Cellular Signalling 21 (2009) 136–142

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

G-protein-coupled receptor-associated A-kinase anchoring proteins AKAP5 and AKAP12: Differential trafficking and distribution

Min-Huei Chen, Craig C. Malbon *

Department of Pharmacology, School of Medicine, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, NY 11794-8651, United States

ARTICLE INFO

Article history:
Received 30 August 2008
Received in revised form 26 September 2008
Accepted 30 September 2008
Available online 10 October 2008

Keywords:
AKAP5
AKAP79
AKAP12
Gravin
G protein-coupled receptors
β_2-adrenergic receptor
Membrane association
receptor resensitization
Receptor recycling

ABSTRACT

A-kinase Anchoring Proteins (AKAPs) define an expanding group of scaffold proteins that display a signature binding site for the RI/RII subunit of protein kinase A. AKAP5 and AKAP12 are multivalent (with respect to protein kinases and phosphatases) and display the ability to associate with the prototypic member of G protein-coupled receptors, the β_2-adrenergic receptor. We probed the relative abundance, subcellular distribution and localization of AKAP5 and AKAP12 in human embryonic kidney HEK293 and epidermoid carcinoma A431 cells. HEK293 cells are relatively rich in AKAP5 (found mostly in association with the cell membrane); whereas A431 cells are rich in AKAP12 (found distributed both in the cytoplasm and in association with the cell membrane). In biochemical analysis of subcellular fractions and in whole-cell imaging, the membrane localization of AKAP5 was decreased in response to treating cells with the beta-adrenergic agonist isoproterenol, whereas membrane association of AKAP12 was increased initially in response to agonist treatment. These data demonstrate quantitatively a clearly different pattern of AKAP-receptor association for AKAP5 versus AKAP12. AKAP5 remains associated with its G-protein-coupled receptor, at the cell membrane, docked with the receptor during agonist-induced internalization and later receptor recycling after agonist wash-out. AKAP12-receptor docking, in contrast, is dynamic, driven by agonist stimulation (accounting for movement of AKAP12 from the cytoplasm to the cell membrane). AKAP12 then is internalized with the β_2-adrenergic receptor, but segregates away from the G-protein-coupled receptor upon recycling of the internalized receptor to the cell membrane. Thus these homologous, AKAPs that dock G-protein-coupled receptors have markedly different patterns of trafficking, docking, and re-distribution.

1. Introduction

The identification of a class of proteins harboring a binding site for the regulatory subunits (*i.e.*, RI/RII) of cyclic AMP-dependent protein kinase A (PKA, A-kinase) was seminal to the understanding of the roles of these scaffolds (termed A-Kinase Anchoring Proteins or AKAPs) in cellular signaling [1]. The ability of AKAP proteins to dock PKA was followed by the discovery that these scaffold act as molecular "tool boxes" that are multivalent and capable of docking PKA, protein kinase C (PKC), as well as phosphoprotein phosphatases, such as PP2B [2]. AKAPs have been shown to participate in macromolecular signaling complexes that include kinases (both serine/threonine and tyrosine kinases), phosphatases, phosphodiesterases (PDE), adaptor molecules, ion channels, and also G protein-coupled receptors (GPCR) [3–6]. Herein, we examine two members of the class of GPCR-associated AKAPs, namely AKAP5 (also known as AKAP79/150) and AKAP12 (also known as gravin and AKAP250), which have been shown by immunoblotting to associate with the β_2-adrenergic receptor in very different manners. AKAP5 has been reported to dock to the receptor, its association apparently little affected by agonist stimulation [7]. The association of AKAP12 with the β_2-adrenergic receptor, in contrast, has been shown to be dynamic, increasing 3-fold in response to β-adrenergic agonist [8]. In the current study we examine the subcellular localization of these two AKAPs in the absence and presence of stimulation with beta-adrenergic agonist. We employed two cell lines that differ in the native abundance of each AKAP. HEK293 cells are rich in AKAP5 and display much less AKAP12 [9]; A431 cells display the opposite, being replete in AKAP12 and much less so in AKAP5 [7,10–12]. The results of biochemical analysis of subcellular fractionations and cell imaging are consistent with AKAP5-receptor in stable association, but internalizing and later recycling to the cell membrane in association with its G-protein-coupled receptor in response to isoproterenol. AKAP12, in contrast, docks to the β_2-adrenergic receptor in response to agonist stimulation, later internalizes with agonist-induced receptors, and finally dissociates from the recycled, cell membrane-bound receptor.

