A Library of 7TM Receptor C-terminal Tails

INTERACTIONS WITH THE PROPOSED POST-ENDOCYTIC SORTING PROTEINS ERM-BINDING PHOSPHOPROTEIN 50 (EBP50), N-ETHYLMALEIMIDE-SENSITIVE FACTOR (NSF), SORTING NEXIN 1 (SNX1), AND G PROTEIN-COUPLED RECEPTOR-ASSOCIATED SORTING PROTEIN (GASP)*

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Adaptor and scaffolding proteins determine the cellular targeting, the spatial, and thereby the functional association of G protein-coupled seven-transmembrane receptors with co-receptors, transducers, and downstream effectors and the adaptors determine post-signaling events such as receptor sequestration through interactions, mainly with the C-terminal intracellular tails of the receptors. A library of tails from 59 representative members of the super family of seven-transmembrane receptors was probed as glutathione S-transferase fusion proteins for interactions with four different adaptor proteins previously proposed to be involved in post-endocytic sorting of receptors. Of the two proteins suggested to target receptors for recycling to the cell membrane, which is the route believed to be taken by a majority of receptors, ERM (ezrin-radixin-moesin)-binding phosphoprotein 50 (EBP50) bound only a single receptor tail, i.e. the β2-adrenergic receptor, whereas N-ethylmaleimide-sensitive factor bound 11 of the tail-fusion proteins. Of the two proteins proposed to target receptors for lysosomal degradation, sorting nexin 1 (SNX1) bound 10 and the C-terminal domain of G protein-coupled receptor-associated sorting protein bound 23 of the 59 tail proteins. Surface plasmon resonance analysis of the binding kinetics of selected hits from the glutathione S-transferase pull-down experiments, i.e. the tails of the virally encoded receptor US28 and the δ-opioid receptor, confirmed the expected nanomolar affinities for interaction with SNX1. Truncations of the NK1 receptor revealed that an extended binding epitope is responsible for the interaction with both SNX1 and G protein-coupled receptor-associated sorting protein as well as with N-ethylmaleimide-sensitive factor. It is concluded that the tail library provides useful information on the general importance of certain adaptor proteins, for example, in this case, ruling out EBP50 as being a broad spectrum-recycling adaptor.

Interaction of receptors with adaptor and scaffolding proteins is important for their biogenesis, their cellular sorting and targeting to the cell membrane, and their function at the membrane in complex with transducer molecules and downstream effector molecules as well as the subsequent internalization and post-endocytic sorting of the receptors (1, 2). These interactions among receptors, adaptors, and scaffolding proteins are highly regulated processes that can be controlled by phosphorylation events (3), expression of receptor activating, or inactivating variants of adaptor proteins (4), by competition among adaptor proteins, and by competition between adaptor proteins and effector molecules (5). For the large family of G protein-coupled seven-transmembrane segment receptors (7TM1 receptors), this field is still in its infancy and only a rather sketchy picture has emerged of relative importance of specific adaptor and scaffolding proteins for the biogenesis, function, and desensitization of these receptors. Methods such as yeast two-hybrid screening, co-immunoprecipitation, and affinity chromatography using immobilized receptor fragments as bait have been used to identify potential receptor-binding proteins. The proposed functional roles of these interacting proteins are very diverse. Examples include promotion or inhibition of agonist-induced receptor internalization (6–8), inhibition of mitogen-activated protein kinase activation (9), regulation of constitutive activity (10), retention of receptors in the endoplasmic reticulum (11, 12), coupling to second messenger systems (13–15), and spatial organization of synapses (16).

Thus, a number of cases have been described where a specific adaptor protein has been biochemically and/or functionally linked to a single or several related receptors. However, to what degree such interactions are of general importance for 7TM receptors or for specific subsets of receptors or, in fact, only a single or a few receptors is in most cases still unclear. To address the question of the importance of specific adaptor scaf-

