Pias1 Interaction and Sumoylation of Metabotropic Glutamate Receptor 8*

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Group III presynaptic metabotropic glutamate receptors (mGluRs) play a central role in regulating presynaptic activity through G-protein effects on ion channels and signal transducing enzymes. Like all Class C G-protein-coupled receptors, mGluR8 has an extended intracellular C-terminal domain (CTD) presumed to allow for modulation of downstream signaling. In a yeast two-hybrid screen of an adult rat brain cDNA library with the CTDs of different components of the sumoylation cascade (ube2a, Pias1, mGluR8a and 8b (mGluR8-C) as baits, we identified sumo1 and four different components of the sumoylation cascade (ube2a, Pias1, Pyax, Piasxβ) as interacting proteins. Binding assays using recombinant GST fusion proteins confirmed that Pias1 interacts not only with mGluR8-C but also with all group III mGluR CTDs. Pias1 binding to mGluR8-C required a region N-terminal to a consensus sumoylation motif and was not affected by arginine substitution of the conserved lysine 882 within this motif. Co-transfection of fluorescently tagged mGluR8a-C, sumo1, and enzymes of the sumoylation cascade into HEK293 cells showed that mGluR8a-C can be sumoylated in vivo. Arginine substitution of lysine 882 within the consensus sumoylation motif, but not other conserved lynes within the CTD, abolished in vivo sumoylation. Our results are consistent with post-translational sumoylation providing a novel mechanism of group III mGluR regulation.

G-protein-coupled metabotropic glutamate receptors (mGluRs)

have been implicated in the regulation of transmitter release, short and long term modulation of synaptic transmission, neuronal development, and synaptic plasticity (1–5). mGluRs are structurally distinct from family A and B G-protein-coupled receptors (GPCRs), as they possess a large extracellular ligand binding domain and an extended intracellular C terminus (4, 6). At least eight different mGluR isoforms have been identified and classified into three subgroups based on sequence homology, downstream effectors, and agonist specificity. Group III mGluRs (mGluR4, -6, -7, and -8) are specifically activated by L(+)-2-amino-4-phosphonobutyric acid (L-AP4), negatively coupled to adenylate cyclase and, apart from mGluR6, exclusively localized presynaptically. Aside from mGluR6, which is only found in the retina, group III mGluRs are expressed throughout the brain.

Compared with other mGluRs, mGluR8 expression is regionally restricted, with highest mRNA levels detected in olfactory bulb, pontine gray, thalamus, and mamillary body (7, 8). Alternative splicing and out-of-frame insertion generates two splice variants of mGluR8, named mGluR8a and 8b, which differ in the last 16 amino acids of the cytoplasmic C-terminal domain (CTD) (9). Both splice variants share similar pharmacological profiles and expression levels throughout the brain, except for the lateral reticular and ambiguous nuclei where only mGluR8a mRNA is found (9). Pronounced mGluR8 expression in the dentate gyrus and CA3 region of the hippocampus (10, 11) and performance deficits of mice lacking mGluR8 in learning tasks (12) suggest that this receptor plays a role in memory formation. In stress-related behavioral tests, mGluR8-deficient mice show increased anxiety, indicating that mGluR8 may alter neurotransmission at synapses that regulate adaptation to novel stressful environments (13).

Sumo1 (small ubiquitin-related modifier 1) is a 101-amino acid protein that can be covalently linked to the ε-amino group of lysine side chains of target proteins. This post-translational modification is catalyzed by an enzymatic cascade termed “sumoylation pathway,” which requires an activation enzyme (E1), conjugase (E2), and for most substrates, ligase (E3) (14–16). This pathway is mechanistically but not functionally related to ubiquitination. Whereas ubiquitination destines target proteins to internalization and degradation, sumoylation can have such diverse effects as promoting transport between the cytoplasm and the nucleus, protection from ubiquitination (15), and regulation of protein-protein interactions (17).

