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Expression of the human activin type I and II receptor extracellular domains in *Pichia pastoris*

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Abstract

Methods for the expression in *Pichia pastoris* and purification of the human activin receptor type I and II extracellular domains (ARIa/ARIb-ECDs, ARIIA/ARIIB-ECDs) are described. Key experimental aspects are also documented of the vector transformation methodology and the binding characteristics of these ECDs with activin A and inhibin. The cDNA constructs for these ECDs contained a C-terminal His_6-tag with either the native signal (*N*) or the yeast α mating factor (αMF) sequence and were introduced into the pPICZ expression vector either as a single-copy or as a four-copy expression cassette. Hyper-resistant transformants (zeo^R: 500 μg/mL) generated from the cassette containing a single copy of the expression vector gave the stronger signal intensity with a DNA dot-blot screening assay. These transformants also produced higher quantities of the corresponding recombinant protein compared to transformants using the four-copy cassette vector. All receptor-ECD proteins expressed were found to be heterogeneously glycosylated, whereby the ARIIA-ECD and ARIIB-ECD had undergone two Asn-linked glycosylation events and the ARIb-ECD a single event. By SDS–PAGE, the de-glycosylated proteins migrated larger than the expected core size, indicating that they may have undergone O-linked glycosylation. Biacore-based procedures with the glycosylated and de-glycosylated ARIIA-ECD were employed to determine the kinetic and equilibrium binding parameters for the interaction with activin A and inhibin. The glycosylated ARIIA-ECD bound to activin A with a K_D of 11.9 nM and inhibin with a K_D of 21.1 nM. Although glycosylation of ARIIA-ECD was not strictly required for high affinity interactions with activin A or inhibin, it markedly improved the overall stability of the ARIIA-ECD.

Keywords: Human activin receptors; Expression; Purification; *Pichia pastoris*; Surface plasmon resonance; Ligand binding; Glycosylation

Activin A is a member of the transforming growth factor-β family of cytokines and is responsible for multiple effects in both reproductive and non-reproductive tissues [1–3]. Activin A and the closely related protein, inhibin, were originally isolated from follicular fluid and found to induce (in the case of activin A) and inhibit (in the case of inhibin) FSH production and secretion from pituitary cells. Inhibin consists of two unrelated subunits (α/β) whilst activin A is the dimer of the common β_A subunit (β_A/β_A)[4–7].

Activin A signals via two classes of cell surface receptors, named I and II. The type II receptors are thought to bind the ligand directly in a high affinity interaction and recruit the type I receptors to form a complex that initiates an intracellular signal. The recruitment of the type I receptors or the existence of a pre-formed type II–type I complex on the cell surface has been implicated from cellular studies where ligand binding is thought to induce re-orientation of the kinase domains for activation of the type I receptor [8]. The type II receptors are single chained serine/threonine kinase proteins comprising 494 amino acid residues with an extracellular domain of 116 amino acids, which contains two putative Asn-linked glycosylation sites. The apparent size of the immunoprecipitated type II receptor has been found to be larger than predicted even after treatment with peptide *N*-glycosidase F, indicating that other post-translational modifications, such as O-linked glycosylation and/or

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phosphorylation of specific serine residues, can occur [9]. There are two sub-types of the human type II activin A receptor (ARIIA and ARIIB), and two potential sub-types of the human type I receptor (ARIa and ARIb).

In the present investigation, the methylotrophic yeast, *Pichia pastoris*, has been used to obtain both the human type I and type II activin receptor ECDs. The human type I activin receptor ECDs (ARIb-ECD and ARIa-ECD) have hitherto not been recombinantly expressed, although a baculoviral system has been employed previously to express the rat ARIIB-ECD for crystallographic studies [10], whilst the mouse ARIIA-ECD has also been successfully expressed using the *P. pastoris* system in high yield for crystallographic [11,12] and other biophysical studies [13]. A number of *P. pastoris* expression vectors are commercially available [14]. The pPICZ vector is based on the strong AOX1 promoter with expression of the recombinant protein achieved by induction with methanol (as the sole carbon source). Given that in vivo the activin receptors are transported through the secretory pathway by a native signal sequence, and yeasts tend to have low specificity for signal sequences [15], clonal constructs were generated containing either the receptor's own signal sequence (*N*) or the yeast signal (αMF) for secretion of these recombinant proteins.