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1 The abbreviations used are: 7TM, seven-transmembrane; GST, glutathione S-transferase; EBP50, ERM-binding phosphoprotein 50; SNX1, sorting nexin 1; GASP, G protein-coupled receptor-associated sorting protein; PAR1, protease-activated receptor 1; NSF, N-ethylmaleimide-sensitive factor; SPR, surface plasmon resonance; cGASP, C-terminal fragment GASP; 5-HT, 5-hydroxytryptamine; KOP, ρ-opioid; H, histamine; M, muscarinic; SST, somatostatin; V, vasopressin; OT, oxytocin; MC, melanocortin; MCH, melanin-concentrating hormone; AT1, angiotensin II receptor type 1; DOP, δ-opioid; MOP, μ-opioid; GLP, glucagon-like peptide; mGlut, metabotropic glutamate; GABA, γ-aminobutyric acid; GABAβ, γ-aminobutyric acid, type A; NK, tachykinin; D, dopamine; ERM, ezrin-radixin-moesin.
folding proteins for the function of 7TM receptors in general, we chose a systematic biochemical approach by establishing a library of 7TM receptor tails fused to glutathione S-transferase (GST). 7TM receptors expose several intracellular loops for potential interaction with intracellular proteins. However, it is especially the C-terminal tail of the receptors that interacts with adaptor and scaffolding proteins. Although, for example, intracellular loop 3 is critically involved in the recognition process between the receptor and transducer/effector molecules such as the heterotrimeric G proteins and arrestins, these proteins also interact with parts of the C-terminal receptor tail (26–30). Thus, the tail contains recognition sequences and epitopes for effector as well as scaffolding proteins. Besides the so-called "helix VIII" region, i.e., a relatively short, amphipathic, and helical segment located between the intracellular end of TM-VII and a frequently occurring palmitoylated Cys motif, very little information is available concerning the secondary and tertiary structures of 7TM receptor tails, which in the available x-ray structures have appeared to be rather unordered (31). Nevertheless, it is known that recognition motifs for adaptor and scaffolding proteins in the tails can be coiled-coil domains or C-terminally located PDZ recognition sequences (32, 33). In this study, proteins proposed to be involved in post-endocytic sorting of receptors were probed for interactions with the library of 7TM receptor tail-fusion proteins. The vast majority of 7TM receptors are internalized upon agonist stimulation. In the classical, arrestin-mediated pathway, the activated receptor is phosphorylated by G protein-coupled receptor kinases, which leads to recruitment of arrestin. Arrestin functions as an adaptor protein interacting with clathrin and AP2, thereby targeting the receptor to clathrin-coated pits and subsequent endocytosis. Following endocytosis, the receptors may enter one of two pathways (see Fig. 1). In the recycling pathway, which has been described for the β2-adrenergic receptor, the μ-opioid receptor, and the tachykinin NK1 receptor, the ligand dissociates in the acidic pH of the endosomal compartment and the receptor is dephosphorylated and subsequently returned to the plasma membrane. In contrast, in the lysosomal pathway used by the δ-opioid receptor and protease-activated receptor 1 (PAR1), the receptor is targeted for degradation in lysosomes. The mechanism behind this targeted sorting of receptors is poorly understood. However, a number of proteins have been proposed to govern the differential sorting event. ERM-binding phosphoprotein 50 (EBP50), also called Na+/H+ exchanger regulatory factor (NHERF) and N-ethylmaleimide-sensitive factor (NSF), have both been suggested to be responsible for the recycling of the β2-adrenergic receptor (21, 22). In contrast, sorting nexin 1 (SNX1), which originally was demonstrated to be required for the lysosomal sorting of the epidermal growth factor receptor, was recently suggested to be involved in the lysosomal sorting of PAR1 as well (24, 34). Protease-activated receptors are irreversibly activated by enzymatic digestion of the N-terminal segment of the receptor, and the sorting of activated receptors to lysosomes rather than recycling is critical for terminating signaling for these receptors. Another protein called G protein-coupled receptor-associated sorting protein (GASP) was recently suggested to be involved in the preferential lysosomal sorting of the δ-opioid receptor (23). As shown in Table I, the four proteins, EBP50, NSF, SNX1, and GASP, which have been proposed to function as adaptor proteins involved in the post-endocytic sorting of 7TM receptors, are structurally very different and have been implicated in various other cellular functions. Here, these proteins are probed for their ability to bind to the C-terminal tails of 59 different 7TM receptors as determined by GST pull-down assays, which routinely have been used to confirm protein interactions identified by co-immunoprecipitation and yeast two-hybrid screening (8, 23, 35–37). In selected cases, interactions were further studied by surface plasmon resonance (SPR) technology or the interaction was characterized in more detail through gradual deletion mutagenesis of the tail protein. 

**EXPERIMENTAL PROCEDURES**

**Materials**—Rat NSF cDNA was provided by Jim Rothman through Bob Lefkowitz (Duke University). Human EBP50 cDNA was provided by Mark von Zastrow (UCSF). Human SNX1, GASP, and cGASP have been described previously (23, 24). Human β2- and β1-adrenergic receptor-activating receptors were from Brian Kobilka (Stanford University). Human 5-hydroxytryptamine receptors 5-HT1A, 5-HT1D, and 5-HT1B, human histamine receptors H1, H2, and H3, and human κ-opioid (KOP) receptor were from Guthrie cDNA Resource Center (www.cdna.org). Human muscarinic acetylcholine receptors M1, M2, M3, M4, and M5 were from Tom I. Bonner (National Institutes of Health, Bethesda, MD). Human tachykinin receptor NK2 was from Norma Gerard (The Children's Hospital, Boston, MA). Human NK3 and NK2 receptors were from Jim Krause (Washington University School of Medicine, St. Louis, MO). Human somatostatin receptors SST1, SST2, SST3, SST5, and SST3 and SST5, and human neuropeptide receptor Y1 were from Carsten Stidsen (Novo Nordisk A/S, Målev, Denmark). Human vasopressin receptor V2 and human oxytocin (OT) receptor were provided by Claude Barberis (INSERM, Montpellier, France). Mouse melanocortin receptor MC4, human melanin-concentrating hormone receptors MCH1 and MCH2, and human ghrelin receptor were from Christian E. Elling (7TM Pharma). Human angiotensin II receptor type 1 (AT1) was from Hans T. Schambye (Maxygen, Hørsholm, Denmark). Human motilin receptor was from Bruce Conklin (UCSF). Mouse δ-opioid (DOP) and mouse μ-opioid (MOP) receptors were described previously (23). Human PAR1 and PAR2 were from Shaun R. Coughlin (UCSF). Human leukotriene LTβ3 receptor was cloned from a human cDNA library. Human chemokine receptors CXCR2 and CXCR4 and human cytomegalovirus chemokine receptors US28 and US27 were from Timothy N. C. Wells (Serono Pharmaceutical Research Institute, Geneva, Switzerland). Human chemokine receptors were from Kuldeep Neote (Pfizer, Groton, CT). Human herpesvirus 8 chemokine receptor ORF74 was from Mette M. Rosenkilde (University of Copenhagen, Copenhagen, Denmark). Human MAS1 oncogene receptor was from Michael R. Hanley (Cambridge University, Cambridge, United Kingdom). Human gastric inhibitory polypeptide receptor GIP1, rat glucagon-like peptide-1 receptor GLP1, rat secretin receptor, rat glucagon receptor, and rat vasoactive intestinal polypeptide receptor VIP1 were from Guthrie cDNA Resource Center (www.cdna.org).
TABLE I