Members of the Pias (protein inhibitor of activated STAT) family promote the conjugation of sumo1 to different proteins and have been classified as E3 protein ligases (18). They have been found to interact with several targets, among them nuclear proteins and steroid receptors (19). Pias proteins contain an N-terminal SAP (SAF-A/B, Acinus, and Pias) domain consisting of a four helix bundle that is responsible for binding to A/T-rich DNA oligomers and p53 tumor suppressor protein (20). The sumo ligase activity of Pias family proteins (but not the Ran-binding protein 2 (RanBP2) sumo1 E3 ligase) requires a conserved C-terminal zinc finger domain, which is related to the essential ring finger motif of many ubiquitin ligases (21).

Known sumo targets are predominantly nuclear proteins, like transcription factors, nuclear body proteins, and viral and nuclear pore complex components, although soluble signal transduction proteins (MAPK kinase 1 (Mek1), calmodulin kinase II (CaMKII)) and other cytoplasmic proteins (e.g. yeast septins) have also been identified (22). Subcellularly, the largest fraction of sumo conjugates localizes to nuclear speckles and the nuclear envelope (23). Sumoylation of plasma membrane proteins has rarely been reported and can have bimodal effects on trafficking

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4 The abbreviations used are: mGluR, metabotropic glutamate receptor; CFP, cyan fluorescent protein; CTD, C-terminal domain; GFP, green fluorescent protein; GLUT, glucose transporter; GPCR, G-protein-coupled receptor; GST, glutathione S-transferase; HEK, human embryonic kidney cell; L-AP4, L(+)-2-amino-4-phosphonobutyric acid; MBP, maltose-binding protein; Pias1, protein inhibitor of activated STAT1; RanBP2, Ran-binding protein 2; RanGAP1, Ran GTPase-activating protein; SBM, sumo-binding motif; SAP domain, SAF-A/B, Acinus, and Pias domain; Siah, seven in absentia homolog; sumo1, small ubiquitin-related modifier 1; ubc9, ubiquitin-conjugating enzyme 9; ubl1, ubiquitin-like 1; YFP, yellow fluorescent protein. © 2005 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
within the same protein family; overexpression of the E2 conjugase ubc9 results in a 4-fold increase of GLUT4 at the membrane, whereas levels of GLUT1 are decreased (24, 25). Recently, sumoylation has been found to silence the plasma membrane leak potassium channel K2P1 (26). To our knowledge, no plasma membrane receptors have yet been identified as targets of the sumoylation machinery. Here we report an interaction of family C GPCRs, namely group III mGlRs, with the E3 ligase Pias1. In addition, we provide evidence that the CTD of mGlRa8 can undergo in vivo sumoylation on a consensus site in HEK cells.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen and Yeast Mating—Yeast two-hybrid screening was carried out using the DupLEX-A system (OriGene Tech. Inc., Rockville, MD) as described previously (27). Briefly, the cDNA fragments encoding the CTDs of mouse mGlRa8a (mGlRa8a-C) and mGlRa8b (mGlRa8b-C) were generated by PCR, cloned into pGilda and used as baits to screen an adult rat brain cDNA library cloned into pG4–5, according to the user’s manual (Version 1.2). Yeast strain EGY48 was transformed using lithium acetate, and protein expression was induced by galactose. For screening with the mGlRa8a and mGlRa8b baits, 3 × 10^5 and 1.25 × 10^5 independent recombinants were examined, and 1,385 and 934 clones, respectively, were identified to be positive by Leu– auxotrophy and α-galactosidase expression. Yeast mating was performed with yeast strains EGY48 and REY206, using the same selection conditions as in the two-hybrid screen. After examining insert sizes and HaeIII digestion patterns, ~100 candidate clones identified with each bait were selected for sequencing.

