

Distinct in vitro interaction pattern of dopamine receptor subtypes with adaptor proteins involved in post-endocytotic receptor targeting

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Abstract The mechanisms underlying targeted sorting of endocytosed receptors for recycling to the plasma membrane or degradation in lysosomes are poorly understood. In this report, the C-terminal tails of the five dopamine receptors (D1–D5) were expressed as glutathione *S*-transferase (GST) fusion proteins and studied for their interaction with ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50) and *N*-ethylmaleimide-sensitive factor (NSF), which are known to be involved in post-endocytic recycling of receptors back to the plasma membrane, and with sorting nexin 1 (SNX1), known to be involved in targeting receptors to lysosomal degradation. EBP50 did not bind any of the dopamine receptor tails. NSF bound strongly to D1 and D5 and only weakly to D2, D3 and D4. However, SNX1 clearly distinguished between D1 and D5, as only D5 bound strongly to this protein. This report shows that there are distinct interaction patterns for NSF and SNX1 to the various dopamine receptor subtypes.

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Key words: Dopamine receptor; Sorting nexin 1; Ezrin–radixin–moesin-binding phosphoprotein 50; *N*-Ethylmaleimide-sensitive factor; Protein sorting

1. Introduction

Cloning of receptors for transmitters and hormones in the eighties and nineties showed that the vast majority belong to the G protein-coupled seven transmembrane segment (7TM) receptor superfamily [1]. Although it was expected for many transmitters that there would be receptor subtypes, the cloning effort resulted in a surprisingly large number of these, for example: five homologous but distinct muscarinic acetylcholine receptors, four different histamine receptors, five different somatostatin receptors and even 15 different serotonin, 5HT receptors. In many cases we still do not have a good functional ‘reason’ for the development of all these receptor subtypes. Although receptor subtypes may bind synthetic ligands or drugs differently, they often recognize the endogenous li-

gand with rather similar affinity. Different receptor subtypes provide the possibility for a given transmitter or hormone to utilize different signalling pathways. However, this specialization does not explain why, for example, there are two homologous dopamine receptors: D1 and D5 with long C-terminal tails that couple to G_{α_s} , and three other, mutually homolo-

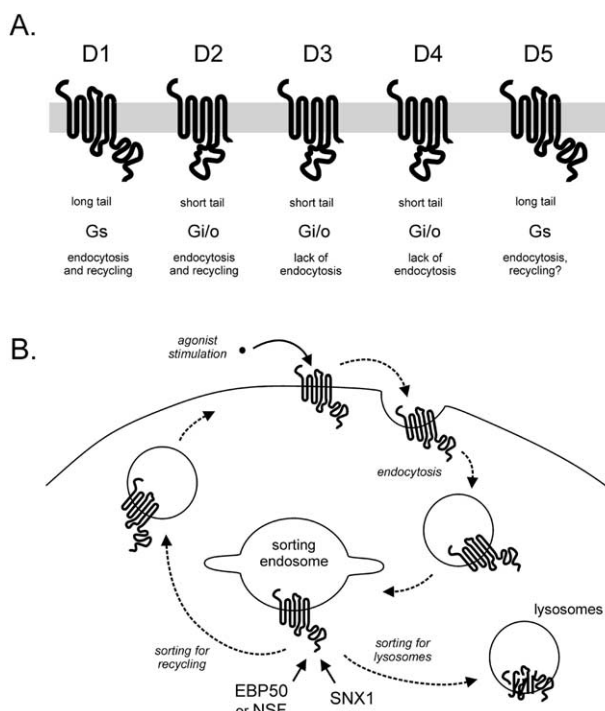


Fig. 1. The D1-like (D1, D5) and D2-like (D2, D3, D4) 7TM receptors have different signaling and endocytotic properties. A: Schematic representation of the dopamine receptors in the plasma membrane. D1, D2 and D5 are endocytosed upon agonist stimulation and D1 and D2 are known to recycle back to the plasma membrane. D3 and D4 are not endocytosed upon agonist stimulation. B: Following agonist stimulation, many 7TM receptors are desensitized and internalized into endosomes. In the sorting endosome, proteins bind to the C-terminal tail of the 7TM receptor and either recycle the receptor back to the plasma membrane (such as EBP50 and NSF in β_2 AR recycling) or target the receptor for degradation in lysosomes (such as SNX1 in PAR1 receptor downregulation).