Sorting proteins interacting with TM receptors

| Oligomer formation | Protein-protein interactions/complexes | Protein-protein interactions with receptors | Proposed role in receptor recycling/degradation | Location in cells |
|--------------------|-------------------------------------|---------------------------------------------|------------------------------------------------|------------------|
| Homooligomers and hetero-oligomers with NH2PF2 (59) | Ezrin (64), moesin (64), merlin (65), radixin (65), NHE3 (39), GRK6A (66), YAP65 (67), β-catenin (68), Go (69), Trp4, Trp5, PLC-β1, PLC-β3 (70), PAG (71), MRP2 (72), V-ATPase B1 (73), NBC7 (74) | DBAR (21), KOP (47), CFT (85), PTHIR (86), PDGRF (87), P2Y1 (88) | Recycling shown for βAR and KOP | Apical membrane of epithelial cells |
| Homohexamers (60, 61) | αSNAP (48), β-arrestin 1 (75), Rab3, Rab4, Rab6 (76), GABARAP (77, 78), GATE-16 (79, 80) | βAR (22), AMPA-R (89–93) | Recycling shown for βAR and AMPA-R | Both cytosolic and membrane-associated |
| Homohexamers and hetero-oligomers with NH2PF2 (59) | αSNAP (48), β-arrestin 1 (75), Rab3, Rab4, Rab6 (76), GABARAP (77, 78), GATE-16 (79, 80) | EGFR (34), PAR1 (24), insulin-R (63), leptin-R (63), PDGFR (63), transferrin-R (63, 94) | Lysosomal degradation shown for EGFR and PAR1 | Endosomal membranes and cytosol |
| Not known | Hrs (81), hVPS35 (82), SNX6 (83), SNX15 (84) | DOP (23), βAR (23), αmAR (23), Di (23), MOP (23) | Lysosomal degradation shown for DOP | Throughout the cytoplasm |

*Domain searches were done by SMART (smart.embl-heidelberg.de) (95) and COILS (www.ch.embnet.org/software/COILS_form.html) (96).
Expression and Purification of SNX1—For use in surface plasmon resonance analyses, SNX1 was expressed in a cell-free E. coli system (RTS 500 expression system, Roche Applied Science) and purified using the ProBond purification system (Invitrogen). The isoelectric point of SNX1 had to be increased from 5.08 to 5.75 by adding 4 lysines and 12 histidines to the C-terminal end of the protein to achieve efficient adsorption/preconcentration at the carboxymethyl dextran chip surface (described below). The coding sequence of SNX1 (with 4 lysines and 12 histidines added to the C-terminal) was amplified by PCR and cloned into the pET101D vector containing a C-terminal six-histidine tag and a T7 promoter using the TOPO expression kit (Invitrogen).

**SPR Analysis**—Experiments were performed on a BIAcore 3000 instrument using carboxymethyl dextran (CM5) chips (Biacore, AB).

### Table II

7TM receptor tails cloned and expressed as GST fusion proteins

| Family A | TM receptor | Abbreviation | Tail length amino acids | Recognitions motifs | Coiled-coils |
|----------|-------------|--------------|-------------------------|--------------------|-------------|
| h\(1\)-Adrenergic receptor | h\(\beta_1\) | 97 | PDZ Type I SKV |
| h\(2\)-Adrenergic receptor | h\(\beta_2\) | 84 | PDZ Type I SLL |
| h-5-Hydroxytryptamine 1A receptor | h\(5-HT_1A\) | 19 |
| h-5-Hydroxytryptamine 1D receptor | h\(5-HT_1D\) | 17 |
| hMuscarnic acetylcholine receptor M1 | h\(M_1\) | 39 |
| hMuscarnic acetylcholine receptor M2 | h\(M_2\) | 23 |
| hMuscarnic acetylcholine receptor M3 | h\(M_3\) | 43 |
| hMuscarnic acetylcholine receptor M4 | h\(M_4\) | 23 |
| hMuscarnic acetylcholine receptor M5 | h\(M_5\) | 34 |
| hHistamine H1 receptor | h\(H_1\) | 17 |
| hHistamine H2 receptor | h\(H_2\) | 70 |
| hHistamine H3 receptor | h\(H_3\) | 28 |
| hTachykinin NK1 receptor | h\(NK_1\) | 97 |
| hTachykinin NK2 receptor | h\(NK_2\) | 88 | PDZ Type II VEI |
| hTachykinin NK3 receptor | h\(NK_3\) | 106 |
| hSomatostatin receptor type 1 | h\(sst_1\) | 64 | PDZ Type I TTL |
| hSomatostatin receptor type 2 | h\(sst_2\) | 66 |
| hSomatostatin receptor type 3 | h\(sst_3\) | 102 | PDZ Type I SYL |
| hSomatostatin receptor type 4 | h\(sst_4\) | 74 |
| hSomatostatin receptor type 5 | h\(sst_5\) | 56 | PDZ Type I SKL |
| hNeuropeptide Y receptor type 4 | h\(Y_4\) | 50 |
| h Vasopressin V2 receptor | h\(V_2\) | 43 |
| hOxytocin receptor | h\(OT\) | 57 |
| mMelanocortin receptor 1 | m\(MC_1\) | 17 |
| mMelanocortin receptor 4 | m\(MC_4\) | 28 |
| hMelanin-concentrating hormone receptor 1 | h\(MCH_1\) | 30 |
| hMelanin-concentrating hormone receptor 2 | h\(MCH_2\) | 30 |
| hAngiotensin II receptor type 1 | h\(AT_1\) | 63 |
| hGrelin receptor | h\(GREL\) | 40 |
| hMotilin receptor | h\(MT\) | 54 |
| mDelta-type opioid receptor | m\(\delta\) | 61 | PDZ Type II AAA |
| mMu-type opioid receptor | m\(\mu\) | 69 | >95% probability |
| hKappa-type opioid receptor | h\(\kappa\) | 47 |
| hProtease-activated receptor 1 | h\(PAR1\) | 51 |
| hProtease-activated receptor 2 | h\(PAR2\) | 50 |
| hLeukotriene B4 receptor | h\(LTB_4\) | 63 |
| hCXCR3 | h\(CXCR3\) | 45 | PDZ Type I TTL |
| hCXCR4 | h\(CXCR4\) | 47 |
| ORF74 (human herpesvirus 8 chemokine-R) | ORF74 | 23 |
| US28 (human cytomegalovirus chemokine receptor) | US28 | 59 |
| US27 (human cytomegalovirus chemokine receptor) | US27 | 64 |
| hMAS1 oncogene receptor | h\(MAS1\) | 41 | PDZ Type I TVV |

