General and Comparative Endocrinology 179 (2012) 88–98

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

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

# Molecular characterization of prostaglandin F receptor (FP) and E receptor subtype 3 ($EP_3$) in chickens

Amy H.Y. Kwok [a], Yajun Wang [b,*], Frederick C. Leung [a,*]

[a] School of Biological Sciences, The University of Hong Kong, Pokfulam Road, Hong Kong, China
[b] Key Laboratory of Bio-Resources and Eco-Environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610064, China

ARTICLE INFO

*Article history:*
Received 6 November 2011
Revised 18 July 2012
Accepted 25 July 2012
Available online 4 August 2012

*Keywords:*
Chicken
Prostaglandin receptors
Cloning
Characterization
$EP_3$
FP

ABSTRACT

Prostaglandin E and F regulate diverse physiological functions including gastrointestinal motility, fever induction and reproduction. This multitude of biological effects is mediated via their four E receptor subtypes ($EP_1$, $EP_2$, $EP_3$ and $EP_4$) and F receptor (FP), respectively. Majority of these studies was performed in mammalian species, while investigations on their roles were impeded by inadequate information on their receptors in avian species. In present study, full-length cDNAs of chicken $EP_3$ ($cEP_3$) and two isoforms of FP – cFPa and cFPb – were cloned from adult hen ovary. The putative $cEP_3$ and cFPa share high amino acid sequence identity with their respective orthologs, while the predicted cFPb is a novel middle-truncated splice variant which lacks 107 amino acids between transmembrane domains 4 and 6. RT-PCR showed that $cEP_3$, cFPa and cFPb are widely expressed in adult tissues examined, including ovary and oviduct. Using a pGL3-CRE luciferase reporter system, $cEP_3$-expressing DF1 cells inhibited forskolin-induced luciferase activity ($EC_{50}$: <1.9 pM) upon $PGE_2$ treatment, suggesting that $cEP_3$ may functionally couple to Gi protein. Upon $PGF_{2\alpha}$ addition, cFPa was shown to potentially couple to intracellular $Ca^{2+}$-signaling pathway by pGL3-NFAT-RE reporter assay ($EC_{50}$: 2.9 nM), while cFPb showed no response. Using a pGL4-SRE reporter system, both $cEP_3$ and cFPa exhibited potential MAPK activation by $PGE_2$ and $PGF_{2\alpha}$ at $EC_{50}$ 0.34 and 13 nM, respectively. Molecular characterization of these receptors paved the road to the better understanding of $PGE_2$ and $PGF_{2\alpha}$ roles in avian physiology and comparative endocrinology studies.



## 1. Introduction

Prostaglandins, in particular prostaglandin E2 ($PGE_2$) and prostaglandin F2α ($PGF_{2\alpha}$), are known to regulate a vast range of body functions, from the cellular level (e.g. apoptosis and proliferation) to the central and tissue-specific level, including fever generation, sleep induction, regulation of gastrointestinal function, and reproduction including ovulation, embryo implantation, maintenance of pregnancy and labour (as reviewed in [5,23,45]), and tumor growth and metastasis in various malignancies like colorectal, breast and ovarian cancers [12,33,53]. Despite their chemical nature as small 20-carbon lipids (eicosanoids), unlike steroids, prostaglandins exist as organic anions at physiological pH and cannot cross the plasma membrane freely. Hence, their actions are mediated primarily via specific cell surface receptors, i.e. the prostaglandin F receptor (FP) for $PGF_{2\alpha}$, and the four prostaglandin E receptor subtypes – $EP_1$, $EP_2$, $EP_3$ and $EP_4$ – for $PGE_2$.