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gous dopamine receptors: D2, D3 and D4 with short C-terminal tails that couple to $G\alpha_{i/o}$ [2] (Fig. 1).

Although G proteins are key players in the function of 7TM receptors, a number of other accessory proteins such as scaffolding and adaptor proteins are also very important in, for example, creating the infrastructure in which the receptors and their downstream effector molecules function. Binding domains in such adaptor proteins interact with recognition motifs often located in the C-terminal tails of the receptors [3,4]. Interestingly, the structural homology among receptor subtypes is highest in the transmembrane segments, whereas especially the C-terminal tail regions can vary considerably. Thus, it could be argued that one of the as yet unnoticed differentiating properties of many receptor subtypes could be that they differ in their interactions with scaffolding and adaptor proteins. One important group of adaptor proteins is involved in the post-endocytotic sorting of receptors either in recycling back to the cell surface, which is the predominating pathway, or in the targeting to lysosomes for degradation. Recently, two proteins have been proposed to be involved in the recycling of the β_2 -adrenergic receptor (β_2 AR). Ezrin–radixin–moesin-binding phosphoprotein 50 (EBP50) is the human homolog of rabbit NHERF (Na^+/H^+ -exchanger regulatory factor) and binds to the cytoplasmic tail of the β_2 AR through a PDZ domain [5]. This interaction and hence the recycling of the receptor back to the membrane is regulated by G protein-coupled receptor kinase 5 (GRK5) phosphorylation of the receptor [5]. *N*-Ethylmaleimide-sensitive factor (NSF), an ATPase involved in membrane vesicle trafficking, also binds to the cytoplasmic tail of the β_2 AR and this interaction has been suggested to be necessary for both internalization and recycling of the receptor [6]. Another protein, sorting nexin 1 (SNX1), which was originally identified to interact with epidermal growth factor receptor was recently shown to be responsible for the efficient targeting of the thrombin receptor PAR1 (protease-activated receptor 1) from sorting endosomes to lysosomes [7]. In the present study we investigate the binding of the purified C-terminal tails of all five dopamine receptors with EBP50, NSF and SNX1.

2. Materials and methods

2.1. Materials

cDNA encoding rat NSF was provided by Jim Rothman by way of Bob Lefkowitz, human EBP50 cDNA was provided by Mark von Zastrow, and the cDNAs for the human D1 to D5 receptors were provided by Hubert H.M. Van Tol.

2.2. Construction of GST fusion protein expression clones

To generate fusion proteins encoding the receptor C-terminal tails and glutathione *S*-transferase (GST), the D1[334–446], D2[430–443], D3[389–400], D4[450–467], D5[361–477] and β_2 -adrenergic[330–413] receptor tails were amplified by PCR, digested with *Bam*HI and *Xho*I and ligated into the pGEX-4T-1 vector. Whereas non-fused GST consists of 239 amino acids, the fusion constructs consist of the first 224 amino acids of GST (including the *Bam*HI site) followed by the receptor C-terminal tails. This means that the molecular weights of the GST tail fusions of D2, D3 and D4 with short C-terminal tails are similar to the molecular weight of non-fused GST (Fig. 2). Plasmids were transformed into XL1-Blue bacteria and the DNA sequence verified by automated sequencing. Plasmids were transformed into the *Escherichia coli* strain BL21 for protein expression. Bacterial fusion protein production was induced by addition of 0.2 mM isopropyl-1-thio- β -D-galactopyranoside for 4 h at 37°C. Fusion proteins were purified on glutathione Sepharose 4B beads as described by the manufacturer (Amersham Pharmacia). The amount

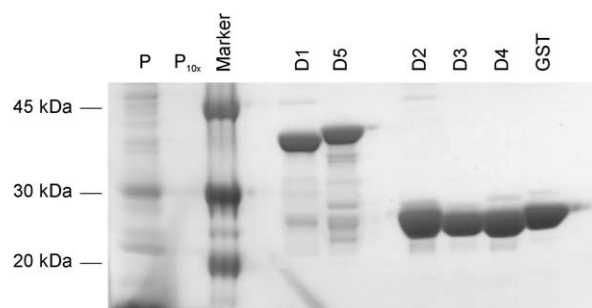


Fig. 2. Purified dopamine receptor C-terminal tails fused to GST. Proteins were visualized by Coomassie staining. Theoretical molecular weights are GST–D1 (38.6 kDa), GST–D5 (39.5 kDa), GST–D2 (28.0 kDa), GST–D3 (27.8 kDa), GST–D4 (28.5 kDa) and GST (27.9 kDa) (see Section 2).

