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Biochemical Pharmacology 67 (2004) 1853–1858
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Human α_{1D}-adrenoceptor phosphorylation and desensitization

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Received 14 November 2003; accepted 23 January 2004

Abstract

Rat-1 fibroblast were transfected with a plasmid containing the cDNA of the human α_{1D}-adrenoceptor. A cell line was isolated that stably expressed the receptor as evidenced by BMY 7378-sensitive noradrenaline-induced increases in intracellular calcium concentration. The effect of noradrenaline was blocked by active phorbol esters; such blockade was mediated by protein kinase C (PKC) as evidenced by its inhibition by staurosporine or the downregulation of this protein kinase. Radioligand binding experiments showed expression of receptors with high affinity for [^{3}H]tamsulosin (K_D 0.30 ± 0.05 nM) but low density (B_{max} 35 ± 4 fmol/mg protein). The receptors had the expected orders of potency for agonists (adrenaline = noradrenaline > oxymetazoline) and antagonists (BMY 7378 > 5-methyl-urapidil = phentolamine). Photoaffinity labeling identified the receptor as a band of M_r 70–80 kDa, which could be immunoprecipitated with a selective anti-α_{1D}-adrenoceptor antiserum. In cells metabolically labeled with radioactive phosphate the adrenoceptor was identified as a phosphoprotein whose phosphorylation state was increased by the agonist, noradrenaline, and by phorbol myristate acetate. The data indicate that the human α_{1D}-adrenoceptor function was regulated through phosphorylation by PKC.

Keywords: α_{1D}-Adrenoceptor; α_1-Adrenergic receptor; Human adrenergic receptor; Protein kinase C; Desensitization; Receptor phosphorylation

1. Introduction

α_1-adrenergic receptors (Ars) are a heterogeneous subfamily of G protein-coupled receptors comprising three isoforms, i.e. the α_{1A}-, α_{1B}-, and α_{1D}-ARs. These receptors mediate many actions of adrenaline and noradrenaline, including regulation of smooth muscle contraction, modulation of renal and hepatic metabolism, cell growth and proliferation, among many others; they also take part in the pathogenesis of diseases, such as hypertension and benign prostatic hypertrophy [1–3]. Therefore, knowledge on their structure, function, and regulation is important for basic medical sciences and everyday clinical practice.

Attenuation of receptor function (desensitization) is characteristic of G protein-coupled receptors and can be elicited by activation of the same receptor (homologous desensitization) or of unrelated receptors (heterologous desensitization). Such desensitizations are associated to receptor phosphorylation and key roles have been suggested for G protein-coupled receptor kinases in homologous desensitization and second messenger-activated kinases in the heterologous form [4]. Protein kinase C (PKC) seems to play a key role in heterologous desensitization of α_1-ARs [2].

The hamster α_{1B}-AR was the first receptor of this subfamily to be cloned and it has been studied much more extensively than the other subtypes. There is a large amount of evidence showing that the function of this receptor is modulated through the action of PKC [2,5,6]; phosphorylation sites have been identified through site-directed mutagenesis [6]. The human isoform is also modulated through PKC-catalyzed phosphorylation [7]. Bovine α_{1A}-ARs are also subjected to regulation through phosphorylation [8] and the data have been confirmed and extended using the human receptors [9]. We have recently reported that rat α_{1D}-ARs are also phosphoproteins whose function is regulated through phosphorylation [10]. In the present manuscript, we show that human α_{1D}-ARs expressed in Rat-1 fibroblast can be desensitized when PKC is activated by phorbol esters. Receptors were identified by photoaffinity labeling and we were able to immunoprecipitate them with a selective antibody; experiments in which cells were labeled with radioactive phosphate showed that α_{1D}-ARs

Abbreviations: AR, adrenergic receptor; PKC, protein kinase C; TPA, tetradecanoyl phorbol acetate

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doi:10.1016/j.bcp.2004.01.025

were phosphorylated in the basal state and that such phosphorylation was further increased when the cells were incubated with adrenaline or phorbol esters.

2. Materials and methods

(−)-Adrenaline, (−)-noradrenaline, oxymetazoline, 5-methyl-urapidil, BMY 7378, lysophosphatidic acid (LPA), tetradecanoyl phorbol acetate (TPA), and protease inhibitors were obtained from Sigma Chemical Co. Phentolamine was a generous gift from Ciba-Geigy. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin, antibiotics, and other reagents used for cell culture were from Life Technologies. [^{32}P]P_i (8500−9120 Ci/mmol) and [*aryl*-^{125}I]azido-prazosin (2200 Ci/mmol) were from Perkin Elmer Life Sciences. [^{3}H]Tamsulosin (56.3 Ci/mmol) was a generous gift from Yamanouchi Europe. Sepharose-coupled protein A was from Upstate Biotechnology and Fura-2/AM from Molecular Probes; DNA purification kits were from Qiagen.