**Family B**

| TM receptor | Abbreviation | Tail length amino acids | Recognitions motifs | Coiled-coils |
|-------------|--------------|-------------------------|--------------------|-------------|
| hGastric inhibitory polypeptide receptor | h\(GIP\) | 68 | PDZ Type II AAA |
| rGlucagon-like peptide-1 receptor | r\(GLP\) | 55 |
| rSecretin receptor | secretin | 57 |
| rGlucagon receptor | glucagon | 80 |
| rVasoactive intestinal polypeptide receptor 1 | r\(VIP\) | 64 | PDZ Type I SLV |

**Family C**

| TM receptor | Abbreviation | Tail length amino acids | Recognitions motifs | Coiled-coils |
|-------------|--------------|-------------------------|--------------------|-------------|
| rGABA-B 1A receptor | r\(GABA_B1\) | 107 | >95% probability |
| rMetabotropic glutamate receptor 1a | r\(mGlu_1a\) | 354 | PDZ Type I STL/Homer EVHI, SH3 PPSPFR |
| rMetabotropic glutamate receptor 1b | r\(mGlu_1b\) | 66 |
| rMetabotropic glutamate receptor 2 | r\(mGlu_2\) | 53 | PDZ Type I SSSL |
| rMetabotropic glutamate receptor 3 | r\(mGlu_3\) | 51 | PDZ Type I SSSL |
| rMetabotropic glutamate receptor 4 | r\(mGlu_4\) | 65 |
| rMetabotropic glutamate receptor 5a | r\(mGlu_5a\) | 353 | PDZ Type I SSL/Homer EVHI, SH3 PPSPFR |
| rMetabotropic glutamate receptor 6 | r\(mGlu_6\) | 32 |
| rMetabotropic glutamate receptor 7 | r\(mGlu_7\) | 65 | PDZ Type II LVI |
| rMetabotropic glutamate receptor 8 | r\(mGlu_8\) | 65 |

*a Abbreviations according to (97).

*b Recognition motifs in 7TM receptor tails.

*c Coiled-coils were predicted by COILS (www.ch.embnet.org/software/COILS_form.html) (96).

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Runs were conducted at 25 °C with HBS-EP buffer (10 mM Hepes, pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant P20) at a flow rate of 10 μl/min. SNX1 was covalently attached to the carboxymethyl dextran surface using standard amine coupling. CM5 chips were activated by a 7-min injection of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccimide. SNX1 (100 μg/ml) was immobilized by injection in 10 mM sodium acetate, pH 4.0, for 40 min to 2000 response units (2 ng/mm²) at a flow rate of 8 μl/min. Finally, unreacted succimide esters were blocked by a 7-min injection of 1 M ethanolamine hydrochloride, pH 8.5. GST tail-fusion proteins of US28, DOP, and KOP and non-fused GST protein as the control were passed over the surfaces at concentrations ranging from 50 to 1000 nM, allowing an association time of 12 min and a dissociation time of 12 min. The surfaces were regenerated between runs by a 1-min injection of 10 mM glycine-HCl, pH 3.0, at a flow rate of 30 μl/min. Sensorgram curves were fitted to a 1:1 kinetic binding model with the BIAevaluation 3.0 software.

RESULTS

The receptors, which were included in the library of 7TM receptor C-terminal tails used in this study, were selected from all three major classes of 7TM receptors, i.e. 44 Family A, rhodopsin-like receptors, 5 Family B receptors, and 10 Family C receptors (Table II). The Family A receptors comprise 13 biogenic amine receptors, 21 peptide receptors, 2 protease-activated receptors, 1 lipid messenger receptor, 3 chemokine receptors, and 3 virally encoded chemokine receptors plus one orphan receptor. The library was somewhat biased as the complete set of receptor subtypes for a particular ligand in some cases were included in order to be able to probe for potential subtype-specific interactions with the adaptor proteins (for example, all five muscarinic receptors and all five somatostatin
Receptors). The length of the tails varied considerably between the Family A and B receptors from only 17 residues (5-HT1D, H1, and MC1) to 106 amino acid residues (NK3). Among the Family C receptors, the length varied from 32 to 354 residues (Table II).

### FIG. 3. **7TM receptor tail interactions with endosomal sorting proteins.** Panel A, EBP50. Panel B, NSF. Panel C, SNX1. Panel D, cGASP.

The relative binding of receptor tails to sorting proteins in GST pull-down experiments are shown as the mean and mean ± S.E. Band intensities were normalized to the band intensity of a reference lane so that 100% binding corresponded to the retention of all of the added radioactive sorting protein (see Fig. 2). The cut-off values between binders and non-binders for NSF (5.5%), SNX1 (4.1%), and cGASP (6.3%) are marked by dotted lines. Only the β2 receptor bound to EBP50, and therefore, no cut-off line is marked for EBP50.