Expression Constructs—Glutathione S-transferase (GST) fusion proteins of the CTDs of mGlRa4, -4, -7a, -7b, -8a, and -8b, and the mGlRa7a truncations GST-7a-N38 and GST-7a-C27 have been described previously (28). The EcoRI- and SalI-flanked truncated mGlRa8a tail constructs GST-8a-N24 and GST-8a-C44 were generated by standard PCR and inserted into pGEX-5X-1 (Amersham Biosciences). A site-directed mutagenesis kit (Stratagene, Amsterdam, Netherlands) was used to introduce lysine to arginine substitutions. Flanking EcoRI and SalI sites were used to shuttle inserts between pGEX-5X-1 and pEGFP-C2 to generate bacterial or mammalian fusion proteins. MBP-ube2a and MBP-Piasy were generated by transferring in-frame inserts between EcoRI and Xhol sites from the identified clones to pMAL-c2 (New England Biolabs, Frankfurt, Germany). The ube2a fragment contained the full-length coding sequence including 69 bp of the 5′-untranslated region. The Piasy fragment encoded the 157 C-terminal amino acids of rat Piasy. MBP-Pias1, GFP-Pias1, and CFP-Pias1 were constructed by transferring the full-length Pias1 cDNA from pCMV5-FLAG-Pias1 (gift from Dr. Shuai Ke, University of California, Los Angeles) into pMAL-c2, pEGFP-C2, and pECPF-C1 (Clontech, Heidelberg, Germany), respectively. BamHI and HindIII flanked full-length cDNAs of ube2a and sumo1 were generated by PCR and subcloned into the BglII and Hind III sites of pEVFP-C1 (Clontech) and pECPF-C1, respectively. Full-length His6-aos1 and uba2 expression constructs were similarly generated by PCR using pET28a-His6-aos1 and pET11d-uba2 (gifts from Dr. Frauke Melchior, University of Goettingen, Germany), respectively, as templates, and subcloned between the EcoRI and Xhol sites of pcDNA3 (Invitrogen), and the EcoRI and Xhol sites of pcDNA3.1, respectively.

Protein Expression and Binding Studies—Expression of GST and MBP fusion proteins was performed in Escherichia coli BL21 (Stratagene) as described (28). Bacteria were lysed in phosphate-buffered saline containing the protease inhibitor mixture CompleteTM (Roche Diagnostics) via passage through a French press. The supernatant was collected after centrifugation at 100,000 × g for 45 min at 4°C. GFP, CFPR, or YFP fusion proteins were expressed in HEK293 cells and processed as described (27). Protein expression in the bacterial lysates and cell homogenates was confirmed by Western blotting with anti-GST, anti-MBP, and anti-GFP antibodies. For pull-down assays, the lysates were incubated with 25 μl of glutathione-agarose beads (Amersham Biosciences) preadsorbed with GST-mGlRa8-C in incubation buffer (PBS containing 0.1% (v/v) Triton X-100, 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, and protease inhibitor mixture). After 2 h of rotary agitation, beads were collected by centrifugation and washed three times with incubation buffer. After elution with SDS sample buffer, eluted proteins were resolved by SDS-PAGE, followed by Western blotting with monoclonal anti-MBP (New England Biolabs) or polyclonal rabbit anti-GFP (Clontech). About one-fifth of the glutathione-agarose beads preadsorbed with GST-mGlRa8-C were loaded onto another gel and used to evaluate the amount of GST fusion protein bound. After transfer to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), proteins were stained for 4 min with 2% (w/v) Ponceau in 3% (v/v) trichloroacetic acid.

RESULTS

Yeast Two-hybrid Screen and Yeast Mating—To identify mGlRa8-C-interacting proteins, we performed a yeast two-hybrid screen of an adult rat brain cDNA library, using the entire CTD coding regions of the mGlRa8a and mGlRa8b cDNAs as baits. Twelve and thirteen clones encoding the C-terminal region of protein inhibitor of activated STAT1 (Pias1) were identified with mGlRa8a-C and 8b-C, respectively (TABLE ONE). The shortest cDNA fragment isolated for Pias1 comprised only 106 candidate proteins, except Piasx, in bacteria as MBP fusion proteins and used them with GST-mGlRa8-C fusion proteins in pull-down assays (Fig. 1). Batch adsorption onto glutathione-agarose, followed by SDS-PAGE and Western blotting with an antibody against MBP
Pias1 Interaction with Group III mGluRs