2.1. Cell line

Rat-1 fibroblasts were cultured in glutamine-containing high-glucose DMEM supplemented with 10% fetal bovine serum. Cells were transfected with pcDNA, containing the cDNA of the human α_{1D}-AR (generously provided to us by Dr. Marvin L. Bayne (Merck)) [11], using lipofectamine 2000, as recommended by the manufacturer. Cells were growth in selection medium containing 1 mg/ml of the neomycin analog, G-418 sulfate; colonies were isolated and further cultured in medium containing 300 μg/ml G-418 sulfate, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37 °C under a 95% air/5% CO_2 atmosphere as described before [5,8]. Functional expression of α_{1D}-ARs was determined by studying the increase in intracellular calcium observed in response to noradrenaline.

2.2. Intracellular calcium concentration ($[Ca^{2+}]_i$)

Confluent fibroblasts were loaded with 5 μM Fura-2/AM as described [5,8]. Fluorescence measurements were carried out, with an Aminco-Bowman Series 2 Spectrometer with excitation monochromator set at 340 and 380 nm, with a chopper interval 0.5 s, and the emission monochromator set at 510 nm. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. [12] using the software provided by Aminco-Bowman; traces were directly exported to the graphs.

2.3. [^{3}H]Inositol phosphate production

Cells were labeled with [^{3}H]inositol (5 μCi/ml) for 18–24 h in inositol-free DMEM containing 1% fetal bovine serum. Cells were washed and preincubated in buffer containing 10 mM LiCl. Incubations with noradrenaline and/ or PMA were for 15 min and were ended by the addition of ice-cold perchloric acid. Supernatants were neutralized and [^{3}H]inositol phosphates were separated by Dowex AG1-X8 chromatography [13].

2.4. Receptor binding

Membranes were prepared as described [5,8]. [^{3}H]Tam-α_{1A}- and α_{1D}-ARs and was selected for our studies [14,15]. Binding studies were performed by incubating the radioligand (0.025–8 nM in saturation experiments and 1 nM in binding competition studies) with membranes (500 μg of protein) in a final volume of 0.25 ml of binding buffer for 60 min at 25 °C in a water bath shaker. Incubation was ended by addition of 5 ml of ice-cold buffer and filtration through GF/C filters using a Brandel harvester. Filters were washed twice, dried, and radioactivity was measured in a liquid scintillation counter. Nonspecific binding was determined in the presence of 10 μM phentolamine; specific binding was >90% of total binding at the K_D. The EBDA program (Biosoft-Elsevier) was used to analyze saturation and competition curves; K_i values were calculated according to Cheng and Prusoff [16].

2.5. Photoaffinity labeling and phosphorylation

Membranes (250 μg protein), were incubated in the dark with 6 nM of [*aryl*-^{125}I]azido-prazosin and exposed to UV light as described [5,8,10]. After this treatment membranes were centrifuged, washed, solubilized, and electrophoresed in 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions.

Receptor phosphorylation studies were performed as described in detail [8] with the exception that, due to the low receptor density of the cells, extracts from six 10-cm culture dishes were pooled for each condition. In brief, cells incubated in medium containing [^{32}P]P_i (0.03 mCi/ml) for 3 h at 37 °C. Labeled cells were stimulated, washed, and solubilized [5,8,10]. Extracts were centrifuged and the supernatants transferred to tubes containing a rabbit antiserum generated against the rat α_{1D}-adrenoceptor [10] and Sepharose-coupled protein A and immunoprecipitated as described. The amount of phosphorylated receptor was determined by PhosphorImager analysis.

3. Results and discussion

As it can be observed in Fig. 1 (upper panels) in the cell line selected noradrenaline increased the intracellular calcium concentration. No effect was observed in the parental Rat-1 cell line (data not shown). The magnitude of the increases and their speeds (slopes) were dose dependent;