#### Mapping Interactions of Receptor Tail with Sorting Protein by GST Pull-down Assay—Fig. 2 shows autoradiographic images and the corresponding quantitative data obtained in the GST pull-down experiments with a representative selection of GST tail-fusion proteins and the four proposed 35S-labeled sorting proteins. The volume intensity of the radioactive bands was determined, and the relative amount of the bound radioactive protein was quantified by dividing the volume intensity of each band by that of the probe band, which was defined as the relative binding. Using relative binding instead of absolute band intensity values allowed for a comparison between experiments performed on different days and analyzed in different gels. Unspecific binding of the radioactive probe (sorting protein) to the GST beads was estimated by running the GST protein alone without tail-fusion in parallel in all of the assays,
which typically gave a relative apparent binding of 0–0.5% and always below 1%. Similar experiments were performed for the full GST tail protein library. Positive interactions were repeated at least twice in independent experiments, and the relative binding was averaged.

Adaptor Proteins Suggested to be Involved in Receptor Recycling—EBP50 has been proposed to be involved in the recycling of the \( \beta_2 \) receptor through a phosphorylation-sensitive binding to a C-terminal PDZ recognition sequence (39). Accordingly, it was found that the GST fusion of the C-terminal tail of the \( \beta_2 \) receptor strongly bound the \(^{35}\)S-labeled EBP50 protein (Fig. 3, panel A). However, as shown in Fig. 3A, surprisingly, the \( \beta_2 \) receptor tail was the only tail protein among the 59 fusion proteins tested, which bound EBP50 convincingly. A small signal was observed also for the mGlu1b tail-fusion and a few other tail proteins; however, in view of the strong signal from the \( \beta_2 \) receptor tail and the fact that the mGlu1b tail, for example, does not have a PDZ recognition sequence, only the \( \beta_2 \) receptor was considered a true hit (Fig. 3, panel A).

NSF is another protein that has been suggested to be required for the recycling of the \( \beta_2 \) receptor (22). Also, in this case, the \( \beta_2 \) receptor tail protein served as a convenient positive control binding strongly to the \(^{35}\)S-labeled NSF protein (Fig. 3, panel B). However, in contrast to EBP50, NSF bound to a number of the other tail-fusion proteins, albeit not as strongly as the \( \beta_2 \) receptor tail. For an additional number of tail-fusion proteins, a weak signal was observed. Statistical analysis revealed that the data could be considered to be composed of two Gaussian distributions of what could be coined “non-binders” and “binders,” respectively, and that a cut-off value in relative

![Graphs showing binding of \(^{35}\)S-labeled proteins to 7TM receptor tails](http://www.jbc.org/)
binding, which would ensure <1% contamination of the binders with non-binders, would be 5.5% for NSF (Fig. 3B, dotted line; see Supplemental Data). According to this finding, the following tail proteins, besides the β2 receptor, bound to NSF: the muscarinic M1, M4, and M5 receptors; the tachykinin NK1 and NK2 receptors; the somatostatin SST1 receptor; the DOP receptor, and as the virally encoded chemokine receptors US27 and US28 from human cytomegalovirus. However, a couple of tail proteins fall just under the relatively stringent cut-off value ensuring less than 1% false-positives, i.e. PAR1 and mGlu4, and US28 (see Fig. 3B). These receptors should probably also be considered true NSF binders (see Supplemental Fig. S1 and “Discussion”).

Adaptor Proteins Suggested to Be Involved in Lysosomal Receptor Targeting—SNX1 has been proposed to be responsible for the targeting of PAR1 to lysosomes (24). Pull-down analyses between 35S-labeled SNX1 and the library of 7TM tail-fusion proteins showed a number of strong binders (Fig. 3C). Surprisingly, however, the GST fusion protein of the C-terminal tail of the human PAR1 bound only weakly to SNX1 in comparison to the virally encoded US28 receptor tail, for example. Statistical analysis of the relative binding data gave a cut-off value of 4.1% ensuring <1% false positive binders (see Supplemental Data). According to this cut-off value, the following tail proteins were considered to bind to SNX1: the muscarinic M1, M4, and M5 receptors; the tachykinin NK1, NK2, and NK3 receptors; the oxytocin receptor; the DOP receptor; US28; and the GLP1 receptor (Fig. 3C). Four receptors can be considered to be borderline binders that are probably positive: the muscarinic M3 receptor; the CXCR2 chemokine receptor; and the mGlu1a and mGlu1b metabotropic glutamate receptors (Fig. 3C and Supplemental Fig. S1).

GASP is a large protein of 1394 amino acids that has recently been implicated in selective lysosomal sorting of the DOP receptor as opposed to the MOP receptor (23). Originally, yeast two-hybrid screening with the DOP receptor tail gave four positive clones, all of which contained sequences exclusively from the C-terminal 497 amino acid fragment of GASP (called cGASP).

As shown in Fig. 3D, the binding profile for cGASP was clearly the broadest among the tested sorting proteins. Approximately one-third, i.e. 23 of the 59 tail-fusion proteins, bound 35S-labeled cGASP with a specific binding above the cut-off value of 6.3% (see Supplemental Data). The positive receptors were as follows: the β1- and β2-adrenergic receptors but not the three 5-HT receptors tested; the muscarinic M1, M4, and M5 receptors; the three tachykinin receptors; the oxytocin but not the V1 receptor; the AT1 receptor; the motilin but not the homologous ghrelin receptor; the DOP but not the MOP receptor (which are, respectively, the positive and negative controls) (23); PAR1 and PAR2; CXCR2; ORF74 and US28; the GLP1 and the VIP receptor but not the secretin and glucagon receptors; and, finally, the mGlu1a, mGlu5a, and mGlu receptors. The M4 and the mGlu1b receptors could be considered as borderline binders of cGASP.

