Trout red blood cell arrestin (TRCarr), a novel member of the arrestin family: cloning, immunoprecipitation and expression of recombinant TRCarr

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Arrestins are cytosolic proteins involved in the desensitization of G-protein-coupled receptors. We report the cloning of trout red blood cell arrestin which shows 76, 82 and 52 % identity with bovine β -arrestin1, β -arrestin2 and retinal arrestin respectively. Antibodies were generated against the C-terminus of trout red blood cell arrestin. These antibodies detected arrestin in erythrocyte cytosol and were able to precipitate the native protein. The Na^+/H^+ antiporter of trout red blood cell is activated by β -adrenergic stimulation and is then desensitized whereas the transmembrane signalling pathway is not. To investigate the subcellular distribution of arrestin on β -adrenergic activation

and desensitization of the antiporter, precipitation experiments were carried out on trout erythrocytes. A desensitization-dependent shift in cytosolic arrestin to the membranes could not be detected using the immunoprecipitation technique but we cannot exclude the possibility that a small number of cytosolic arrestins might be involved in the regulation of membrane proteins in trout erythrocyte. Recombinant trout arrestin was produced in a protease-deficient *Escherichia coli* strain and its functionality was tested in a reconstituted rhodopsin assay. The recombinant protein provides a suitable tool for investigating the target for arrestin in trout red blood cell, which still remains to be identified.

INTRODUCTION

A decrease in cellular responsiveness to persistent external stimuli, termed desensitization, is the result of complex regulatory processes at different levels of the transmembrane transduction cascade of a variety of visual, chemical and hormonal signals. Over the last decade a growing number of receptors and channel systems has been reported to be regulated by activation-dependent desensitization phenomena [1–3]. However, as a model, two G-protein-coupled receptor systems have mainly contributed to our knowledge of mechanisms implicated in the desensitization process: photoactivation of cGMP phosphodiesterase via the transducin-coupled rhodopsin [4] and hormonal activation of adenylate cyclase via the G_s -protein-coupled β -adrenergic receptor [5,6]. In both systems, agonist-mediated homologous desensitization is characterized by a two-step process leading to uncoupling of the receptor from its G-protein. First, specific kinases (rhodopsin kinase or β -adrenergic receptor kinase) phosphorylate the agonist-occupied forms of the receptors [7,8]. This phosphorylation allows subsequent stoichiometric binding of an inhibitory cytosolic protein, which is arrestin in the case of rhodopsin [4,9] and β -arrestin for the β -adrenergic receptor [10,11]. Coupling of the respective arrestins precludes receptor–G-protein interactions and thus interrupts the activation process [12]. In addition, arrestins are supposed to interfere with regulatory receptor kinases and phosphatases as their binding prevents further kinase-mediated phosphorylation as well as the dephosphorylation of occupied receptors by blocking the access of protein phosphatases [13,14].

Retinal arrestin was the first member of the arrestin family to be genetically identified [9], and was shown to be identical with a highly autoantigenic 48 kDa protein already described in the cytosol of photoreceptor cells [15,16]. This so called ‘S-antigen’ was considered to be responsible for immunogenic ocular inflammatory diseases [17,18]. Further immunocytochemical studies indicated retinal localization of arrestin in different species [18–20]. Initially these proteins were thought to be specific for photosensitive cells. However, antibodies directed against retinal arrestin also showed immunological reactivity in histological preparations from a variety of mammalian, avian and fish tissues and cells not known to be photosensitive [21–23], suggesting that members of this gene family might also be implicated in the regulation of signals other than light-induced transmembrane signals. Cloning of the first arrestin homologue from non-photosensitive cells in the bovine brain, termed β -arrestin [10], finally provided evidence for the existence of arrestin-like proteins in extraretinal tissues. So far, four members of the arrestin gene family have been cloned from vertebrates, including retinal arrestin [9], retinal cone (C)-arrestin [24], β -arrestin1 [10] and β -arrestin2 [25–27]. In addition, two arrestin-like *Drosophila* proteins have been found for invertebrates [28–30].

An arrestin-like protein has been previously detected in cytosolic extracts of nucleated trout and turkey erythrocytes utilizing antibodies raised against bovine retinal arrestin [22]. Moreover, trout arrestin-enriched cytosolic fractions bind to bovine retinal rhodopsin when it is photoactivated and phosphorylated by rhodopsin kinase [31]. The ability of trout arrestin to substitute for retinal arrestin suggests that it is

Abbreviations used: GST, glutathione S-transferase; mAb, monoclonal antibody; ORF, open reading frame; TRC, trout red cell; TRCarr, TRC arrestin; rTRCarr, recombinant TRCarr; IPTG, isopropyl 1-thio- β -D-galactopyranoside; ROS, rod outer segment; PRD, phosphorylation-recognition domain.

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The sequence data for the three isoforms of trout red cell arrestin will appear in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession numbers U48410, U48411 and U48412.

involved in a desensitization process in trout erythrocytes. The appropriate target for this arresting protein, however, has not yet been identified. After stimulation of trout red blood cells by catecholamines, the β -adrenergic signalling pathway remains activated for hours whereas the catecholamine-activated cAMP-dependent Na^+/H^+ exchanger is rapidly desensitized [32,33]. We have speculated that arrestin could be involved in the desensitization of the membrane transport system [34]. To evaluate the functional significance of the red blood cell arrestin, a series of biochemical and immunological approaches has been engaged.

Here we describe cloning of trout red cell arrestin (TRCarr), a novel member of the arrestin family. Highly sensitive antibodies were generated against a bacterial fusion protein containing C-terminal amino acids derived from TRCarr cDNA which allowed us to perform immunoprecipitation experiments on native trout erythrocytes. In addition, recombinant TRCarr (rTRCarr) was produced in the protease-deficient *Escherichia coli* strain BL-21 and shown to be functionally active in a reconstituted bovine retinal system. This recombinant protein should allow a more detailed investigation of the role of native arrestin in the trout red blood cell.