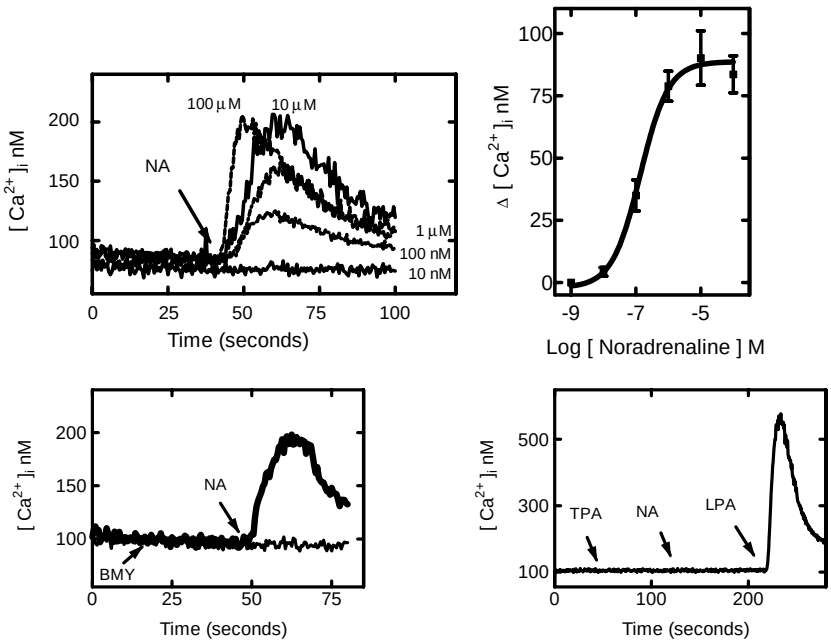

Fig. 1. Effect of noradrenaline on intracellular calcium ($[Ca^{2+}]_i$). Upper left panel: representative traces of the changes in $[Ca^{2+}]_i$ in response to different concentrations of noradrenaline (NA). Upper right panel: concentration–response curve to noradrenaline for the changes in $[Ca^{2+}]_i$. Plotted are the means and vertical lines represent the S.E.M. of five determinations using different cell preparations. Lower left panel: representative traces of the changes in $[Ca^{2+}]_i$ in response to 10 μM noradrenaline (NA) in the absence (thick trace) or presence of 100 nM BMY 7378 (thin trace). Lower right panel: representative trace of the absence of change in $[Ca^{2+}]_i$ in response to 10 μM noradrenaline (NA) in cells treated with 1 μM tetradecanoyl phorbol acetate (TPA); 1 μM lysophosphatidic acid (LPA).

the maximal increase observed was approximately twofold and the EC_{50} 150 ± 30 nM (mean ± S.E.M., $n = 5$). Several other lines were tested but neither responded to noradrenaline in a bigger or more consistent fashion (data not shown). The effect of 10 μM noradrenaline was completely blocked by the selective α_{1D}-AR antagonist, BMY 7378 (100 nM), and also by 1 μM TPA (Fig. 1, lower panels). The effect of TPA was mediated by PKC as evidenced by its inhibition by 300 nM staurosporine or the downregulation of this protein kinase (overnight treatment with TPA) (data not shown). TPA did not block the ability of the cells to respond to other agents as evidenced by the large effect of LPA (Fig. 1, lower right panel). Neither the antagonist nor TPA induced changes in basal intracellular calcium.

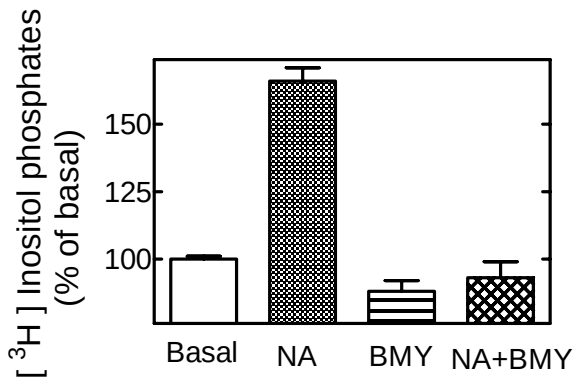

Fig. 2. Effect of noradrenaline on [^{3}H]inositol phosphate production. [^{3}H]Inositol-labeled cells were challenged with 10 μM noradrenaline (NA), 10 μM BMY 7378 (BMY), or both agents (NA + BMY) and [^{3}H]inositol phosphate production quantified. Plotted are the means and vertical lines represent the S.E.M. of four experiments using different cell preparations. Data are present as percent of basal production of [^{3}H]inositol phosphates.

As shown in Fig. 2, 10 μM noradrenaline was able to increase the production of [^{3}H]inositol phosphates and such effect was blocked by 10 μM BMY 7378. The antagonist alone was without effect on the basal production of [^{3}H]inositol phosphates.