of fusion protein on the beads was determined by comparison with BSA standards on Coomassie-stained Bis–Tris polyacrylamide gel electrophoresis (PAGE) gels (12% NuPage Bis–Tris gel, Invitrogen).

2.3. Synthesis of 35 S-labeled sorting proteins

[35 S]Methionine was incorporated into full-length EBP50, NSF and SNX1 in a coupled *in vitro* transcription and translation reaction according to the manufacturer's instructions (Rabbit Reticulocyte Lysate System, Promega L5010).

2.4. GST fusion protein binding assay

GST fusion protein on beads (3 μ g on 15 μ l settled beads, adjusted with empty beads) was incubated with 500 μ l blocking buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 1% ovalbumin) for 30 min at room temperature. Beads were collected by centrifugation (500 $\times$ g for 5 min) and 2 μ l of the *in vitro* translation reaction mixture was added along with 18 μ l wash buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 0.1% Triton X-100). This mixture was vigorously mixed for 1 h at room temperature. Beads were washed three times for 5 min in ice-cold wash buffer and collected by centrifugation, eluted in 4 $\times$ sodium dodecyl sulfate sample buffer, and separated by Bis–Tris PAGE (12% NuPage Bis–Tris gel, Invitrogen). Gels were Coomassie stained and dried over night (DryEase Gel Drying System, Invitrogen). Fig. 2 shows that each of the dopamine tail fusion constructs were present in roughly equal amounts. Finally, radioactive bands on the gel were visualized on a phosphor imager screen (Molecular Dynamics) and developed in a molecular imager (BioRad). Quantitative determination of radioactive band intensities was done with molecular imager software (BioRad) using background subtraction. Band intensities were subsequently normalized to the band intensity of the reference lane that was loaded with 2 μ l of the *in vitro* translation reaction.

3. Results

3.1. EBP50 binding

EBP50 has been reported to bind to the β_2 AR [5]. We confirmed this interaction in our system as the *in vitro* 35 S-labelled EBP50 protein bound strongly to the GST fusion of the tail from the β_2 AR (Fig. 3). In contrast, the five different GST-fused C-terminal tails from the dopamine receptors did not pull down any significant amount of 35 S-labelled EBP50 protein when compared with GST alone (Fig. 3). This suggests that EBP50 is not involved in the recycling of any of the five dopamine receptor subtypes.

3.2. NSF binding

35 S-labeled NSF protein bound strongly to the GST-fused C-terminal tails of both the D1 and the D5 receptors (Fig. 4). The GST fusions of the tails from the D2-like receptors, i.e. D2, D3, and D4, did not pull down the 35 S-labeled NSF