EXPERIMENTAL

Isolation and nucleotide sequencing of TRCarr cDNA

Synthetic oligonucleotides were deduced from the bovine retinal S-antigen cDNA sequence [9] and labelled with [γ - ^{32}P]ATP by phosphorylation with bacterial T4 polynucleotide kinase. They were used as a probe to screen a commercially available bovine retinal cDNA λ gt11 library (Clontech, Palo Alto, CA, U.S.A.). A 520 bp arrestin cDNA fragment (931–1450) was isolated and subcloned into the *Eco*RI restriction site of pBluescript II (Stratagene, San Diego, CA, U.S.A.) and its nucleotide sequence was checked by the dideoxynucleotide chain-termination method [35]. Total RNA from adult trout circulating red blood cells and cephalic kidney were isolated by standard procedures [36,37]. mRNAs were selected by chromatography on oligo(dT)–cellulose and reverse-transcribed using an oligo(dT) primer. The resulting cDNAs were size-selected on electrophoresis gels and two libraries were constructed from the fragments above 1 kb, a red blood cell library (λ ZAP kit; Stratagene) and a cephalic kidney library (λ gt10 kit; Stratagene). The retinal arrestin 520 bp fragment, labelled with [α - ^{32}P]dCTP by random priming (Boehringer, Mannheim, Germany), was used as a probe to screen 6.5×10^5 plaques from the red blood cell library and 4.3×10^5 plaques from the cephalic kidney library, for 16 h at 65 °C [38]. Positive clones were plaque-purified and the phagemids containing the inserts were rescued from the recombinant λ ZAP using the helper phage R408 or subcloned from the recombinant λ gt10 into pBluescript II (Stratagene). The nucleotide sequences of both strands of the cDNA inserts were determined using the dideoxynucleotide chain-termination method.

Preparation of antibodies against the C-terminus of trout arrestin (anti-TRCarrC)

We had previously used the mouse monoclonal antibody (mAb) S2D2 to detect TRCarr [22]. The S2D2 antibody (kindly provided by Dr. M. Mirshahi and Professor J. P. Faure, Institut Biomédical des Cordeliers, Paris, France) is directed against the N-terminus of bovine retinal arrestin. To confirm the ability of S2D2 to detect specifically the N-terminal part of TRCarr, and to obtain antibodies against the C-terminus of TRCarr, we

constructed fusion proteins utilizing the glutathione S-transferase (GST) gene fusion vector pGEX-1 λ T (Pharmacia). For expression in the *E. coli* strain XL1-Blue, N- and C-terminal coding sequences of cDNA designated TRCarr2S (see the Results section) were ligated in-frame with the 3' end of the coding sequence of GST. DNA fragments encoding amino acid sequences 1–153 (N-terminus) and 329–405 (C-terminus) were synthesized with flanking *Eco*RI restriction sites at both ends for subcloning by PCR. The restricted PCR fragments were inserted into the pGEX-1 λ T vector to form the plasmids pGEX-N-arr and pGEX-C-arr. Both constructs were verified by the dideoxynucleotide chain-termination method. Expression of fusion proteins was induced at 30 °C by adding isopropyl 1-thio- β -D-galactopyranoside (IPTG) to a final concentration of 1 mM. At 3 h after induction, cells were harvested on ice, centrifuged once at 4000 *g* at 4 °C for 10 min and resuspended in 0.1 vol. of ice-cold PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.3). Bacteria were lysed with a French Press (American Instrument Company) at 82.8 MPa in the presence of 20 $\mu\text{g}/\text{ml}$ DNase and 2 mM MgSO_4 . After addition of 0.2 mM PMSF, 5 mM EDTA and 1% Triton X-100, the lysate was centrifuged at 10000 *g* at 4 °C for 15 min. Expression of fusion proteins was verified by SDS/PAGE followed by Coomassie Blue staining. After cell lysis the supernatant with the soluble C-terminal TRCarr fusion protein (GST–TRCarrC) was filtered through 0.45 μm Minisart filters (Sartorius) and loaded on a glutathione–Sepharose 4B affinity-chromatography column (Pharmacia). Affinity-purified GST–TRCarrC was eluted with an excess of GSH (10 mM GSH in 50 mM Tris/HCl, pH 8.0). N-Terminal TRCarr fusion protein (GST–TRCarrN) formed inclusion bodies and remained in the pellet after cell lysis and centrifugation. The pellet was resuspended in tenfold concentrated PBS and kept at –20 °C overnight. After thawing, the pellet was washed in ice-cold PBS at least five times. Rabbits were inoculated intravenously and then boosted after 2, 4 and 8 weeks with 150 μg of the C-terminal fusion protein in incomplete Freund's adjuvant. Sera were collected 6 and 12 weeks after injection and assayed for activity by immunoblotting. IgG fractions were isolated from crude whole sera using octanoic acid. Briefly, most serum proteins except the IgGs were precipitated by dropwise addition of 750 μl of octanoic acid to 10 ml of crude rabbit serum, diluted threefold in 60 mM sodium acetate buffer, pH 4.0. Serum was stirred for 30 min at room temperature and centrifuged once at 12000 *g* at 4 °C for 15 min. Supernatant was filtered through glass-wool fibres and dialysed overnight against PBS. The *E. coli* strain XL1-Blue was transformed with the pGEX vector and production of GST was induced by 1 mM IPTG. Cells were harvested, lysed by French Press and centrifuged as described above. The soluble material from the supernatant was cross-linked to CNBr-activated Sepharose 4B gel (Pharmacia) according to the manufacturer's instructions. This GST–*E. coli*–Sepharose affinity column was finally used to eliminate antibodies against *E. coli* GST and other bacterial contaminants from the IgG fractions. Specific antibodies against the C-terminus of TRCarr, referred to as anti-TRCarrC, were collected from the flow-through fractions and then tested by Western-blot analysis.