2. Materials and methods

2.1. Cell culture

Human epidermal carcinoma cells (A431) and human embryonic kidney (HEK293) cells were grown in the Dulbecco's modified Eagle's

* Corresponding author. Tel.: +1 631 444 7873; fax: +1 631 444 7696.
E-mail address: craig@pharm.sunysb.edu (C.C. Malbon).

0898-6568/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.cellsig.2008.09.019

medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (60 μg/ml), and streptomycin (100 μg/ml) and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

2.2. Establishment for stable cell lines and challenge with β-adrenergic agonist

Both A431 and HEK293 were transfected with expression constructs harboring either GFP-tagged AKAP5 or AKAP12 by the method of Lipofection® (Life Technologies, Inc.), according to the manufacturer's protocol. Stable clones were selected with 300 μg/ml of neomycin analogue G418 for several rounds within roughly 60 days, isolating clones that stably express levels of GFP-tagged AKAPs that were no greater in abundance than 10–15% of endogenous levels. Stable G418-resistant clones expressing either AKAP5-GFP or AKAP12-GFP were propagated in a Petri dish on the bottom of which was a poly-L-lysine-coated glass coverslip. Clones were serum-starved overnight (at least 16 h) in DMEM. On the next day, cells were either untreated or stimulated with β-adrenergic agonist (10 μM isoproterenol, ISO) for 10 min. By 30 min, agonist promotes essentially full internalization of β_2-adrenergic receptor, at which time the cells are chilled on ice to stop endocytosis, homogenized for subcellular analysis or employed for cell imaging. For experiments in which the recovery of the cells from a 30-min β-agonist treatment was investigated, the cultures were washed free of isoproterenol with DMEM and then allowed to remain under incubation for an additional 30 to 60 min (*i.e.*, wash-out and recovery).

2.3. Confocal microscope

Images of autofluorescently-tagged AKAPs (or GFP-tagged G-protein-coupled receptors) were acquired under the Zeiss LCM510 confocal scanning microscope equipped with an argon and helium-neon laser. Serial sections were collected as a file, and maximum projection images were created in which single images were assembled from the brightest pixels in a stack (Z-series). Z-stacks of images were exported as TIFF files, and individual sections were analyzed for fluorescent signal overlap in Adobe Photoshop 5.5.

2.4. Cell fractionation

Cell culture medium was aspirated and the cultures rinsed with phosphate-buffered saline (PBS, pH 7.4) twice. An aliquot (5 ml) of PBS/EDTA buffer was added and the cultures allowed to remain at 37 °C for 10 min, and then collected at 1000 rpm for 3 min. The cell mass was gently resuspended in 1 ml of HME buffer (20 mM HEPES, pH7.4, 2 mM $MgCl_2$, 5 mM EDTA) containing a protease inhibitor cocktail (10 mM dithiothreitol, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 100 μg/ml bacitracin, 100 μg/ml benzamidine. Cells were placed on ice for 15 min and then subjected to homogenization on ice by 10 strokes of a chilled Dounce glass pestle homogenizer. The homogenate was subjected to centrifugation at 2000 rpm for 5 min at 4 °C. The nuclei-free supernatant from the homogenized cells was transferred to a 5 ml polycarbonate tube, subjected to centrifugation at 15,000 rpm for 30 min at 4°C with Beckman JA-21 rotor. The resultant supernatant was termed "cytosol-enriched fraction". The pellet was termed "membrane-enriched fraction". The protein concentration/content was determined according to the Lowry method. The samples were aliquoted based upon protein equivalence and subjected to SDS-PAGE. The resolved proteins were transferred to nitrocellulose blots electrophoretically and the blots probed with specific antibodies, *i.e.*, subjected to western blot analysis with specific antibodies or immunoblotting. In each case the ECL-based exposed films were scanned by calibrated Umax 1000 absorbance scanner equipped with SilverFastAi software (LaserSoft Imaging Inc. Longboat Key, FL). The bands were quantified by use of Aida software (Raytest, Germany). The results are displayed as representative blots. Graphs quantifying the relative amounts of each AKAP were prepared from the data obtained from at least 4 separate experiments, each performed on separate occasions.

