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Selective cell-surface labeling of the molecular motor protein prestin

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ABSTRACT

Prestin, a multipass transmembrane protein whose N- and C-termini are localized to the cytoplasm, must be trafficked to the plasma membrane to fulfill its cellular function as a molecular motor. One challenge in studying prestin sequence-function relationships within living cells is separating the effects of amino acid substitutions on prestin trafficking, plasma membrane localization and function. To develop an approach for directly assessing prestin levels at the plasma membrane, we have investigated whether fusion of prestin to a single pass transmembrane protein results in a functional fusion protein with a surface-exposed N-terminal tag that can be detected in living cells. We find that fusion of the biotin-acceptor peptide (BAP) and transmembrane domain of the platelet-derived growth factor receptor (PDGFR) to the N-terminus of prestin-GFP yields a membrane protein that can be metabolically-labeled with biotin, trafficked to the plasma membrane, and selectively detected at the plasma membrane using fluorescently-tagged streptavidin. Furthermore, we show that the addition of a surface detectable tag and a single-pass transmembrane domain to prestin does not disrupt its voltage-sensitive activity.

1. Introduction

In response to changes in transmembrane potential, mammalian sensory outer hair cells (OHCs) change length [1–3]. This motion enhances sound-induced vibrations within the cochlea and improves hearing sensitivity [4]. The transmembrane protein prestin is critical for this signal amplification process [5]. Prestin is a molecular motor within the plasma membrane that responds to changes in transmembrane potential, and the electrical activity of prestin is tightly coupled to changes in the length of OHCs [6,7]. The physiological role of prestin's electromechanical coupling (molecular piezoelectricity) function in hearing has been clearly delineated [8,9]. Prestin responds to cellular depolarization by reducing cell length, whereas prestin increases cell length when cells become hyperpolarized. Currently, the molecular mechanism of voltage-to-length conversion within this multipass transmembrane protein is not understood.

In contrast to other widely studied molecular motors (e.g., myosin, kinesin, and dynein), prestin does not require ATP or calcium for motor function [3,10]. Prestin belongs to a family of anionic membrane transport proteins, solute carrier family 26 (SLC26), for which little structural information is available. Like all membrane proteins, prestin function requires proper insertion into the plasma membrane. This latter property makes prestin challenging to study upon heterologous expression in cultured cells, since transiently transfected cells exhibit heterogeneity in both the level of prestin expressed and the amount of prestin trafficked to the plasma membrane [11,12]. In addition, mutation of prestin can alter these properties as well as electromechanical coupling, making it difficult to interpret the effects of amino acid substitutions or deletions [13]. Since mutagenesis coupled with functional analysis is widely used to study prestin [11,14–21], an ability to selectively identify, and potentially quantify, the membrane-localized fraction would greatly enhance the biochemical and biophysical information gleaned from these studies.

To establish a strategy for studying the function of membrane-localized prestin, we investigated whether prestin could be metabolically tagged in living cells with biotin, a vitamin that binds with high affinity [22,23] to streptavidin-conjugated fluorophores. The lack of endogenous surface biotinylation in mammalian cells [24,25], and the impermeability of cell membranes to streptavidin conjugates makes this strategy compatible with selective labeling and purification of surface expressed membrane proteins. We based our reporter construct on the most extensively studied endogenously biotinylated protein, the 1.3 S subunit of *Propionibacterium shermanii* transcarboxylase (commonly called PSTCD) [26]. The biotin acceptor peptide (BAP) sequence from PSTCD has been fused to the C-terminus of multiple cytoplasmic enzymes and demonstrated to undergo in vivo metabolic biotinylation in both bacteria and yeast [27]. Parrot and Barry extended this technology to metazoans [28], and they demonstrated that both secreted and simple, single-pass integral membrane proteins can

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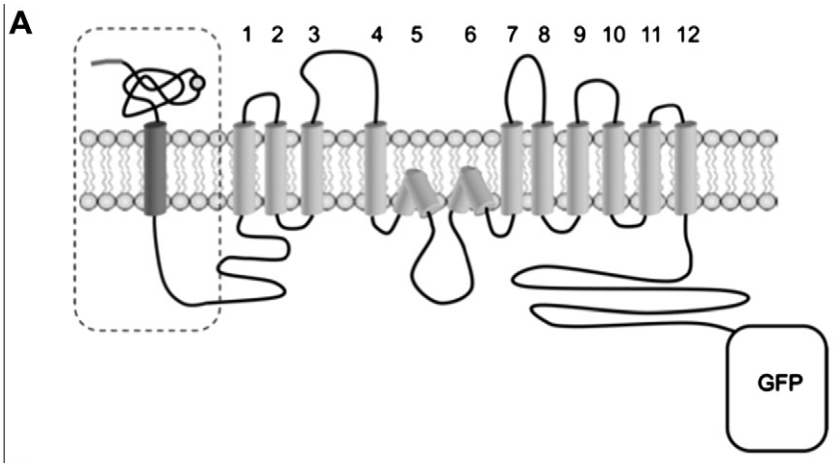
be biotinylated [29]. One of their constructs, BAP fused to the N-terminus of the platelet-derived growth factor receptor (PDGFR) transmembrane domain, was used to introduce streptavidin conjugated contrast agents to the surface of tumor cells and enhance magnetic resonance imaging (MRI) [30]. Herein, we demonstrate the first use of BAP-PDGFR for extracellular biotinylation of a multi-pass transmembrane protein.