Preparation of trout erythrocyte membrane and cytosolic extracts

Trout and turkey erythrocytes were prepared as previously described [22] and lysed on ice in 10 vol. of isotonic lysis buffer (145 mM NH_4Cl , 4 mM KCl, 10 mM NaHCO_3 , 15 mM Hepes, 5 mM CaCl_2 , 1 mM MgSO_4 , 1 mM PMSF, 0.1 mM Tos-Phe- CH_2Cl , pH 7.5). Cell nuclei and cell debris were removed by

centrifugation at 4000 *g* at 4 °C for 10 min and the supernatant was centrifuged at 24000 *g* at 4 °C for 30 min to separate membranes from cytosolic proteins. The membrane fraction was resuspended in one initial cell volume of lysis buffer. The protein concentration of both membrane and cytosolic fractions was quantified before immunoprecipitation experiments. As a negative control, a corresponding amount of BSA was subjected to immunoprecipitation. To investigate whether stimulation of β -adrenergic receptors has an effect on the subcellular distribution of TRCarr, trout erythrocytes were stimulated with 0.55 μ M isoprenaline. This dose of catecholamine induces a maximal response of the Na⁺/H⁺ antiporter [39]. Cells were divided into three equal portions, which were stimulated for 0 (control), 10 or 30 min, and immediately processed as described above.

Western-blot analysis

For Western-blot analysis 1 ml aliquots of induced and non-induced bacteria were centrifuged once and resuspended in 150 μ l of ice-cold PBS. From erythrocytes 150 μ l aliquots of tenfold diluted cytosol were obtained as described above. The samples were diluted in an equal volume of SDS-loading buffer (2 % SDS, 20 % glycerol, 60 mM Tris, 0.002 % Bromphenol Blue, 2 % 2-mercaptoethanol, pH 6.8) and run on SDS/12 % polyacrylamide gels. The gels were transferred to nitrocellulose membranes (Hybond C; Amersham) and incubated with the purified anti-TRCarrC antibodies diluted 1:3000. Immunoreactive bands were visualized by chemiluminescence (ECL; Amersham) after incubation with horseradish-peroxidase-conjugated goat anti-rabbit secondary antibodies (Sigma). Bovine retinal arrestin (a gift from Dr. M. Mirshahi and Professor J. P. Faure) and recombinant bovine brain β -arrestin (a gift from Professor M. J. Lohse, Genzentrum München, Martinsried, Germany) were also included as controls.

Immunoprecipitation of native TRCarr

The capability of the purified fusion protein antibodies to immunoprecipitate TRCarr from trout erythrocytes was assayed by addition of 1–10 μ l of anti-TRCarrC to 1 ml of both membranes and cytosolic fractions, prepared as described above. After mixing of the samples overnight at 4 °C, immunocomplexes were collected on 100 μ l of Protein A-Sepharose 6MB (Pharmacia) for 2 h at 4 °C and then washed five times with ice-cold immunoprecipitation buffer N⁺ (150 mM NaCl, 3 mM KCl, 60 mM Hepes, 5 mM EDTA, 0.1 mM PMSF, 0.1 mM Tos-Phe-CH₂Cl, 1 % Triton X-100, pH 7.4). Antigens were released by incubation of the Sepharose for 10 min at 85 °C with 100 μ l of SDS-sample buffer (SDS-loading buffer without 2-mercaptoethanol). The Sepharose was removed by centrifugation and the supernatant was analysed by SDS/PAGE (12 % gel). Western-blot analysis was carried out as described above, except that the mouse mAb S2D2 was used to detect immunoprecipitated TRCarr. Horseradish-peroxidase-conjugated goat anti-mouse secondary antibodies (Bio-Rad) were used to visualize immunoreactive bands by chemiluminescence.

Expression of rTRCarr in *E. coli*

The pET expression system (Novagen) was used to produce rTRCarr in the *E. coli* strain BL-21, which lacks *lon* and *ompT* proteases. TRCarr cDNA was amplified by PCR with either *Nde*I or *Bam*HI restriction sites just upstream of the start codon and a *Xho*I restriction site just upstream of the stop codon. The restricted PCR fragments were ligated into the pET21a vector,

previously digested with either *Nde*I and *Xho*I or *Bam*HI and *Xho*I, to give the plasmids pET-TRCarr-His and pET-T7-TRCarr-His respectively. Both constructs were verified by the dideoxynucleotide chain-termination method. The created plasmids encode TRCarr with an additional six-histidine tag at the C-terminus; in addition, pET-T7-TRCarr-His encodes an N-terminal T7 tag, which can be detected by mAb T7 (provided by Novagen). The plasmids were introduced into *E. coli* strain BL-21, and expression of the recombinant proteins was induced at 30 °C by adding IPTG to 1 mM final concentration. At 3 h after induction cells were harvested on ice, centrifuged at 4000 *g* at 4 °C for 10 min and then resuspended in 0.1 vol. of ice-cold binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9). Bacteria were lysed with a French Press at 82.8 MPa in the presence of 40 μ g/ml DNase, 0.2 mM PMSF, 0.1 mM Tos-Phe-CH₂Cl and 5 mM EDTA. After the addition of 1 % Triton X-100, the lysate was centrifuged at 10000 *g* at 4 °C for 15 min. The supernatant was filtered through 0.45 μ m filters and loaded on a Ni²⁺-affinity-chromatography column (His-Bind® metal chelation resin; Novagen). Recombinant proteins were eluted with an excess of imidazole (1 M imidazole, 500 mM NaCl, 20 mM Tris/HCl, pH 7.9). Expression of the recombinant protein was monitored by samples taken from induced and non-induced whole cells, supernatant and pellet fractions after cell lysis and the eluates from the His-Bind resin. The samples were size-fractionated on SDS/12 % polyacrylamide gels followed by Coomassie Blue staining and by Western blotting using the polyclonal antibody anti-TRCarrC (1:2000), the monoclonal retinal arrestin antibody S2D2 (1:30) or, to identify T7-rTRCarr, the commercially available antibody mAb T7 (1:1000). Suitable horseradish-peroxidase-conjugated anti-species secondary antibodies were used to reveal immunoreactive bands by chemoluminescence. As a negative control, *E. coli* BL-21 was transformed with the pET 21a plasmid and treated in parallel as described above.