The binding of 35S-labeled cGASP and similarly labeled full-length GASP was compared in ten receptor tails (Fig. 4). Although the overall picture was rather similar, cGASP gave a somewhat higher binding signal with most of the receptor tails. Interestingly, for the oxytocin and GLP1 receptor tails, this difference was large and the binding of full-length GASP was borderline or would not be considered to be significant.

Table III lists all of the positive hits among members of the 7TM receptor tail library with the four different adaptor proteins proposed to be involved in receptor sorting. None of the tail proteins were positive for all four adaptor proteins. However, a group of tail proteins were positive for NSF, SNX1, and cGASP, i.e. the M1, M4, and M5 receptors; the NK1 and NK2 receptors; the DOP receptor; and the virally encoded US28. It should be noted that these tail proteins, with respect to their size, cover the full range of the library, because they include some of the shortest (the M4 tail being only 23 residues long) as well as some of the longest tails (the NK1 and NK2 being 97 and 88 residues long, respectively) (Table II).

**Fig. 4. Binding of cGASP versus full GASP to receptor tails.** Relative binding of a selection of 7TM receptor tails to full-length GASP (1395 amino acids) and cGASP (C-terminal 497 residues of GASP). Band intensities were normalized to the band intensity of a reference lane so that 100% binding corresponded to the retention of all of the added [35S]cGASP or [35S]GASP. The mean and mean ± S.E. are shown.

**SPR Affinity Measurements**—This study constitutes a “horizontal” analysis of a large library of 7TM receptor tails with a selection of adaptor proteins as determined in pull-down experiments. However, to confirm a few of the positive hits and quantify the binding affinities, US28 and DOP binding to SNX1 were analyzed by SPR analysis. Preliminary experiments showed that preconcentration of SNX1 at the chip surface was not efficient, which was most probably caused by its low isoelectric point. Therefore, the isoelectric point of SNX1 was increased from 5.08 to 5.75 by adding 4 lysines and 12 histidines to the C-terminal end of the protein. In Fig. 5 are shown the association and dissociation kinetics for the US28 and DOP fusion proteins to SNX1 immobilized on the chip. The KOP fusion protein did not bind to SNX1 and is shown as a negative control. When the data were fitted to a 1:1 kinetic-binding model (A + B ↔ AB) with the BIAevaluation 3.0 software, it was found that the tail of the virally encoded receptor US28 bound to SNX1 with a KD value of 49 nM (chi2 = 12). The tail of the DOP receptor bound with an even higher affinity, i.e. a KD value of 20 nM (chi2 = 11).

**Mutational Analysis of the NK, Tail Sequence Required for Binding to NSF, SNX1, and cGASP**—The structural basis for the strong binding of one of the tails, i.e. the one from the NK1 receptor, which binds to three of the four adaptor proteins, was characterized through systematic deletion mutagenesis. Five truncated versions of the NK1 receptor tail were analyzed in GST pull-down experiments: NK1Δ4; NK1Δ21; NK1Δ42; NK1Δ63; and NK1Δ84, where the number following the Δ refers to the number of amino acids deleted from the C-terminal end of the full tail sequence, i.e. residues 311–407 in the NK1 receptor (Fig. 6A). As shown in Fig. 6B, a fairly similar picture was obtained for all three adaptor proteins. Deletion of the last four residues, which would have eliminated the tail binding if it had been recognized through a PDZ-domain type of recognition, had no or very little effect on the binding of the NK1 tail to NSF, SNX1, and cGASP (Fig. 6B). Deletion of the last 21 residues clearly diminished the binding to all three adaptor proteins.
proteins, and subsequent further deletions gradually diminished the binding. It should be noted that the shortest version of the NK₁ tail, NK₁Δ84, which basically only consists of the helix 8 motif, still showed binding albeit weak binding to all three adaptor proteins (Fig. 6). These data indicate that the binding epitope for NSF, SNX1, and cGASP to the NK₁ tail is large and covers major parts of the tail structure.

**DISCUSSION**

Adaptor and scaffolding proteins are highly important for the function of membrane receptors, for example, in determining their targeting to specific locations in the cell membrane (16, 40), in determining the spatial and thereby functional association of receptors with various co-receptors (41), transducer proteins, and downstream effector molecules (13, 14, 16, 42), and in determining post-signaling events such as receptor sequestration and post-endocytotic sorting (21–24). The interactions of 7TM receptors with adaptor and scaffolding proteins are to a large degree governed by epitopes located in their C-terminal intracellular tails (17–25). We have established a library of C-terminal tails from a series of selected 7TM receptors covering families A, B, and C and representing their various subfamilies. In this study, this receptor tail library was screened for binding to four proteins, which have been proposed already determined through some other means, for example, may yield different results. For all four proposed sorting proteins, several unambiguous positive hits (for EBP50, NSF, SNX1, and cGASP). Only hβ₂ bound to EBP50, and therefore, no cut-off value was determined for EBP50.