2. Materials and methods

2.1. Plasmid construction

The 736 bp BAP-PDGFR reporter gene was synthesized from a 24 primer set, composed primarily of 50 bp oligonucleotides, designed using DNAWorks (Helix Systems, NIH) and the PCR-based synthesis methods of Hoover and Lubkowski [31].The gene includes a 5′ NheI restriction site and a 3′ HindIII restriction site, and it encodes the murine Ig κ-chain leader sequence, the hemagglutinin A (HA) epitope tag, a biotin acceptor peptide (BAP), the transmembrane domain from platelet-derived growth factor receptor (PDGFR), and a 19 amino acid cytoplasmic linker region. Fig. 1A illustrates the protein fusion expressed by this construct. The BAP coding sequence was derived from base pairs 1–390 of the PinPoint Xa vector (Promega, Madison, WI). The murine Ig κ-chain leader sequence, which targets the fusion to the secretory pathway [32], and the PDGFR transmembrane domain, which ensures exoplasmic display of BAP [33], were isolated from base pairs 737–799 and 907–1053 (respectively) of the pDisplay vector (Invitrogen, Carlsbad, CA). All nucleotide segments were codon



B

METDTLLLWV LLLWVPGSTG DYPYDVPDYA AAAAMKLKVT VNGTAYDVDV
DVDKSHENPM GTILFGGGTG GAPAPAAGGA GAGKAGEGEI PAPLAGTVSK
ILVKEGDTVK AGQTVLVLEA MKMETEINAP TDGKVEKVLV KERDAVQGGQ
GLIKIGDLEL IEGRAAAAAV GQDTQEVIVV PHSLPFKVVV ISAILALVVL
TIISLIILIM LWQKKPRGSA GSAGSAGSAD DDDKGSAKAS NSTMDHAEEN
EIPVATQKYH VERPIFSHPV LQERLHVKDK VSESIGDKLK QAFTCTPKKI
RNIIYMFLPI TKWLPAYKFK EYVLGDLVSG ISTGVLQLPQ GLAFAMLAAV
PPVFGLYSSF YPVIMYCFFG TSRHISIGPF AVISLMIGGV AVRLVPDDIV
IPGGVNATNG TEARDALRVK VAMSVTLLSG IIQFCLGVCR FGFVAIYLTE
PLVRGFTTAA AVHVFTSMLK YLFGVKTKRY SGIFSVVYST VAVLQNVKNL
NVCSLGVGLM VFGLLLGGKE FNERFKEKLP APIPLEFFAV VMGTGISAGF
NLHESYSVDV VGTLPLGLLP PANPDTSLFH LVYVDAIAIA IVGFSVTISM
AKTLANKHGY QVDGNQELIA LGICNSIGSL FQTFSISCSL SRSLVQEGTG
GKTQLAGCLA SLMILLVILA TGFLFESLPQ AVLSAIVIVN LKGMFMQFSD
LPFFWRTSKI ELTIWLTTFV SSLFLGLDYG LITAVIIALL TVIYRTQSPS
YKVLGQLPDT DVYIDIDAYE EVKEIPGIKI FQINAPIYYA NSDLYSNALK
RKTGVNPALI MGARRKAMRK YAKEVGNANI ANAAVVKVDG EVDGENATKP
EEEDDEVKYP PIVIKTTFPE ELQRFMPQTE NVHTIILDFT QVNFIDSVGV
KTLAVMVKEY GDVGIYVYLA GCSPQVVNDL TRNRFFENPA LKELLFHSIH
DAVLGSHVRE AMAEQEASAP PPQDDMEPNA TPTTPEARDP PVAT*MVSKGE*
ELFTGVVPIL VELDGDVNGH KFSVSGEGEG DATYGKLTLK FICTTGKLPV
PWPTLVTTLT YGVQCFSRYP DHMKQHDFFK SAMPEGYVQE RTIFFKDDGN
YKTRAEVKFE GDTLVNRIEL KGIDFKEDGN ILGHKLEYNY NSHNVYIMAD
KQKNGIKVNF KIRHNIEDGS VQLADHYQQN TPIGDGPVLL PDNHYLSTQS
KLSKDPNEKR DHMVLLEFVT AAGITLGMDE LYK

Fig. 1. Predicted BAP-prestin-GFP fusion protein topology .A, The biotin acceptor peptide (BAP) was fused to the exoplasmic face of the platelet-derived growth factor receptor (PDGFR) transmembrane domain and attached to a 20 amino acid cytoplasmic linker. This engineered reporter gene and the 12-pass motor protein prestin were cloned to the pEGFP-N1 plasmid (Clontech) to form a fusion (BAP-prestin-GFP) with a convenient external binding moiety. The protein segment from the BAP-PDGFR reporter gene is shown in dashed outline and the transmembrane helices of the prestin-GFP fusion are numbered. BAP can be cleaved from PDGFR by Factor Xa protease and prestin can be liberated from PDGFR by severing the cytoplasmic linker with Enterokinase. B, Translated sequence of BAP-prestin-GFP. The BAP-PDGFR reporter encompasses amino acids 1-237 (bold), prestin 244-987 (gray), and GFP 995-1233 (italics). The BAP-PDGFR reporter contains a leader sequence (amino acids 1–21), hemagluinin tag (22–30), BAP (35–164), PDGFR transmembrane domain (169–216), and cytoplasmic linker (218–237). Biotinylation occurs at Lys 122 (box). The proteolytic cleavage sites (underlined) allow cleavage of the fusion following residue 164 or 234 by Factor Xa or Enterokinase, respectively. GFP contains A207 K (residue 1201 of overall fusion) modification to prevent formation of non-obligate oligomers.