Reconstituted rhodopsin assay

Rod outer segment (ROS) disc membranes were prepared from fresh bovine retina as previously described [31,40] and stored under nitrogen at –80 °C. For the assay one batch of ROS was homogenized in the dark, extensively washed in 10 mM Tris/HCl/2 mM EDTA, pH 7.4, to remove endogenous retinal arrestin and resuspended in a 70 mM sodium phosphate buffer, pH 7.0, containing 0.1 mM EDTA and 8 mM MgCl₂. The protein concentration of the ROS preparation was 185 μ g/ml. The binding of rTRCarr to bovine rhodopsin was assayed in the presence of 3 mM ATP and 0.1 mM guanosine 5'-[γ -thio]-triphosphate (to keep transducin in the active form) by mixing in the dark the arrestin-free ROS preparation, containing rhodopsin and membrane-bound rhodopsin kinase, with the soluble cytosolic fraction of induced *E. coli* BL-21, expressing rTRCarr with or without an N-terminal T7 tag. The amount of rTRCarr in the soluble fractions was estimated in Western blots using purified recombinant protein as a standard. Each sample contained about 2 μ g (40 pmol) of rTRCarr and 18.5 μ g (450 pmol) of rhodopsin in a volume of 200 μ l. In addition, the effect of 1 mg/ml heparin (Sigma) on the binding of rTRCarr to rhodopsin was analysed. The assay was carried out in parallel using recombinant bovine β -arrestin1 (30 pmol in each sample) as a positive control. The mixtures were either kept in the dark or exposed to light for 1 h at 30 °C. After centrifugation at 80000 *g* at 4 °C for 3 min, the rhodopsin-arrestin-containing pellets were washed twice in an ice-cold 10 mM Hepes buffer, pH 7.0, and resuspended in 50 μ l of a 500 mM KCl/2 mM

Hepes buffer, pH 7.2. The samples were shaken overnight at 4 °C to remove bound arrestins from the rhodopsin [40,41]. After centrifugation at 25000 *g* at 4 °C for 20 min, the arrestin-containing supernatants were loaded on SDS/11% polyacrylamide gels and tested by Western-blot analysis using anti-TRCarrC (1:1000) and the mAb T7 (1:1000) as primary antibodies. Immunoreactive bands were detected by chemiluminescence using species-specific horseradish-peroxidase-conjugated secondary antibodies.

RESULTS

cDNA cloning reveals the existence of three isoforms of TRCarr

Screening of a trout red blood cell cDNA library for an arrestin homologue led to the isolation and sequencing of 17 positive clones. The inserts ranging from 1.3 to 2.5 kb corresponded to fragments of three different cDNAs. Comparison of the respective sequences revealed the existence of two distinct trout arrestin isoforms designated TRCarr1 [open reading frame (ORF) 1221 bp, encoding 407 amino acids] and TRCarr2 (ORF 1215 bp, encoding 405 amino acids) exhibiting a remarkably high amino acid identity (97.7%). Moreover, a third isoform (ORF 1245 bp, encoding 415 amino acids) could be found, which was identical

with TRCarr1 except for a 24-nucleotide insert in the C-terminal coding sequence (bp 1002–1026, encoding residues 334–341). This insert was also missing from TRCarr2. According to the recently proposed nomenclature for the arrestin/ β -arrestin polypeptides [25], short and long isoforms of TRCarr were named TRCarr1S, TRCarr2S and TRCarr1L respectively (Figure 1). The two short polypeptides differ only in nine amino acids, six of them located at the C-terminus of the protein, including two supplementary amino acids (Lys, Asp) in positions 403 and 404 of TRCarr1S. The third longer isoform TRCarr1L contains an additional C-terminal insert of eight amino acids between residues 334 and 335 (LLSGVLER). In order to check whether any other mRNA encoding for an arrestin-like protein was present in the early developmental stage of erythroblasts and absent from circulating mature nucleated erythrocytes, we also screened a cDNA library from cephalic kidney, which is a haematopoietic tissue in trout. Four additional positive clones that were isolated and analysed were shown to be identical with, or to contain fragments of, the previously identified clones from the red blood cell library.

Sensitivity and specificity of TRCarr antibodies

To check the ability of the mAb S2D2 to detect the N-terminus and to obtain antibodies against the C-terminus of TRCarr, we