TABLE ONE

| Prey (GenBank™ no.) | Predicted no. aa of full-length protein | Fragments identified | Number of clones identified 8a/8b | Yeast mating 8a/8b |
|---------------------|----------------------------------------|---------------------|-------------------------------|-------------------|
| Pias1 (62653796)    | 1–721                                  | 209–721             | 12/13                         | +/+               |
|                     |                                        | 283–721             |                               |                   |
|                     |                                        | 319–721             |                               |                   |
|                     |                                        | 412–721             |                               |                   |
|                     |                                        | 514–721             |                               |                   |
| Piasxβ (Miz1) (16758049) | 1–572                               | 312–572             | 12/9                          | +/+               |
|                     |                                        | 1–158               | 158                           | +/+               |
| Ube2a (ube9) (4079642) | 1–101                                 | 6–101               | 0/2                           | +/+               |
| Sumo1 (ubl1, sentrin) (57528278) | 1–507                               | 351–507             | 0/1                           | +/+               |

Revealed that the MBP-Pias1 could be affinity-purified on GST-mGluR8b-C and, to a lesser extent, on GST-mGluR8a-C, but not on GST alone. Normalization of the GST fusion protein levels retained on the beads by Ponceau staining of the nitrocellulose membrane (data not shown) revealed that the amount of GST-mGluR8a-C adsorbed onto the agarose beads was only about 25% of that of mGluR8b-C. A comparatively weak specific interaction was also detected for the MBP fusion of the C-terminal region of Pias1, which similarly bound to both mGluR8-C isoforms. GST alone failed to bind MBP and all MBP fusion proteins tested. MBP-ube2a did not exhibit detectable binding to any of the GST-mGluR8-C termini. It is, however, important to note that rather stringent washing conditions had to be used in these pull-down assays because of high unspecific binding of MBP (data not shown).

**GFP-Pias1 Interacts with All Group III mGluRs**—Based on its frequent detection in the yeast two-hybrid screen, we next focused on Pias1. To examine whether binding of Pias1 is shared by other group III or even group II mGluR members, we performed binary yeast two-hybrid assays with the C-terminal coding fragment of the Pias1 cDNA and group II/III mGluR-CTDs (except mGluR7b-C and mGluR8b-C; Fig. 2A). In these assays, mGluR8a-C showed the strongest interaction, followed by mGluR7a-C and then mGluR6-C/mGluR4-C. In contrast, neither mGluR2-C nor mGluR3-C interacted with Pias1. Presuming that Pias1 binds in its function as E3 ligase in sumoylation, we compared group II (29) and group III (28) CTD sequences for the presence of candidate acceptor lysine residues (Fig. 2A). Notably, the group II mGluR C termini contain only a single lysine (mGluR2, Lys<sup>B2</sup>; mGluR3, Lys<sup>R3</sup>), just C-terminal of the end of the transmembrane domain 7.

Group III mGluRs do not carry a lysine at this position but display multiple lysine side chains throughout their CTDs.

Next, we investigated whether GFP-Pias1 generated in mammalian cells binds other group III mGluR C termini and tested its interaction with the respective GST fusion proteins (Fig. 2B). Whereas GST failed to bind GFP-Pias1 and conversely GFP did not interact with GST-mGluR7a-C (data not shown), all group III mGluR C termini showed some interaction. Strongest binding was seen with mGluR7a-C, mGluR4-C, and mGluR6-C. The weak band recovered with GST-mGluR8a could be attributed to substantially less GST fusion protein being retained on the agarose beads; a sequential Western blot with anti-GST antibody produced only a signal corresponding to ~25% of the other GST fusion proteins used in this experiment (data not shown). In conclusion, all group III mGluR-CTDs were able to bind GFP-Pias1.