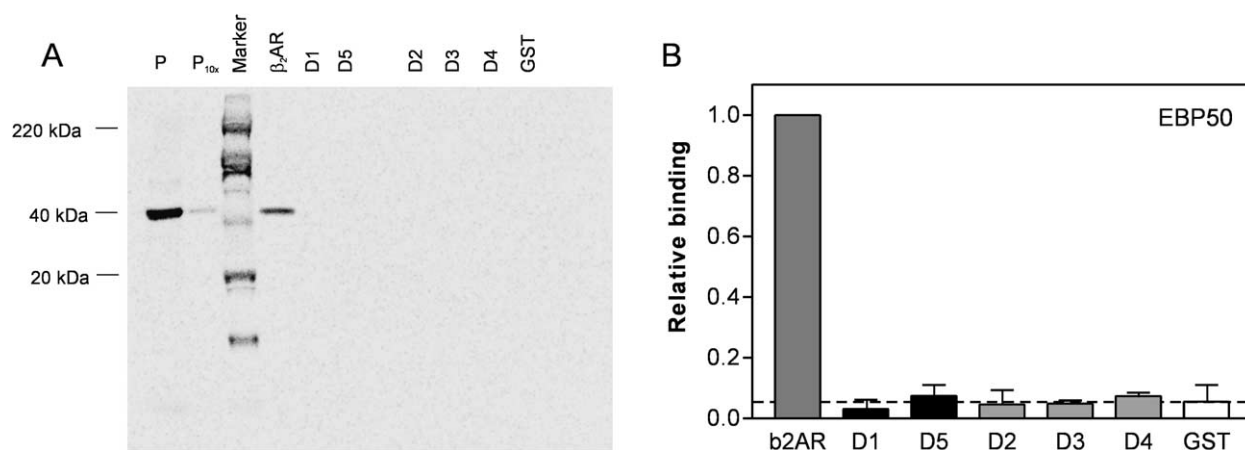


Fig. 3. Dopamine receptor C-terminals do not associate with EBP50 in GST pull-down assays. A: In vitro interaction of the C-terminus of the β_2 AR and the dopamine receptors with 35 S-labeled EBP50. Proteins were resolved by PAGE and radioactive bands visualized on a phosphor imaging screen. The P and P_{10x} lanes were loaded with 2 and 0.2 μ l respectively of the in vitro translation reaction. B: Band intensities were normalized with the band intensity of the β_2 AR lane. The dashed line corresponds to the relative binding of EBP50 to GST protein alone. Results are representative of three independent pull-down experiments.

protein as efficiently as the D1 and D5 tail fusions did. However, the D2-like receptors were able to pull down the 35 S-labeled NSF protein more efficiently than GST alone (Fig. 4). This was clearly not the case when 35 S-labeled EBP50 was used (Fig. 3). Thus, NSF binds efficiently to the homologous D1 and D5 receptors, but only weakly to the D2-like receptors and NSF could therefore possibly be involved in the recycling of especially the D1-like receptors.

3.3. SNX1 binding

SNX1 in contrast to EBP50 and NSF is believed to be involved in lysosomal targeting of receptors. It binds strongly to the C-terminal tail of the PAR1 receptor and targets it for degradation [7]. As shown in Fig. 5, we found that among the dopamine receptors, the 35 S-labeled SNX1 protein bound strongly only to the D5 receptor C-terminal tail. A weak interaction was observed with the tail of the D1 and possibly the D3 receptor (Fig. 5). These findings suggest the D5 receptor, like the PAR1 receptor, may be sorted to lysosomal degradation by SNX1.

4. Discussion

In the present study we have characterized the interaction pattern of all five dopamine receptors with three proteins that have been proposed to be involved in the post-endocytotic sorting of receptors to be either recycled or targeted for degradation. We find that the tails from the different subtypes of receptors interact differently with the adaptor proteins and that, for example, the two homologous D1-like receptors, which both signal through the same signal transduction pathway, differ in their interaction with SNX1, which binds strongly to the D5 receptor as opposed to the D1 receptor.

4.1. Internalization and recycling of dopamine receptors

Detailed information concerning internalization and post-endocytotic events are mainly available for the D1, D2 and D3 receptors (as summarized in Fig. 1). Initially, basic observations concerning desensitization etc. of the receptors were described [8,9]. However, also more direct observations of the agonist-induced endocytosis and recycling of D1 and D2 have