BRETarr	<u>MKANKPAPNH</u>	<u>VIFKKISRDK</u>	<u>SVTIYLGKRD</u>	<u>YIDHVERVEP</u>	<u>VDGVVLVDPE</u>	<u>LVKGKRVYVS</u>	60
TRCarr1S	M-GD:-:GT-	RV::S:PNC	K::V:::	FV::LDQ:D:	:::I:::	YL:DRK:F:T	57
TRCarr2S	:-:-:-:-:-	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::E:::	57
TRCarr1L	:-:-:-:-:-	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::D:::	57
BRETarr	<u>LTCAFRYGOE</u>	<u>DIDVMGLSFR</u>	<u>RDLYFSOVVO</u>	<u>FPPVGAS-GA</u>	<u>TTRLOESLIK</u>	<u>KLGAANTYPFL</u>	119
TRCarr1S	:::~::~:R:	:L::L:::	K::I:TF:A	:::IAEERK:	NS:::R:L:	:::QOAH::Y	117
TRCarr2S	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	117
TRCarr1L	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	117
BRETarr	<u>LTFPDYLPDS</u>	<u>VMLOPAPODV</u>	<u>GKSCGVDFEI</u>	<u>KAFATHSTDV</u>	<u>EEDKIPKSS</u>	<u>VRLILRKVQH</u>	179
TRCarr1S	FTI:QN:::	:T::G:E:T	::A:::	R::CAK:--I	::-::H:RN:	:::V:::Y	174
TRCarr2S	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	174
TRCarr1L	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	174
BRETarr	<u>APRDMGPOPR</u>	<u>AEASWQFFMS</u>	<u>DKPLRLAVSL</u>	<u>SKEIYYHGEP</u>	<u>IPVTVAVTNS</u>	<u>TEKTVKKIKV</u>	239
TRCarr1S	:::EKP:::M	V:TTRS:L::	:RS:H:EA::	D::L:::	:S:N:H::N	ST:::RL:I	234
TRCarr2S	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	234
TRCarr1L	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	234
BRETarr	<u>LVEQVTNVVL</u>	<u>YSSDYIKTV</u>	<u>AAEEAQEKVP</u>	<u>PNSSLTKTLT</u>	<u>LVPLLANNRE</u>	<u>RRGIALDGKI</u>	299
TRCarr1S	S:R:YADIC:	F:TAQ:KCP:	:QV::DDQ:S	SS:TFC:VY:	:T:T:DK::	K::L:::L	294
TRCarr2S	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	294
TRCarr1L	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	:::~::~:	294
BRETarr	<u>KHEDTNLASS</u>	<u>TIIEGIDKT</u>	<u>VMGILVSYQI</u>	<u>KVKLTVS-G-</u>	<u>LLGELTSSEV</u>	<u>ATEVPFRLMH</u>	357
TRCarr1S	:::~::~:	::V:DVIN:E	:L:::RV	:::VI:R:G	-----D:	SV:L::V::	346
TRCarr2S	:::~::~:	:::~::~:	:::~::~:	:::~::~:	-----:	:::~::~:	346
TRCarr1L	:::~::~:	:::~::~:	:::~::~:	:::~::~:	LLSGVLER::	:::~::~:	354
BRETarr	<u>PQP-EDPDTA</u>	<u>KESFQDENFV</u>	<u>FEEFARQNLK</u>	<u>DAGEYKEEKT</u>	<u>DQEAAMDE</u>		404
TRCarr1S	:K:T:L:ISR	PQ:AVPDSDP	PIDTNLIEFE	TNSFSQDDDF	VF:DFARLRL	KGMADDKDDDC	407
TRCarr2S	:::~::~:	:H:::E::	:::~::~:	S:::~::~:	:::~::~:	:::~::~:	405
TRCarr1L	:::~::~:	:Q:::D::	:::~::~:	T:::~::~:	:::~::~:	:::~::~:	415

Figure 1 Amino acid sequence alignment of the three TRCarr isoforms (TRCarr1S, TRCarr2S and TRCarr1L) with bovine retinal arrestin (BRETarr [9])

A colon means identical amino acid with that just above; a hyphen makes a gap to optimize alignment. The primary interaction domain of BRETarr (2–191) is underlined and the putative phosphorylation-recognition domain (162–181) is double underlined [12].

Figure 2 Fusion proteins used to test S2D2 and to generate anti-TRCarrC antibodies

(a) Coomassie-Blue-stained gel: GST, wild-type enzyme; GST + TRCarrC and GST + TRCarrN, fusion proteins between GST and C- and N-terminus of TRCarr2S respectively; tot., *E. coli* total protein (30 μ g); pur., purified fusion protein (8 μ g). (b) Western blots labelled with anti-TRCarrC (left) and S2D2 (right).

created fusion proteins of bacterial GST with N- and C-terminal domains of TRCarr2S (Figure 2a). Subcloning of the shorter more hydrophilic C-terminal fragment yielded a soluble fusion protein (GST + TRCarrC, 35 kDa). In contrast, fusion of the less hydrophilic N-terminus to GST resulted in insoluble aggregates, also containing endogenous bacterial proteins in the inclusion bodies (GST + TRCarrN, 45 kDa). IgG fractions of the polyclonal antisera raised against GST + TRCarrC were purified on affinity columns with immobilized cytosolic proteins of induced *E. coli*, expressing wild-type GST. The absence of antibodies directed against GST or non-specific bacterial proteins in the flow-through of this purification step was tested by Western-blot analysis of induced bacterial whole cells expressing either GST alone (negative control) or the fusion proteins. The labelling by anti-TRCarrC (1:3000) was highly specific because no other bands were recognized (Figure 2b). mAb S2D2 (1:30), raised against the N-terminus of bovine retinal arrestin, was also used to analyse the fusion products. It specifically labelled the N-terminal but not the C-terminal fusion proteins (Figure 2b). S2D2 was subsequently routinely used in Western-blot analysis to label the N-terminal part of TRCarr.

Western-blot analysis using anti-TRCarrC (1:3000) revealed an approx. 47 kDa protein in the cytosol of trout erythrocytes

(Figure 3). In addition, anti-TRCarrC also detected arrestin from nucleated turkey red blood cells. Purified bovine retinal arrestin and recombinant bovine β -arrestin1 were also recognized by anti-TRCarrC with the same high sensitivity (Figure 3).

Immunoprecipitation of native TRCarr from red blood cells

Anti-TRCarrC antibodies were able to immunoprecipitate native arrestin in a crude cytosolic trout red blood cell fraction at a dilution of 1:200. Anti-TRCarrC (1:200) was subsequently routinely used for immunoprecipitation experiments, and Western-blot analysis of anti-TRCarrC precipitates was developed using the N-terminal retinal arrestin mAb S2D2 (1:30). Our detection method did not show any significant non-specific background, as estimated by immunoprecipitation of BSA, adjusted to the same protein concentration, or detection of anti-TRCarrC alone run directly on the gel. Conversely, a strong band at about 47 kDa was revealed in the erythrocyte cytosolic fraction (Figure 4). To analyse possible changes in the subcellular distribution of TRCarr in trout erythrocytes, both cytosolic and solubilized membrane fractions were subjected to immunoprecipitation with anti-TRCarrC. A signal of immunoprecipitated TRCarr was present in the membrane fraction. The membrane

Figure 3 Western-blot analysis of trout and turkey red blood cells labelled with anti-TRCarrC (20 μ l of tenfold diluted cytosol per lane)

arr, 2 μ g of purified bovine retinal arrestin; r β arr, 3 μ g of recombinant bovine β -arrestin1.