The same assay was also used to examine whether the different components of the sumoylation pathway found in the yeast two-hybrid screen can directly interact with GST-mGluR8a-C. For these experiments, we now used cDNA constructs encoding full-length Pias1, ube2a, and sumo1. GFP-sumo1, YFP-ube2a, and GFP-Pias1 all were expressed in HEK293 cells, and Triton X-100 extracts of the transfected cells were used in binding assays (Fig. 3). Interaction could be confirmed for mGluR8a-C and GFP-Pias1, whereas only very little or no YFP-ube2a was recovered in the bound protein fraction. GFP alone did not bind to GST-mGluR8a-C. In contrast to the results obtained in the original two-hybrid screen, GST-mGluR8a failed to bind GFP-sumo1 (molecular mass ~40 kDa) under our assay conditions but enriched two high molecular mass (≥90 kDa) sumo-conjugated proteins from the

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HEK cell lysates. We did not try to disclose the identity of these proteins but an unbiased mass spectrometry-based analysis of sumo-conjugated HEK cell proteins has identified several candidates in the respective molecular mass range (30). Together these results are consistent with Pias1 representing the primary group III mGluR binding partner of the sumoylation machinery.

Mapping of the Pias1 Interaction Domain of mGluR7a-C and mGluR8a-C—To determine which domains of mGluR7a-C and mGluR8a-C interact with Pias1, we tested binding of mammalian expressed CFP-Pias1 to respective truncated GST fusion proteins (Fig. 4B). A schematic drawing of the truncation mutants used is shown in Fig. 4A. The mGluR7a-C truncation constructs did not overlap whereas those for mGluR8a-C overlapped by three amino acids. Also, the positions of the truncations were different in the respective CTDs: for mGluR7a-C, GST-mGluR7a-N38 ends, and GST-mGluR7a-C27 starts, just before the conserved lysine. For mGluR8a-C, GST-mGluR8a-N24 only included the proximal signal transduction domain with the G-protein and Ca²⁺/calmodulin binding sites (28), whereas GST-mGluR8a-C44 contained all conserved lysines outside of this signaling domain. GST fusion protein levels were normalized and tested semi-quantitatively for the amount retained on beads (Ponceau stain on nitrocellulose membrane, Fig. 4B, lower panel). Lower protein levels were seen particularly for GST-mGluR7a-N38, GST-mGluR7a-K889R, GST-mGluR8a-C, and mGluR8a-K882R. Binding of CFP-Pias1 was found with GST-mGluR7a-N38 and GST-mGluR8a-C44, whereas the complementary truncations GST-mGluR7a-C27 and GST-mGluR8a-N24 failed to interact. In sequence alignments (Figs. 2A and 4A), mGluR7a-N38 and mGluR8a-C44 overlap by 17 amino acids but are only identical in the last six residues preceding the consensus sumoylation motif (sequence DRPNGE; see amino acids 875–880 of mGluR8a). We therefore deduce that these residues are important for Pias1 recruitment to group III mGluRs.

In Vivo Sumoylation of mGluR8a-C Requires Lys⁸⁸²—To demonstrate that mGluR8a-C is sumoylated in vivo, GFP-mGluR8a-C and the
following tagged components of the sumoylation pathway were co-expressed in HEK293 cells: CFP-sumo1, E1 components aos1/uba2, YFP-ube2a, and GFP-Pias1. After detergent extraction of the transfected cells in the presence of protease inhibitors and 20 mM N-ethylmaleimide, which blocks sumo1-deconjugating enzymes (31), the extracts were separated by SDS-PAGE and Western-blotted with an antibody directed against the mGluR8 CTD. In the transfected, but not in untransfected cells, a significant fraction of mGluR8a-C immunoreactivity displayed a size shift to ~70 kDa, consistent with the addition of a single CFP-sumo1 molecule (Fig. 5A). Parallel Western blotting with an anti-sumo antibody confirmed that the 70-kDa band was indeed sumoylated (Fig. 5B). We also examined whether co-transfection of all six cDNAs was necessary for this sumoylation and found that only ube2a was not endogenously expressed at levels high enough to yield visible sumoylation of the overexpressed mGluR8a-C (data not shown). Inclusion of the E1 enzyme (aos1/ub2a) proved to be second most important, whereas overexpression of Pias1 appeared not to be required and hence was omitted from subsequent transfections.