In designing a versatile system, which was intended for subsequent use in the expression of other activin receptor-ECD proteins and receptor-ECD mutants, optimisation of the expression system and transformation methodology was required. This work was initially undertaken using the human activin receptor type IIA receptor-ECD (ARIIA-ECD).[1] The overall aim of the experiments described below was thus to first explore various methods that enable the effective transformation and screening of *P. pastoris* transformants that produce the highest levels of activin type I and II receptor-ECD proteins. The second aim was to examine the binding properties of the purified human receptor-ECD proteins with activin A using surface plasmon resonance techniques with the Biacore system, permitting the kinetic and equilibrium parameters (K_A, K_D, k_{on}, and k_{off}) to be determined.

Methods

Receptor constructs

The general structure (Fig. 1) of the cDNA receptor constructs employed in these studies involved a yeast signal sequence (αMF) or the native signal sequence (*N*) preceding the cDNA encoding the receptor-ECD followed by the cDNA encoding a C-terminal His_6 tag.

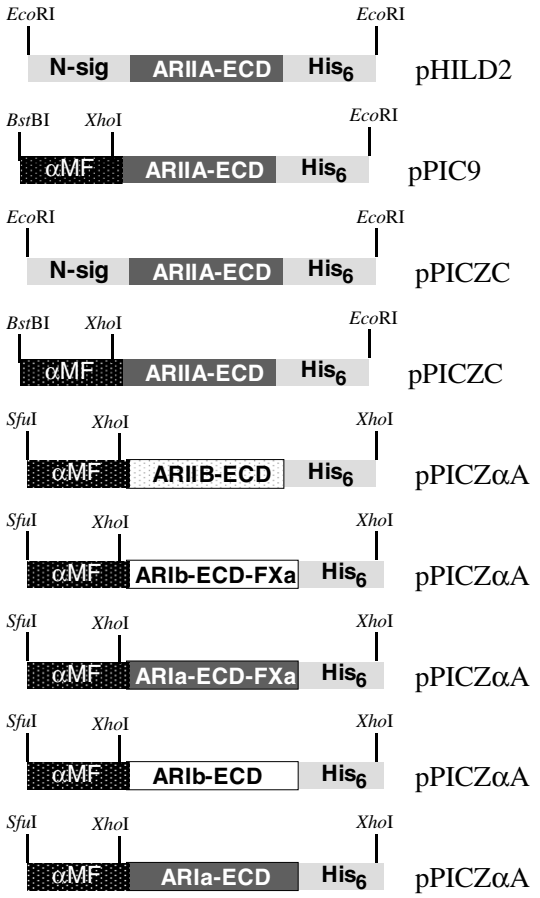

Fig. 1. Structures of the receptor-ECD vector constructs employed in the generation of the ARIa/b and ARIIA/B expression cassettes.

Preparation of the type IIA receptor-ECD constructs

The entire type IIA receptor cDNA sequence was used as a template for PCRs. Two type IIA-ECD cDNA constructs were prepared, with both incorporating a C-terminal His_6 tag, stop codon and *Eco*RI site through the use of the primer, 5′-GAATTCCTA GTGATGGTAGATGGT AGATGGGGTGGCCTTAGGTGTAAC-3′. An *Eco*RI site followed by a kozak consensus sequence and the native signal sequence was introduced for the first construct through the use of the primer 5′-CCAATGAATTCAGGA TGGGAGCTGC TGCAAAGT-3. The second construct incorporated a *Xho*I site and part of the αMF through the use of the primer 5′-CTCGAGAAAAGAGCTATACTTG GTAGATCA-3′. After confirmation by sequencing, the PCR products were subcloned into the *Eco*RI site (for the native signal (*N*) construct) of pHILD2 and the *Xho*I/*Eco*RI (for the αMF construct) of pPIC9.

For expression using the pPICZ vectors, the ARIIA-EC D with the native signal was digested from *N*-ARIIA-EC D-pHILD2 with *Eco*RI and subcloned into the *Eco*RI site of pPICZC. The ARIIA-ECD with the αMF was obtained from α-ARIIA-ECD-pPIC9 by digestion with *Bst*B1/*Eco*RI and subcloned into the *Bst*B1/*Eco*RI site of pPICZC (α-ARIIA-ECD-pPICZC).

[1] *Abbreviations used:* ARIIA-ECD, activin receptor type IIA receptor-ECD; SDM, site-directed mutagenesis; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide; ECDs, extra cellular domains; MW, molecular weight.