Figure 4 Immunoprecipitation with anti-TRCarrC of arrestin from cytosol and membranes of trout erythrocytes

Western-blot analysis was carried out with S2D2. BSA, immunoprecipitation of 24 mg of BSA; ab, 2.25 μ g of anti-TRCarrC antibodies used in the immunoprecipitation procedure run directly on the gel. Immunoprecipitation of trout erythrocyte fractions; cyto., 24 mg of cytosolic protein; mb, 1.5 mg of protein from the membrane fraction; cont., 1.5 mg of cytosolic protein.

fraction signal was compared with the signal obtained with the same amount of protein from the cytosolic fraction. The results show that contaminant cytosolic proteins alone cannot account for the signal observed in the membrane fraction, indicating the presence of membrane-bound arrestin.

In a second step, the distribution of TRCarr upon β -adrenergic-receptor-mediated desensitization of the Na^+/H^+ exchanger from trout erythrocytes was investigated. Immunoprecipitation experiments were carried out on cytosolic and membrane fractions of native red blood cell before and 10 and 30 min after stimulation by 0.55 μM isoprenaline. This stimulating dose induces a maximal response of the Na^+/H^+ exchanger and subsequent desensitization [33]. The Western blot in Figure 5 shows no significant isoprenaline-induced change in the distribution of TRCarr during the time course of cAMP-mediated activation and desensitization of the Na^+/H^+ antiporter protein.

Expression of rTRCarr

Several attempts were made at expressing rTRCarr in bacterial hosts including the use of different expression strategies and

Figure 5 Immunoprecipitation with anti-TRCarrC of arrestin from cytosol and membranes of trout erythrocytes before (control), 10 min and 30 min after β -adrenergic stimulation by 0.55 μM isoprenaline

Western-blot analysis was carried out with S2D2. cyto., 24 mg of cytosolic protein; mb., 1.5 mg of protein from the membrane fraction.

Figure 6 Recombinant TRCarr

(a) Coomassie-Blue-stained gel showing *E. coli* (BL-21 strain) total proteins (25 μ g) after 3 h IPTG induction at 30 $^{\circ}\text{C}$ of cells transformed with wild-type pET21a (control) or pET-TRCarr-His (tot.); rTRCarr, Ni^{2+} -affinity-purified protein (1.5 μ g). (b) Western blots labelled with anti-TRCarrC (left) or S2D2 (right).

different bacterial strains. When utilizing a heat-inducible pT7 expression system [42] to produce recombinant TRCarr in the *E. coli* expression strain XL1-Blue, we could demonstrate the induction-dependent expression of a 47 kDa protein by [^{35}S]Met

Figure 7 Western blots of rhodopsin-bound proteins

Light-dependent interaction between bovine rhodopsin and recombinant trout arrestin (rTRCarr-T7) and bovine β -arrestin1 (r β arr) was assayed in the absence or presence of 1 mg/ml heparin. rTRCarr-T7 was labelled with anti-(T7 tag) and r β arr with anti-TRCarrC.

labelling. This protein was identified on autoradiograms of induced bacterial samples and shown to be proteolytically degraded within 5 min of induction. With the pT7 expression system, induction of transformed bacteria is achieved by shifting the culture growth temperature to 42 °C. Kinetics of [35 S]Met labelling revealed that in *E. coli* X11-Blue the amount of labelled TRCarr decreased after 2 min, whereas in the *Ion* protease-deficient *E. coli* strain Y-1091 the induction-dependent 35 S radioactivity was shown to increase for 20 min. In both strains, with lower induction temperature the production period was only slightly prolonged but proteolytic degradation was slowed down. Thus we decided to use an IPTG-inducible expression system (pET21a vector) allowing production of the subcloned TRCarr2S at a constant temperature of 30 °C. Even with these more stringent temperature conditions we were able to produce rTRCarr only when using the *E. coli* strain BL-21, which lacks both endogenous *Ion* and *ompT* proteases. Tests were made using various IPTG concentrations and expression times. Best results were obtained for 1 mM IPTG and a maximum of 3 h of induction at 30 °C. On Coomassie-Blue-stained SDS/polyacrylamide gel, expression of rTRCarr was detectable only after the Ni^{2+} -affinity-purification step (1.5 μg of rTRCarr; Figure 6a). However, Western-blot analysis of induced cells revealed the expression of an approx. 47 kDa protein in pET-TRCarr-His-transformed BL-21, which is specifically labelled by either anti-TRCarrC or S2D2 (Figure 6b). The apparent molecular mass and the labelling by the two antibodies, specific for either C- or N-terminal arrestin domains, indicate that the expression product probably represents the integral TRCarr protein. In addition, the commercially available mAb T7 labelled an identical 47 kDa expression product when inducing BL-21 transformed with pET-T7-TRCarr-His (not shown).

rTRCarr functionally binds to light-activated and phosphorylated rhodopsin

To analyse whether rTRCarr was functionally active, crude soluble fractions from bacteria expressing rTRCarr (with or without an N-terminal T7 tag) were assayed for activity in a reconstituted bovine rhodopsin system. As shown in Figure 7, rTRCarr-T7 bound to phosphorylated and light-activated rhodo-

psin from bovine retinal ROS. In the presence of endogenous membrane-bound rhodopsin kinase, however, negligible binding of rTRCarr-T7 to the non-photoactivated rhodopsin was observed. The same results were obtained with rTRCarr (without the T7 tag; not shown). The absence of endogenous arrestin (which might have contributed to the signals) was confirmed by control samples without the addition of rTRCarr (not shown). The Western blots shown in Figure 7 clearly demonstrate that binding of rTRCarr to the light-activated rhodopsin was much more pronounced than to non-photoactivated rhodopsin. Moreover, the addition of 1 mg/ml heparin to the samples, previously shown to inhibit arrestin–rhodopsin interaction, completely blocked the binding of rTRCarr to rhodopsin, even after photoactivation. Recombinant bovine β -arrestin1 was assayed in parallel and showed a similar pattern of interaction with rhodopsin to that of rTRCarr. The signals obtained in Western blots with β -arrestin1 were also abolished in the presence of 1 mg/ml heparin.