Substitutions of target lysines by equally charged arginines are commonly used to identify motifs for sumoylation, whereas corresponding alanine substitutions have been shown to result in reduced binding of E2 to substrate proteins like Ran GTPase-activating protein (RanGAP1) (32). Here, sumoylation of mGluR8a-C was also abolished upon replacing specific lysines by arginines within the CTD (for positions of lysine substitutions, see Fig. 4A). Substitutions were selected based on two criteria: location within C44 of mGluR8a and conservation in both mGluR8a and 8b. We found that single or triple arginine substitutions, including Lys882, abolished sumo-conjugation, whereas single or combined substitution of Lys882 and Lys872 did not interfere with this modification (Fig. 5A). Notably, sumo-conjugation did not occur on the neighboring lysines Lys868 or Lys872 when the consensus sumoylation lysine 882 had been substituted. Also, K882R substitution or the triple mutation K868R/K872R/K882R did not lead to sumoylation of one of the remaining four lysines in the CTD of mGluR8a (Fig. 5A). Thus, in transfected cells, sumoylation of mGluR8a-C occurs specifically at lysine 882 located within the conserved consensus sumoylation motif. Notably, arginine substitution of Lys882 in mGluR8a-C and of the homologous lysine Lys886 in mGluR7a-C did not affect binding of CFP-Pias1 in the GST pull-down assay (Fig. 4B). This further confirms that the interaction of Pias1 with mGluRs does not depend on an intact sumoylation consensus motif in the CTD.

**DISCUSSION**

In this study, we identified components of the sumoylation pathway as interactors of the two splice variants of the C terminus of mGluR8. Sumoylation is a highly conserved protein modification that has been shown to be essential for cell cycle function in yeast (22). The following sumo-associated proteins were found to bind to the CTD of mGluR8 in the yeast two-hybrid assays: Pias1, Piasy, Piasxβ, ube2a, and sumo1. Based on the number of clones that were found in the two-hybrid screen, we focused on the involvement of Pias1 in group III mGluR sumoylation. The interaction of Pias1 and mGluR8-C was confirmed in vitro and upon transfection into mammalian cells, whereas recombinant ube2a and sumo1 failed to bind in GST pull-down assays. This may indicate that Pias1 is the only protein of the sumoylation machinery that binds with high affinity to mGluR8-C, consistent with a more general role of the E3 ligase in mGluR regulation. Binding of Pias1 could be demonstrated for all group III mGluC termini in vitro. Thus, sumoylation may be a common post-translational modification of these pre-synaptic receptors.