Prostaglandin receptors belong to family A G protein-coupled receptors (GPCRs) (rhodopsin receptor-like family, or rhodopsin family alpha group in GRAFS system), and they couple to different G proteins and elicit specific cellular response. In mammals, $EP_3$ primarily couples to $G_i$ protein and leads to inhibition of adenylyl cyclase (AC) and decline in intracellular cyclic AMP (cAMP) level, while FP couples to $G_q$ protein and cause elevation of intracellular $Ca^{2+}$ level [43,44]. The multitude of target-specific responses of $PGE_2$ and $PGF_{2\alpha}$ are associated with expression and variability in signaling properties of these receptors, as well documented in mammals, especially in knockout mice studies [20,47,51]. In non-mammalian species such as birds, some physiological effects of $PGE_2$ and $PGF_{2\alpha}$ have been described for decades e.g. oviposition

*Abbreviations:* c-, Chicken *Gallus gallus*; h-, human *Homo sapiens*; m-, mouse *Mus muculus*; r-, rat *Rattus norvegicus*; x-, African clawed frog *Xenopus laevis*; b-, cattle Bos taurus; d-, dog *Canis lupus familiaris*; o-, rabbit *Oryctolagus cuniculus*; PGs, prostanoids; PG-Rs, prostanoid receptors; $EP_1$, prostaglandin E receptor subtype 1; $EP_2$, prostaglandin E receptor subtype 2; $EP_3$, prostaglandin E receptor subtype 3; $EP_4$, prostaglandin E receptor subtype 4; DP, prostaglandin D receptor; IP, prostacyclin receptor; FP, prostaglandin F receptor; TP, thromboxane receptor; cAMP, cyclic AMP; AC, adenylyl cyclase; CRE, cAMP response element; $Ca^{2+}$, calcium ions; NFAT-RE, nuclear factor of activated T-cells response element; SRE, serum response element; MAPK, mitogen-activated protein kinases; DF-1, chicken embryonic fibroblast.

* Corresponding authors. Fax: +86 852 2559 9114 (F.C. Leung), +86 28 85414886 (Y. Wang).

*E-mail addresses:* veekwok@hkusua.hku.hk (A.H.Y. Kwok), cdwyjhk@gmail.com (Y. Wang), fcleung@hkucc.hku.hk (F.C. Leung).

0016-6480/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ygcen.2012.07.019

(egg-laying) [2,58]. However, the mechanisms behind remain poorly understood. Ensuing from our previous study of chicken $EP_2$ and $EP_4$, in present study, we aimed to clone chicken $EP_3$ and FP and characterize their expression by RT-PCR and functions by *in vitro* expression and luciferase reporter systems.

## 2. Materials and methods

### 2.1. Animal tissues

Adult chickens were provided by Kadoorie Agricultural Research Center (Hong Kong). Adult chickens of 25 weeks old were killed and 12 tissues (including brain, pituitary, lung, heart, liver, kidney, intestine, pancreas, breast muscle, spleen, ovary, and testis) and five parts of the oviducts (including infundibulum, magnum, isthmus, shell gland, cloaca; obtained from egg-laying hen) were collected for total RNA extraction. Whole ovary was used in the present study, though the largest five preovulatory follicles (F5 to F1) were removed due to difficulty encountered in RNA extraction. All experiments were performed under license from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of The University of Hong Kong.

### 2.2. Cloning of full-length chicken prostaglandin E receptor subtype 3 ($EP_3$) and F receptor (FP) cDNA

According to the predicted partial cDNA sequences of chicken $EP_3$ (Accession No.: XM_426672) deposited in GenBank, four gene-specific primers were designed to amplify 5′-cDNA and 3′-cDNA ends of $cEP_3$ from chicken ovary using SMART™-RACE cDNA Amplification Kit (RACE) (Clontech, Palo Alto, CA) (Table 1). The amplified PCR products were cloned and sequenced. Based on the cDNA sequences obtained by RACE PCR, new primers were designed to amplify the full-length cDNAs of $cEP_3$ by using high fidelity Taq DNA polymerase (Roche Diagnostics, Basel, Switzerland) (Table 1). The amplified PCR products were cloned into pBluescript SK(+/−) vector (Stratagene, La Jolla, CA) through T/A cloning and sequenced by Genetic Analyzer ABI3100 (PerkinElmer, Foster City, CA). Based on the predicted cDNA sequences for chicken FP (Accession No.: XM_430161), the full-length cDNAs of cFP were also cloned from chicken ovary and sequenced (Table 1). The full-length cDNAs of $cEP_3$ and cFP were finally determined by sequencing (PerkinElmer) at least three independent clones containing whole open reading frames (ORFs).