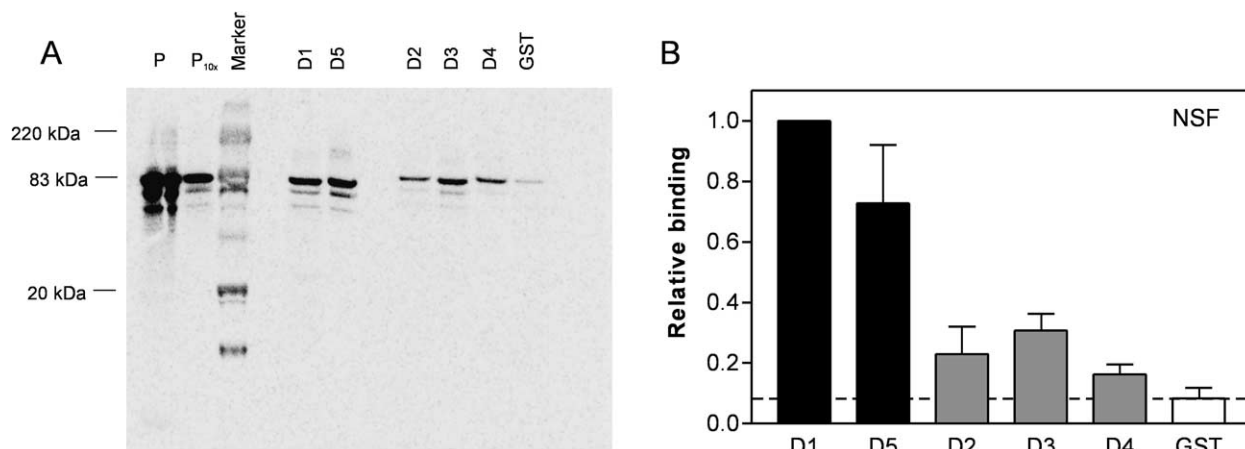


Fig. 4. Dopamine receptor C-terminals associate differently with NSF in GST pull-down assays. A: In vitro interaction of the C-terminus of the dopamine receptors with 35 S-labeled NSF. Proteins were resolved by PAGE and radioactive bands visualized on a phosphor imaging screen. The P and P_{10x} lanes were loaded with 2 and 0.2 μ l respectively of the in vitro translation reaction. B: Band intensities were normalized with the band intensity of the D1 lane. The dashed line corresponds to the relative binding of NSF to GST protein alone. Results are representative of three independent pull-down experiments.

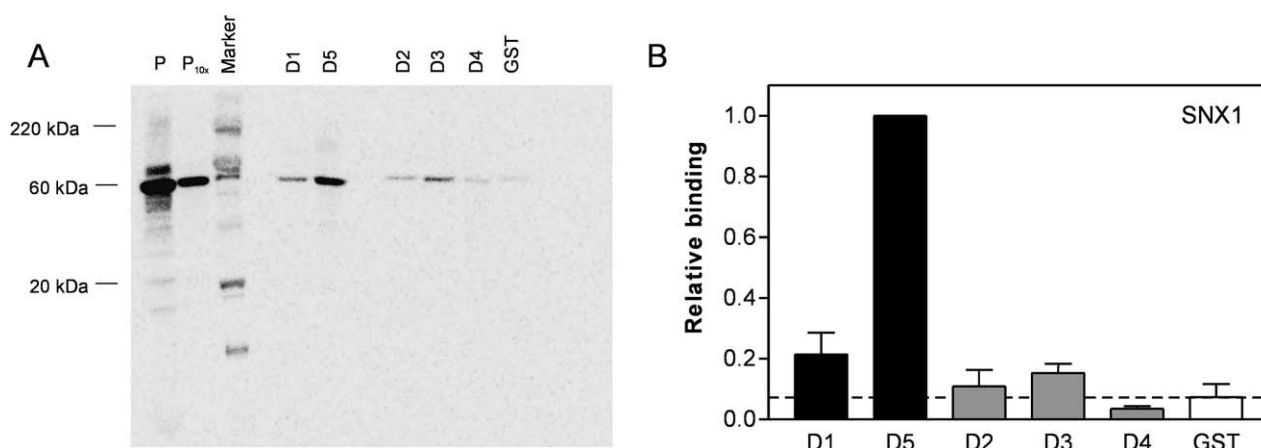


Fig. 5. Dopamine receptor C-termini associate differently with SNX1 in GST pull-down assays. A: In vitro interaction of the C-terminus of the dopamine receptors with ^{35}S -labeled SNX1. Proteins were resolved by PAGE and radioactive bands visualized on a phosphor imaging screen. The P and P_{10x} lanes were loaded with 2 and 0.2 μl respectively of the in vitro translation reaction. B: Band intensities were normalized with the band intensity of the D5 lane. The dashed line corresponds to the relative binding of SNX1 to GST protein alone. Results are representative of three independent pull-down experiments.

been reported [10]. Analysis of surface biotinylated receptors demonstrated that both the D1 and the D2 receptors after endocytosis were efficiently recycled to the plasma membrane and with similar kinetics [10]. Interestingly, when studied in Neuro2A neuroblastoma cells, the D1 and the D2 receptors were targeted to different endocytic vesicles. In this system it appeared that the D2 receptor to a certain degree was constitutively internalized. Compared to the D2 receptor, the homologous D3 receptor is much less phosphorylated upon agonist stimulation, has a very low ability to mobilize arrestin to the plasma membrane and does not undergo agonist-induced internalization [11]. The molecular pharmacological phenotype of the D2 and D3 receptors could be reversed with respect to endocytosis through exchange of the second and third intracellular loops of these two receptors [11]. The D4 receptor does not internalize upon agonist stimulation [12].