Our pull-down assays with truncation or point mutants of the mGluR7a- and mGluR8a-CTDs show that binding of Pias1 to the receptor C termini can occur independently of the presence of the actual sumoylation site (mGluR7a-N38) or the target lysine residue (mGluR8a-K882R, mGluR7a-K889R). We therefore suggest that a min-
imal binding sequence must exist outside of the consensus conjugation site. Using partial constructs of mGluR8a-C and mGluR7a-C, this minimal binding sequence could be shown to reside within six amino acids preceding the consensus conjugation site (mGluR8a 875–880, DRPENGE), a motif that is conserved among mGluR7 and -8 isoforms and, to a lesser extent, in mGluR4. Notably, Pias1 binding was also found with mGluR6 in both yeast two-hybrid and pull-down assays. Among group III mGluRs, mGluR6 is unusual for several reasons: it is localized postsynaptically, is exclusively expressed in retina, and lacks the ability to interact with Ca<sup>2+</sup>/calmodulin, which recognizes all other group III mGluRs (27, 33, 34). As mGluR6 also lacks the consensus sumo-conjugation motif and harbors only two (Pro, Asn) of the six amino acids within the DRPENGE motif common to all other group III mGluRs, we speculate that these two amino acids, which are separated by a gluta- mine, may be sufficient to mediate Pias1 binding. Alternatively, other more homologous regions of the receptor CTDs that are also present in mGluR6 may contribute to the binding of Pias1, although our studies using truncations of mGluR7a and mGluR8a do not provide evidence for the existence of additional interacting sequences. Group II mGluRs, which lack both, the consensus sumoylation site and the Pias1 interaction domain, did not show an interaction with Pias1 in the yeast two-hybrid system. To investigate whether the sequence DRPENGE could be a common binding motif for Pias1, we performed a data base search for short, nearly exact matches. This revealed that only one other mamma- lian gene family, the ING tumor suppressor protein family, contains a >80% identical sequence (i.e. DRPENG). ING proteins are proteins rich in non-consensus lysines and belong to the zinc finger protein family. Zinc finger proteins (22), but not ING proteins, have been reported to constitute a target of Pias1-mediated sumoylation. In fact, one of the lysines in the vicinity of the DRPNGE motif is predicted to be available for sumoylation (www.abgent.com/doc/sumoplot). The domains of Pias1, which mediate the interaction with group III mGluRs, are not yet defined. The fact that our two-hybrid screen isolated a Pias1 fragment encoding only the C-terminal amino acids 514–721 suggests that bind- ing to the target sequence occurs downstream of the zinc finger domain of Pias1 (residues 401–453) that carries the sumo ligase activity. Thus, the interaction and catalytic domains of Pias1 seem to be distinct.

Overexpression of components of the sumoylation machinery in HEK293 cells allowed us to demonstrate conjugation of sumo1 to GFP-mGluR8a-C, provided protease inhibitors that prevent de-sumoylation (31) were added during extract preparation. The fact that sumoylation was also seen without Pias1 co-transfection is in agreement with the ubiquitous expression of this protein and of other Pias family members in many cell types, including HEK293 cells where Pias1 is readily detected by Western blotting. Convincing evidence for a crucial role of Pias1 in sumo1 conjugation in vivo comes from experiments in COS-7 cells in which sumoylation of STAT1 was decreased upon co-expression of a dominant-negative Pias1 mutant (35). Also, in vitro bacterially expressed GST-Pias1 has been shown to produce a dose-dependent stimulation of sumo-conjugation of in vitro translated p53 (21). In conclusion, all presently available data are consistent with an essential role of Pias1 in diverse sumoylation reactions, and hence we propose that the sumoylation of GFP-mGluR8a-C demonstrated here depends on its interaction with Pias family members.

Whereas ubiquitination of GPCRs is a well documented phenomenon (36) that appears to play a role in group I mGluR degradation (37), the related but functionally distinct sumoylation cascade has not yet been linked to any plasma membrane receptor. To our knowledge, our results represent the first evidence for sumo-conjugation of a GPCR. All group III mGluRs except mGluR6 contain a consensus sumoylation motif ΦKX(D/E)(Φ, hydrophobic; K, acceptor lysine; D/E, acidic; Φ, any residue). Arginine substitution of the lysine 882 residue within the consen- sus sumoylation motif of the GFP-mGluR8a-C protein revealed that sumoylation occurs at the predicted consensus site. Consensus site sumoylation has been documented for many sumo targets, but sumoy- lation at lysines outside of consensus motifs has also been described for polypeptides such as huntingtin (38), the promyelocytic leukemia gene product (39), or proliferating cell nuclear antigen (40). In contrast to ubiquitination, sumo1 conjugation never results in formation of sumo chains at the conjugation site. The size shift seen here in SDS-PAGE (molecular mass of sumo1 ~15 kDa) upon overexpression of components of the sumoylation cascade is consistent with the attachment of a single CFP-tagged sumo1 molecule to GFP-mGluR8a-C. Whereas in huntingtin all three non-consensus lysines could be conjugated in HeLa cells and identification of the most relevant residue was possible only by multiple lysine substitution (38), single or combined mutations of the different lysine residues in the C-terminal portion of the mGluR8a CTD reliably produced sumoylation of only the consensus lysine 882. Inversely, substitution of Lys<sup>882</sup> did not redirect the sumoylation machinery to neighboring lysines, which underlines the sequence specific- ity of this covalent modification. Thus, the sumoylation complex, once attached to the mGluR8-CTD, does not modify non-consensus lysine side chains upon substitution of the primary target residue. This cannot be attributed to a masking of alternative acceptor sites by con- structively bound Ca<sup>2+</sup>/calmodulin, because our experiments were performed in the presence of divalent cation chelators, which fully prevent calmodulin binding to group III mGluR CTDs (28).