**Binders versus Non-binders?—** Pull-down experiments using GST fusion proteins is an established in vitro method for assessing protein-protein interactions. However, it is generally performed as part of a study where the interaction of interest is being illuminated through a series of complementary biochemical and cell biological approaches. In such “vertical” studies, where only one or a few receptors are being studied, the issue of “binding versus non-binding” is usually determined solely by comparison with a negative control, GST alone. Furthermore, the pull-down experiments are usually made either as the initial method through which the interaction partner is identified as a positive hit or it is applied to confirm an interaction already determined through some other means, i.e., focus is normally directed toward positive results. In this case, we are performing a horizontal study in which we evaluate among a large number of receptor tail-fusion proteins, which are binding to certain adaptor/sorting proteins and which are not. Although this study predicts a number of protein-protein interactions, studies performed with more physiological systems wherein intact receptors are used in co-immunoprecipitation studies, for example, may yield different results. For all four proposed sorting proteins, several unambiguous positive hits (for EBP50, only one hit) clearly stood out among the multitude of fusion proteins, many of which showed little or no binding (Fig. 3A). Nevertheless, if the binding to GST alone was used as the strict negative control, most of the tail proteins would in fact be considered to be positive hits because the weak bands, although

### Table III

**Significant interactions between sorting proteins and 7TM receptor tails**

| Binding to sorting protein | EBP50 | NSF | SNX1 | CGASP | Post-endocytic sorting properties |
|---------------------------|-------|-----|------|-------|----------------------------------|
| hβ₂                       | hβ₂   | hβ₂ | Recycled (22) |
| hM₁                       | hM₁   | hM₁ | Recycled (99, 100) |
| hM₄                       | hM₄   | hM₄ | Recycled (99, 100) |
| hNK₁                      | hNK₁  | hNK₁| Recycled (50, 51) |
| hNK₂                      | hNK₂  | hNK₂| Recycled (51) |
| hOT                       | hOT   | hOT | Degraded (109) |
| mDOP                      | mDOP  | mDOP| Degraded (110) |
| hMotilin-R                | Not known |
| hM₅                       | hM₅   | Not known |
| ORF74                     | Not known |
| rmGlu₁₃a                  | Not known |
| rmGlu₂₃a                  | Not known |
| rmGlu₃₃a                  | Not known |

Transactions, in particular, GASP, at first does not fit well into the picture. However, mutations of the recognition sequence for PDZ domain binding found in the C-terminal end of the β₂ receptor or phosphorylation of this recognition sequence, which disrupts EBP50 (21) and NSF (22) binding, resulted in lysosomal targeting and degradation of the receptor. Thus, it could be hypothesized that some receptors such as the β₂ receptor are “dual fate” receptors that are able to interact with several proteins and that, after endocytosis, they are sorted either for recycling or to the lysosomes depending on the phosphorylation state and on which sorting proteins are available for interaction with the receptor.

**TABLE III**
weak but slightly stronger than the GST band, were observed for most of the GST fusion proteins. However, the tail-binding results for each adaptor protein were in fact distributed in two Gaussian populations and we have chosen to use a cut-off value in relative binding, which would ensure <1% contamination of the binders with non-binders (see Supplemental Data). In doing so, we took into account that a certain degree of variable nonspecific protein-protein interaction probably is responsible for the low degree of binding observed with some tail-fusions. It is possible that we hereby exclude certain tail proteins, which in a cellular context may in fact form a physiologically important albeit weak interaction with a particular adaptor protein, from being considered as true binders. It will be interesting to test to what degree post-translational modifications such as the phosphorylation of particular residues in the tail will turn such weak binders into true hits with strong binding. Accordingly, it will be interesting to determine the effect of in vitro phosphorylation on the binding properties of the tail library. However, it is important to note that the effect of phosphorylation often does not result in an “all or none binding phenomenon.” For example, the effect of phosphorylation of receptors on the affinity for arrestin is only 5–10-fold (43, 44).

Although this study as described above is primarily a horizontal analysis of the receptor tail library, a few vertical experiments were included to substantiate the results. Thus, for two of the tail-fusion proteins, the virally encoded receptor US28 and the DOP receptor, the strong interaction with one of the adaptor proteins, SNX1, which had been identified through the pull-down experiments, was further analyzed by SPR to quantify the affinity of the protein-protein interaction. The observed nanomolar affinities correspond to affinities previously reported for PDZ interactions (45, 46). This type of analysis will be valuable, for example, in the analysis of potential effects of post-translational modifications on the interaction of the tail library with adaptor proteins.

In another vertical analysis, the structural basis for the interaction of one of the apparently more promiscuous tail-fusion proteins, i.e. the NK1 receptor, with the three adaptor proteins, NSF, SNX1, and cGASP, was dissected through a series of systematic deletion mutants. It should be noted that the NK1 tail does not bind totally promiscuously, because it does not bind to EBP50 (Fig. 3A) or certain other adaptor proteins (data not shown). Although certain differences could be pointed out for the three tail proteins, the structural analysis of the NK1 tail gave a rather similar picture for all three proteins as a gradual loss of binding was observed in parallel with the gradual truncation of the tail from its C-terminal end (Fig. 6). Thus, it appears that the interaction of NSF, SNX1, and cGASP to the NK1 receptor depends on an extended epitope covering most of the tail structure. This is particularly interesting because the binding of SNX1, for example, was also observed with the very short tail from the M4 receptor (shown in Supplemental Fig. S2), which is only 23 residues long. This corresponds to the two most truncated NK1 tail constructs (NK1Δ63 and NK1Δ84), both of which had lost most of their binding. Further mutational analysis is required to identify the epitopes and residues that determine the interactions of especially the short tail proteins. Some of these are so short that the helix 8 region may very well be involved in adaptor protein binding.

Adaptor Proteins Suggested to Be Involved in 7TM Receptor Recycling—Both EBP50 and NSF have been proposed to be responsible for the recycling of 7TM receptors as demonstrated in both cases initially for the β2 receptor.