4.2. Protein interaction with dopamine receptors

A number of proteins have been shown to interact with the intracellular domains of the dopamine receptors, for example: ABP-280 (actin-binding protein 280) [13,14], spinophilin [15], and a novel protein called DRiP78 (dopamine receptor interacting protein of 78 kDa), which appears to be involved in receptor transport from the endoplasmic reticulum to the cell surface [16]. By yeast two-hybrid assays, neurofilament-M was recently shown to bind to the intracellular loop 3 of the D1 and D5 receptors, but not to the D2-like receptors [17]. It was suggested that neurofilament-M is involved in cell surface expression and regulation of the D1-like receptors.

The present study adds NSF and SNX1 to the group of 'DRiPs', i.e. proteins that interact with the C-terminal tails of the dopamine receptors. Based on the previously proposed functional roles of these proteins, it is very likely that NSF and SNX1 are involved in endocytosis and in post-endocytotic sorting of the dopamine receptors, i.e. especially the D1 and D5 receptors. It is very interesting that a similar interaction is observed for the D1 and D5 receptors with NSF but that SNX1 exclusively interacts with the D5 receptor (Fig. 5). Very little is yet known about the cellular handling of the D5 receptor besides that it undergoes ligand-induced endocytosis similarly to the D1 receptor (Marc Caron, personal com-

munication). Our results could suggest that after internalization, the D5 receptor may in the right cellular setting be sorted to the lysosomal pathway in a similar way as the PAR1 receptor [7]. Within neurons, the two D1-like receptors are distributed differently as the D1 receptor is found predominantly in dendritic spines as opposed to the D5 receptor, which accumulates in dendritic shafts [18,19]. Whether this difference has anything to do with the differential interaction of the D1 and D5 receptors with SNX1 remains to be investigated.

4.3. Biochemical versus cell biological studies

The biochemical studies in the present report obviously cannot substitute for thorough cell biological studies of the post-endocytotic sorting process as such. Thus, the present biochemical studies should function as inspiration for subsequent direct cellular studies. However, certain issues require that a direct biochemical approach be taken – at least initially. For example, it is very hard to map on a receptor a recognition motif for an adaptor molecule involved in the post-endocytotic sorting by monitoring the cell biological event as such because there may be an overlap with a recognition motif for another adaptor protein involved in an earlier part of the overall process. For example, if a mutation eliminates arrestin binding, endocytosis will be blocked, and the effect of the mutation on the binding and function of a downstream adaptor protein cannot be determined.

It should be noted that the interaction between receptor and adaptor protein is highly regulated by receptor phosphorylation. This is the case for the EBP50/ $\beta_2\text{AR}$ interaction which is specifically disrupted as a result of GRK5-mediated receptor phosphorylation [5]. In the dopamine receptor field, it has been demonstrated that protein kinase A phosphorylation of a specific threonine residue in intracellular loop 3 regulates a late step in the post-endocytotic targeting of the receptor to a perinuclear region but is not required for the initial ligand-mediated internalization as such [20].

Post-endocytotic sorting of receptors either to the rapid recycling pathway or to lysosomes probably cannot be explained by the interaction of the receptor tails or intracellular loops with a single adaptor protein. It is likely that several

proteins are involved in this sorting process and that the end result for a given receptor depends on multiple factors including the competition of several adaptor proteins at several locations in the endocytotic pathway. It should be noted that GST pull-down assays require binding affinities in the range of 100 nM to detect binding, and therefore some low-affinity protein–protein interactions may escape detection by this approach. Recently a new protein called GASP (G protein-coupled receptor-associated sorting protein) was described by Whistler and co-workers, which should be added to the list of proteins involved in this important process [21].

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