DISCUSSION

Relationship between TRCarr polypeptide variants and β -arrestins

Amino acid sequence analysis reveals a close relationship between TRCarr1S and β -arrestins (82 and 76 % identity with bovine β arr2 and β arr1 respectively) and a 52 % identity with bovine retinal arrestin. However, we could demonstrate that both the native and recombinant protein were able to functionally replace the retinal homologue in a reconstituted rhodopsin system. In analogy to retinal arrestin, both native and recombinant TRCarr bound to light-activated and phosphorylated bovine rhodopsin [31]. Multiple functional domains have been found to be involved in the interaction of visual arrestin with rhodopsin [12]. The primary interaction domains are supposed to be localized within the first 191 residues, comprising a 'phosphorylation-recognition' domain (PRD), probably involving residues 162–181 which are well conserved in all members of the arrestin family, and an 'activation-recognition' domain. Interestingly, the N-terminal portion of TRCarr shares about 64 % identity with retinal arrestin and 67 % identity within the PRD. Similar values can be obtained by comparison of the sequences of β -arrestin1 with retinal arrestin. The first 191 N-terminal residues share about 59 % and the PRD 67 % identical amino acids. In addition, β -arrestin was also found to bind to photoactivated and phosphorylated rhodopsin [26]. Taken together, these results suggest a very close functional relationship between genetically distinct arrestins. Although retinal arrestin and β -arrestin are interchangeable, it has been demonstrated that they are more potent in desensitizing their appropriate receptor, i.e. the light and β -adrenergic receptors respectively [26,43]. The appropriate target for TRCarr in trout erythrocytes still remains to be identified.

Two of the cloned TRCarr isoforms, designated TRCarr1S and TRCarr1L, have been shown to be identical except for an extra eight amino acid insert at the C-terminus. It is noteworthy that human and bovine β -arrestin1 have an identical eight amino acid insert present or absent at position 334, also giving S- and L-isoforms. Analysis of human gene structure has shown that β -arrestin S- and L-isoforms are the products of a single gene, suggesting that two transcripts may be produced by alternative splicing of the respective gene [44]. In the human retinal arrestin gene, the 24 homologue nucleotides represent exon 13 [45]. It is tempting to speculate that polypeptide variants TRCarr1L and TRCarr1S might be created by the same 'cassette exon' mechanism of alternative splicing of the TRCarr gene. The existence

of polypeptide variants appears to be a common phenomenon within members of the arrestin gene family. Immunohistological studies demonstrated that most of the respective isoforms co-localize [25,26,46], whereas some of them exhibit clearly distinct tissue-distribution patterns [26,47]. Polypeptide diversity could thereby not only enhance targeting specificity but might also affect the degree of regulatory and/or arresting capacity of these widespread cytosolic proteins. However, the exact functional role of polypeptide variants remains to be elucidated.

Immunological detection and immunoprecipitation of TRCarr

The anti-TRCarrC fusion protein antibody presented here was sensitive enough to detect trout arrestin in crude cytosolic extract even in the presence of a huge amount of haemoglobin, and partial enrichment of cytosolic fractions by FPLC gel filtration [31] and/or immunoaffinity fractionation [22] before Western blotting was not required. $(\text{NH}_4)_2\text{SO}_4$ precipitation of the cytosolic proteins to eliminate haemoglobin, which was one of the procedures necessary to detect TRCarr with bovine retinal arrestin antibodies, did not significantly improve results. Moreover, it is noteworthy that, despite a considerable C-terminal variability in the arrestin gene family, anti-TRCarrC detected turkey red blood cell arrestin and bovine arrestin with the same high sensitivity in control blots (Figure 3).

Anti-TRCarrC was used to immunoprecipitate native TRCarr from trout erythrocyte cytosols and solubilized membranes, and the precipitates were analysed by SDS/PAGE. In the absence of 2-mercaptoethanol, the majority of immunoglobulin bands shifted away from the 48 kDa range, thus allowing detection of immunoprecipitated TRCarr on Western blots. However, to reliably identify the proteins immunoprecipitated by the C-specific anti-TRCarrC antibody, S2D2, which was shown to detect specifically the N-terminus of TRCarr, was used as primary antibody in Western blots. To avoid any cross-reactivity between secondary antibodies used in the Western-blot procedure and rabbit IgGs used in immunoprecipitation, we used horseradish-peroxidase-conjugated goat anti-mouse secondary antibodies, purified by immunoabsorption on rabbit IgG columns (Bio-Rad). Parallel controls using the same amount of pure anti-TRCarrC as in immunoprecipitation experiments directly run on the gel did not reveal cross-reactivity of secondary anti-mouse IgG antibodies with our rabbit IgGs (Figure 4).

We must emphasize that Western-blot analysis of arrestin revealed an unreproducible high-molecular-mass (about 120 kDa) band, which corresponds to aggregated arrestin, as indicated by our selective immunoprecipitation experiments. This was commonly observed with native and recombinant arrestins from different species (Figure 3, Figure 4 mb fraction, Figure 5).

Because of the relatively strong signal of TRCarr in the cytosol, we could not exclude the possibility that at least a part of the membrane signal was from contaminating cytosolic TRCarr. Thus control samples were run in parallel to estimate this potential cytosolic contamination of the membrane extracts. Assuming that all TRCarr in the membrane fraction represents contamination from cytosol, we would expect a signal corresponding to the same amount of cytosolic proteins (see the Experimental section). However, the signal obtained from immunoprecipitated TRCarr in membrane fractions was clearly stronger than that obtained by immunoprecipitation of the same amount of protein from the cytoplasmic fractions, indicating that only a negligible part of the membrane signal could stem from cytosolic contamination (Figure 4).