In accordance with previously published results, we found that the tail of the β2 receptor bound strongly to EBP50. This binding has been demonstrated to be caused by the interaction of the PDZ domain in EBP50 with a type 1 PDZ recognition sequence SLL located at the far C-terminal end of the receptor.
Although 13 of the tails in the library end in a type 1 PDZ recognition sequence (Table II), none of these tails bound EBP50. It has been suggested that EBP50 could be involved in the recycling of the KOP receptor, which does not contain a PDZ recognition sequence, as demonstrated by co-immunoprecipitation experiments (47). However, neither the KOP receptor tail nor any of the other tails in the library bound EBP50 (Fig. 3A). Thus, we concluded that, although EBP50 may be involved in the recycling of the β2 receptor and possibly a few other receptors, this protein is not an important protein for the recycling of 7TM receptors, in general.

NSF is a hexameric ATPase involved in vesicular transport and fusion throughout the exocytotic and endocytotic pathways (48, 49). Recently, Cong et al. (22) demonstrated that the β2 receptor binds directly to NSF through an epitope involving the three last residues of the receptor tail, although NSF does not hold any PDZ domains, and that this interaction was required for receptor internalization and subsequent recycling (22). In this study, we found that NSF bound 11 of the 59 tail-fusions. As shown in Table III, most of these receptors are known to recycle to the membrane after endocytosis. The binding of NSF to the NK1 receptor, which internalizes rapidly and is recycled and resensitized within 30 min after agonist stimulation (50, 51), was not primarily dependent on the far C-terminal segment of the tail, in contrast to the β2 receptor (Fig. 6). NSF was also found to bind to the DOP and US28 receptor tails, i.e. receptors that are both known to accumulate in the lysosomal compartment upon endocytosis, although rapid recycling has also been demonstrated to occur for US28 in the same cells (52). It is possible that the binding of receptor tails to NSF could be improved upon phosphorylation, although this clearly is not required for the binding of the β2 receptor and the 11 other positive receptors identified in this study.

Adaptor Proteins Suggested to Be Involved in Lysosomal Targeting of 7TM Receptors—Both SNX1 and GASPD have been proposed to be responsible for lysosomal targeting of 7TM receptors as demonstrated in biochemical and cell biological studies using PAR1, DOP, and MOP receptors as the main model systems (23, 24).

SNX1 is a member of a relatively large family of sorting nexins, which are cellular trafficking proteins, all having a phospholipid-binding domain and a strong predisposition to form protein-protein complexes mainly through coil-coil formation (Table I) (53). SNX1, which is ubiquitously expressed, is found together with the homologous SNX2 in endosomes and was originally identified as being involved in the endocytotic processing of the epidermal growth factor receptor (34, 54). Recently, Trejo and colleagues showed that the sorting of activated PAR1 from endosomes to lysosomes is regulated by SNX1 (24). SNX1 co-localizes with internalized PAR1 on early endosomes, and SNX1 is found associated with activated PAR1 in cellular lysates. Moreover, SNX1 deletion mutants cause significant inhibition of agonist-induced PAR1 degradation. However, in the initial report, a direct interaction between SNX1 and PAR1 was in fact not demonstrated. Subsequent studies have shown that depletion of SNX1 by small interfering RNA knockdown also causes significant inhibition of agonist-induced PAR1 degradation; however, experiments using the yeast two-hybrid system failed to detect a direct interaction between the PAR1 C-tail and SNX1. This study also did not detect a direct interaction between the PAR1 C-tail and SNX1 using GST pull-down assays. Together, these findings suggest that SNX1 is critically involved in targeting the PAR1 receptor to the lysosomal sorting pathway for degradation, perhaps through an indirect interaction with the receptor or other important lysosomal sorting machinery. This means that PAR1 should not be taken as a positive control for direct SNX1 binding. In this study, we found that SNX1 bound to 10 of the 59 tails tested. Previously, we have reported that SNX1 binds to the dopamine D3 but not any of the other four dopamine receptors (55). Interestingly, standard software for prediction

Fig. 6. Mutational analysis of the NK1 tail sequence required for binding to NSF, SNX1, and cGASP. Panel A, schematic of NK1 mutants analyzed. The number following the Δ refers to the number of amino acids deleted from the C-terminal end of the full tail NK1 sequence, i.e. residues 311–407 of the NK1 receptor. Panel B, relative binding of receptor tails to sorting proteins in GST pull-down experiments. Band intensities were quantified and normalized to the band intensity of the wild type NK1 lane.

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2 J. Trejo, personal communication.
7TM Receptor Tails and Post-endocytic Adaptor Proteins

of coiled-coil domains, which could identify the three coiled-coil domains in SNX1, did not indicate that such a motif occurred frequently either among the 7TM receptor tails, in general, or among the positive hits for SNX1. Among the receptor tails that bound to SNX1, the oxytocin receptor, the DOP receptor, and the virally encoded US28 receptor are all known to be targeted to the lysosomal pathway, which would fit with the expected role of SNX1 (24, 34). In Mutagenesis experiments and overexpression of a dominant D

GASP was recently identified as a cytoplasmic protein that selectively interacts with the DOP receptor versus the M4 receptor. Mutagenesis experiments and overexpression of a dominant D

The 7TM Receptor Tail Library—

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A Library of 7TM Receptor C-terminal Tails: INTERACTIONS WITH THE PROPOSED POST-ENDOCYTIC SORTING PROTEINS ERM-BINDING PHOSPHOPROTEIN 50 (EBP50), N-ETHYLMALEIMIDE-SENSITIVE FACTOR (NSF), SORTING NEXIN 1 (SNX1), AND G PROTEIN-COUPLED RECEPTOR-ASSOCIATED SORTING PROTEIN (GASP)

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