3. Results

To monitor the subcellular localization of AKAPs using confocal microscopy, A431 and HEK293 cells were stably transfected with expression vectors harboring either GFP-tagged AKAP5 or AKAP12. Stable clones were selected that displayed expression of exogenous AKAP that was <15% expression of that measured for the endogenous counterpart (Fig. 1). A representative blot stained for AKAP12 and for AKAP5 displays both the endogenous AKAP and also the slower migrating GFP-tagged fusion protein. Note that endogenous expression of AKAP5 is greater in HEK293 than A431 cells, while endogenous expression of AKAP12 is greater in the A431 than HEK293 cells (Fig. 1A). In order to provide relative quantification of expression of each AKAP (both for the endogenous as well as the exogenously-expressed forms), we set the expression of the endogenous AKAP12 observed in A431 cells

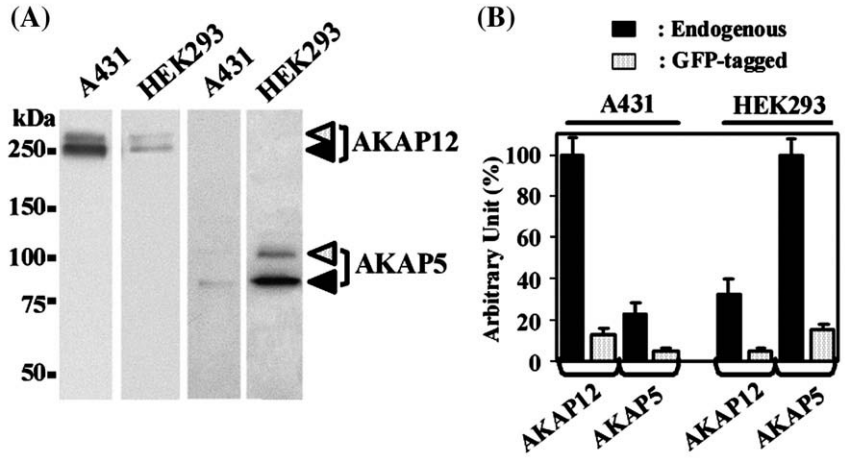

Fig. 1. Distribution of AKAP5 and AKAP12 in A431 and HEK293 cells. (A) Crude total proteins were extracted from either A431 or HEK293 clones stably expressing either AKAP5-GFP or AKAP12-GFP. The samples were analyzed by SDS-PAGE and immunoblotting with anti-AKAP5 or anti-AKAP12 antibodies. Equal amounts of protein (10 μg protein/PAGE lane) were loaded. Endogenous AKAP5 and AKAP12 is labeled with black arrow; exogenously expressed GFP-tagged AKAP5 and GFP-tagged AKAP12 is labeled with open arrows. (B) Quantification of the level of expression of endogenous and GFP-tagged AKAP5 versus AKAP12 in both A431 and HEK293 cells. The results shown in panel A are representative and the summations of the analyses from multiple independent experiments (±S.E.M.) are shown in panel B.