Desensitization-dependent changes in the subcellular distribution patterns of native TRCarr are not detected by immunoprecipitation

In trout red blood cells, the appropriate target for arrestin has not yet been identified but the presence of β -adrenergic receptors in the membrane of trout erythrocytes suggests that TRCarr might be involved in their desensitization. This seems unlikely because no significant desensitization of β -adrenergic receptors is observed in trout erythrocytes. After β -adrenergic stimulation, intracellular cAMP increases dramatically and levels remain elevated for hours. The persistent high level of cAMP immediately falls when a β -blocker is added and this fall is sensitive to phosphodiesterase inhibitors. This indicates that the constant high level of cAMP observed results from a continuous stimulation of the β -adrenergic receptor G-protein cascade [32]. Thus the exact function of TRCarr in the trout red blood cell remains unclear.

After β -adrenergic stimulation, the activity of a cAMP-dependent Na^+/H^+ exchanger, which is localized in the membrane of trout erythrocytes, increases dramatically and reaches a maximum after 2–3 min. Then it decreases exponentially towards the initial level despite the persistent elevated concentration of cAMP [33]. It is possible to bypass the β -adrenergic receptors by stimulating the antiporter either by addition of exogenous cAMP to the erythrocyte suspension or by stimulation of adenylate cyclase with forskolin. In both cases, the antiporter activity follows the same pattern as after stimulation by β -adrenergic agonists: Na^+/H^+ exchange decreases over time and cannot be re-activated by a fresh challenge of cAMP [33,39]. Thus the decrease in exchange responsiveness to persistent stimulus corresponds to a desensitization of the transport system itself and not to a desensitization of some element of the transmembrane transduction cascade such as β -adrenergic receptors. The transport protein called βNHE has been identified and characterized [48]. Two protein kinase A consensus sites, located at the C-terminus, were shown to be crucial for the exchanger activation by cAMP [49]. The activation and subsequent desensitization of βNHE is controlled by a complex phosphorylation/dephosphorylation process [39], and internalization of the exchanger could occur simultaneously with desensitization [50]. We speculated that an arrestin-like protein could be involved in the desensitization of membrane proteins other than receptors, mainly transport proteins [34]. The catecholamine-dependent $\text{Na}^+-\text{K}^+/\text{2Cl}^-$ co-transport system of turkey red blood cells has also been reported to be desensitized by catecholamine activation [51], and we detected arrestin-like proteins in both trout and turkey erythrocytes [22]. Cloning of both TRCarr and βNHE from trout erythrocytes and generation of specific antibodies raised against the two proteins will allow us to test the putative implication of arrestin in the desensitization of a membrane transport protein.

We used immunoprecipitation experiments in order to study the subcellular distribution of TRCarr in trout erythrocytes. We made an attempt to investigate the potential distribution of TRCarr on exposure of trout erythrocytes to β -adrenergic receptor agonists. We used a concentration of isoprenaline that was shown to cause a maximal stimulation of the Na^+/H^+ antiporter followed by desensitization. In our experiments no detectable isoprenaline-induced change in the distribution of TRCarr during the time course of cAMP-mediated antiporter activation and desensitization was observed. It is noteworthy that, although we attempted to process all the samples the same way, we observed different aggregated arrestin signals (high-molecular-mass band) in the cytosolic fractions. Thus either our

immunoprecipitation technique was not sensitive enough to detect a desensitization-dependent shift of cytosolic TRCarr to the membranes or such a shift does not occur as a result of our stimulation protocol. However, we cannot exclude the possibility that only a few arrestins from the large cytoplasmic pool are implicated in the regulation of an as yet unidentified target in the erythrocyte membrane. Alternatively, although we have screened cDNA libraries from circulating red blood cells as well as haematopoietic tissue, we cannot exclude the possibility that we have failed to detect an arrestin subtype that may be involved in the inactivation of β NHE.

Generation and functional analysis of TRCarr

For a variety of experimental approaches it is necessary to have access to large amounts of TRCarr. However, the isolation of native TRCarr from trout erythrocytes was shown to provide denatured and aggregated proteins. Although we were able to demonstrate the existence of a relatively large cytoplasmic TRCarr pool, the absolute amount of TRCarr in crude cytosol is only of the order of 100 pg/g of total protein, as estimated from FPLC-enriched cytosolic fractions [31]. Thus we made an attempt to produce larger amounts of TRCarr using a recombinant expression strategy with bacterial hosts. In spite of the use of protease inhibitors, rTRCarr seems to be rapidly attacked by endogenous proteases. This was circumvented by using an IPTG-inducible rather than a heat-inducible expression system, together with *E. coli* strain BL-21, which is devoid of endogenous *lon* and *ompT* proteases, cultured at constant low temperature.

The functional integrity of the recombinant protein was then checked in a reconstituted rhodopsin assay. The bovine ROS membrane preparations used were devoid of endogenous arrestin. rTRCarr with or without the N-terminal T7 tag bound to the phosphorylated light-activated rhodopsin from bovine retina, as has been shown for the native protein [31]. In addition, a small amount of binding of rTRCarr to rhodopsin was always observed in the dark, indicating that some phosphorylation of rhodopsin by membrane-bound rhodopsin kinase occurred independently of light activation. rTRCarr as well as β -arrestin1 binding to rhodopsin was completely abolished in the presence of high concentrations of heparin (1 mg/ml), which have been shown specifically to block arrestin–rhodopsin interaction irrespective of light activation and/or the degree of phosphorylation [52]. This indicates that rTRCarr is a suitable tool for investigating further the functional role of TRCarr in the trout erythrocyte and may be helpful in the identification of its appropriate target in these cells.

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