### 2.3. RNA extraction and RT-PCR assay

Total RNA was extracted from the 12 different adult tissues and the five parts of the oviduct using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Reverse transcription was performed at 42 °C for 2 h in a total volume of 10 μl consisting of 2 μg of RNA, 1x PCR buffer, 10 mM dithiothreitol, 0.5 μM of each dNTP, 0.5 μg of oligo(dT), and 100 U of Superscript II (Invitrogen, Carlsbad, CA). One microliter of the first-strand cDNA was used as the template for each PCR reaction. According to our previously established methods [54–57], RT-PCR assays were performed to examine relative mRNA levels of $EP_3$ and FP in various tissues. PCR was performed under the following conditions: 2 min at 95 °C for denaturation, followed by 21 cycles (for β-actin: 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C), 35 cycles (for $EP_3$: 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C), 35 cycles (for FP: 30 s at 95 °C, 30 s at 54 °C, and 60 s at 72 °C) of reactions, ending with a 10-min extension at 72 °C. The primers used are listed in Table 1. The PCR products were electrophoresized in 2% agarose gels, stained with ethidium bromide, and visualized under UV illumination (Bio-Rad, Hercules, CA). Their identities were then confirmed by sequencing (PerkinElmer).

### 2.4. Quantitative real-time PCR assay of cFPa and cFPb mRNA expression

Primers for β-actin, cFPa and cFPb were designed for real-time PCR with EvaGreen (Biotium, Haywards, CA) (Table 1). Opti-

**Table 1**
Primers used[a].

| Gene | Sense /antisense | Primer sequence[a] | Size (bp) |
|---|---|---|---|
| | | *Primers for rapid amplification of 5′-cDNA end* | |
| $cEP_3$ | Antisense | 5′-GTCCAGACGCGGTCGGACAGAT-3′ | 400 |
| | Antisense | 5′-CGGACAGATACACGGCGATGACGA-3′ | 387 |
| | | *Primers for rapid amplification of 3′-cDNA end* | |
| $cEP_3$ | Sense | 5′-GACCATGATGATCACGGGCATCGT-3′ | 1159 |
| | Sense | 5′-CGCGCTGGCCATGCTGCTGGTGT-3′ | 1129 |
| | | *Primers for RT-PCR assay* | |
| $cEP_3$ | Sense | GCCGCTGCTGGTAACGAT | 310 |
| | Antisense | CTCGAGCCGTGCTCCATCACGC | |
| cFP | Sense | GCAGCGTAATGGCTGTTGA | 684 (cFPa) |
| | Antisense | TGGAACTTACGGGTGACTCA | 363 (cFPb) |
| | | *Primers for Real-time PCR* | |
| cFPa | Sense | CAAAGACAAGGCAGATCGCAT | 113 |
| | Antisense | ATCCCAATTCTGGCCATTGA | |
| cFPb | Sense | GCAGCGTAATGGCTGTTGA | 112 |
| | Antisense | TCTGGCCATTGACACCATA | |
| | | *Primers for both RT-PCR assay and Real-time PCR* | |
| β-actin | Sense | CCCAGACATCAGGGTGTGATG | 123 |
| | Antisense | GTTGGTGACAATACCGTGTTCAAT | |
| | | *Primers for constructing expression plasmids*[b] | |
| $cEP_3$ | Sense | GAATTCCGCCGCCATGAGCC | 1142 |
| | Antisense | CTCGAGCCGTGCTCCATCACGC | |
| cFP | Sense | GGATCCTAACCATGAACAGT | 1119 |
| | Antisense | CTCGAGTTTAATTTTATTGCCCT | |

[a] All primers were synthesized by Tech Dragon Ltd. (Hong Kong).
[b] Restriction sites added in 5'-end of the primers are underlined.