The physiological consequences of sumo-modification of mGluR8 are presently unknown. If sumoylation would merely antagonize ubiqui- titination, mGluRs should be subject to ubiquitination reactions. Indeed, a group I mGluR has been shown to bind a protein involved in photoreceptor cell differentiation via the ubiquitin/proteasome path- way (41). This protein, named mammalian homologue of Drosophila seven in absentia (Siah-1A), has also been found to regulate Ca<sup>2+</sup>/calmodulin binding to group I mGluRs, and thus to modulate signal transduction. The functional significance of this finding was questioned because of the fact that truncated versions of Siah-1A lacking domains required for the ubiquitin/proteasome pathway displayed similar regulatory effects (42). However, a recent report (37) provides clear evidence that Siah-1A indeed mediates ubiquitination and subsequent degrada- tion of group I mGluRs. Thus, the regulation of mGluR ubiquitination could be one of the functional roles of sumo-conjugation to group III mGluR CTDs.

Recent studies indicate that sumoylation may also have regulatory effects on protein-protein interactions (17). A sumo-binding motif (SBM) in RanBP2 can bind the sumo-conjugated RanGAP1. This SBM consists of a (V/I)X(V/I)(V/I) sequence, a motif found in many proteins that are related to sumo-dependent processes. Among the proteins known to interact with some of the group III mGluR-C termini, only syntenin (43) and filamin A (44) contain one, or six, bona fide SBMs, respectively. The fact that these proteins only interact with the non- conserved, isoform-specific extreme C termini of some group III recep- tors is inconsistent with Pias1 binding to regions conserved in all group III mGluR CTDs as described here. Thus, sumo-interacting proteins recognizing these conserved regions of mGluR CTDs remain to be identified.

A yet unsolved cell biological question is where mGluRs and the sumoylation cascade are likely to meet. There is circumstantial evidence

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5 Z. Tang, unpublished results.
that sumoylation can happen at the plasma membrane. Dynamin has been shown to interact with ubc9, sumo1, and Pias1 without being sumoylated itself (45), and to be involved in internalization of the group III mGluR4 (46). Furthermore, overexpression of the sumoylation cascade in mammalian cells can down-regulate the dynamin-mediated endocytosis of other proteins, e.g. transferrin (45). It therefore is tempting to speculate that dynamin serves to localize the primarily nuclear sumoylation machinery in the vicinity of target plasma membrane proteins. A convincing demonstration that components of the sumoylation machinery are indeed present at the cytoplasmic face of the plasma membrane comes from confocal imaging of Xenopus oocytes expressing the sumo1 conjugation enzyme ubc9 (26). In oocytes, ubc9 shows a uniform, nonpolarized distribution beneath the plasma membrane, thereby confirming that this protein actually reaches this cellular compartment.

In summary, sumo modification of G-protein-coupled receptors may have different regulatory functions in targeting and modulating receptor signaling. Further studies will be needed to understand the specific roles, regulation, and kinetics of sumo-conjugation to group III mGluRs.

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