain); tyrphostins A23, A46, and A47 are 3,4-dihydroxylated on their phenyl ring; and tyrphostins A25 and A51 are 3,4,5-trihydroxyphenyl compounds (Fig. 2). The reason for the failure of tyrphostins with three-ring hydroxyl groups (e.g., A25 and A51) to inhibit the interaction between YXXϕ motifs and μ2 results from the fact that the third hydroxyl group would necessarily be forced into the hydrophobic part of the cleft (Banbury et al. 2003). Thus, it appears that the tyrosine-binding cleft in μ2 can accommodate a 3,4-dihydroxy derivative, but not a 3,4,5-trihydroxy derivative, of a phenyl ring. In fact, addition of an extra hydroxyl group in the 3-position of the phenyl ring is beneficial for the interaction with μ2. Thus, a 3,4-dihydroxyphenyl compound (such as tyrphostin A23 or A46) is predicted, by molecular modelling studies, to both fit well in the tyrosine-binding cleft of μ2 and be stabilized in that binding by hydrogen bonding and other interactions (Banbury et al. 2003). This is in agreement with in vitro studies showing that tyrphostin A23 inhibits the interaction between μ2 and a YXXϕ motif more efficiently than tyrphostin A8 (which is structurally identical to tyrphostin A23 apart from the fact that A8 is a 4-monohydroxyphenyl compound and A23 is a 3,4-dihydroxyphenyl compound) (Crump et al. 1998).

By inhibiting the interaction between μ2 and YXXϕ motifs, tyrphostins are potentially very useful and specific inhibitors of RME, provided that the receptor molecule involved in the process contains a YXXϕ internalization signal in its cytosolic domain. Although this is not the only internalization signal described so far, it is one of the most widely distributed among PM receptors internalized by endocytosis in mammalian cells (Kirchhausen 1999). One of the best characterized is the YTRF motif found in the cytosolic tail of the human transferrin receptor, which has been shown to interact with the μ2 subunit of the AP2 complex (Collawn et al. 1989, 1993). Therefore, the hTfnR has been a good candidate to test the effects of tyrphostins. In a very elegant study, tyrphostin A23 has been shown to inhibit the internalization of the transferrin receptor, without having discernible effects on the morphology of endosomal compartments. However, control tyrphostins, like A51 or A63, which also inhibit tyrosine kinase activity but are incapable of inhibiting the YXXϕ–μ2 interaction, did not inhibit transferrin receptor internalization. These data indicate that A23 can specifically interfere with the interaction of the medium subunit of the AP-2 adaptor complex and YXXϕ motifs in intact cells (Banbury et al. 2003).

Preliminary experiments suggest that tyrphostins may also function to inhibit RME in plant cells. In this respect, tyrphostin A23 (but not tyrphostin A51) has been found to inhibit the internalization of the human transferrin receptor transiently expressed in *Arabidopsis thaliana* protoplasts (Elena Ortiz-Zapater, Dolores Ortiz, Fernando Aniento, and María Jesús Marcote, unpubl. observations), suggesting that the endocytosis signal in the cytosolic tail of this receptor is recognized by the endocytic machinery of plant cells.

The same differential effect of tyrphostins has been observed in the internalization of biotinylated markers (Elena Ortiz-Zapater and Fernando Aniento, unpubl. observations), suggesting that the process may involve a PM receptor containing a tyrosine-based endocytosis signal.

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