[^{3}H]Tamsulosin bound to a homogeneous membrane receptor population, as evidenced by the Rosenthal transformation of the binding isotherm (Fig. 3, upper panels). These receptors had high affinity (K_D 0.30 ± 0.05 nM) for the radioligand. The density of receptors obtained with this cell line was low, i.e. B_{max} 35 ± 4 fmol/mg protein (data are the means ± S.E.M. of five experiments using different membrane preparations). Other clones have also low receptor densities and no specific binding was observed in the parental Rat-1 cells (data not shown). As expected the receptors had the pharmacological profile of α_{1D}-ARs, i.e. in binding competition experiments, the order of potency for agonists was adrenaline (440 ± 100 nM) = noradrenaline (475 ± 25 nM) > oxymetazoline (4825 ± 340 nM), and that for antagonists BMY 7378 (8±2 nM) > 5-methyl-urapidil (55 ± 5 nM) = phentolamine (58 ± 5 nM) (see Fig. 3, lower panels; data are the means ± S.E.M. of seven experiments using different membrane preparations).

The low receptor density in the cell line was unsatisfying but not unexpected. It has been observed that α_{1D}-ARs

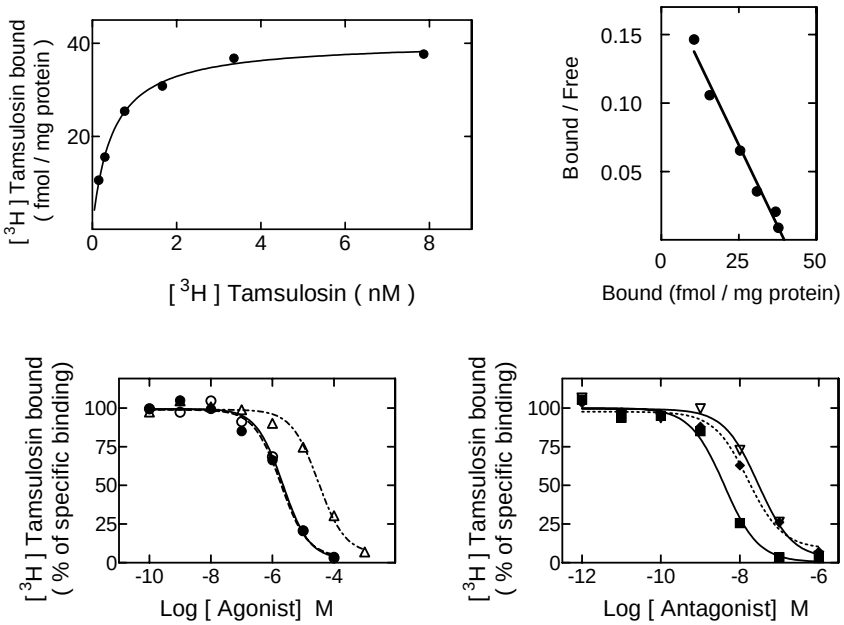

Fig. 3. Radioligand binding characterization of α_{1D}-ARs. Upper left panel: representative saturation isotherm. Upper right panel: Rosenthal transformation of the saturation data. Lower left panel: agonist binding competition: adrenaline (solid circles), noradrenaline (open circles), oxymetazoline (open triangles). Lower right panel: antagonists binding competition: BMY 7378 (solid squares), 5-methyl-urapidil (solid diamonds), phentolamine (inverted open triangles). Representative experiments are shown.

expression is not high in vivo, to the extent that even their presence in rat tissues had been questioned [17]. It is also low in transfected cell lines and it has been observed that truncation of the N-terminus increases the detection of receptors (but not protein) which has lead to the suggestion that the processing of this receptor might encounter difficulties in some cells [18]. This could be related to the finding that a large proportion of receptor protein has been detected intracellularly in cells expressing this receptor subtype [19]. α_{1D}-ARs are constitutively active in arteries where they play a key role in maintaining the vascular tone [20]: similarly, constitutive activity has been detected in transfected cells [19,21]. In the present studies, we were unable to detect such constitutive activity likely due to the low level of receptor expression.

Photoaffinity labeling using [*aryl*-^{125}I]azido-prazosin allowed detection of a major broad band of M_r 70–80 kDa; the labeling of this band was competed by BMY 7378 showing that it does correspond the α_{1D}-ARs. This is similar to what is observed with the rat α_{1D}-ARs [10], which is also presented in this work for comparison (Fig. 4, upper right panel). Photolabeled membranes were solubilized and the ability of the antiserum (generated with the GST-rat α_{1D}-AR fusion protein) [10] to immunoprecipitate the receptor was tested. The rat α_{1D}-AR fragment present in the fusion protein (LREWRLLGPLQR) is similar but nor the same as that in the human receptor (FREWRLLGPFRR) [10]. In spite of the difference, the antibody was able to immunoprecipitate the photolabeled receptors (Fig. 4, upper right panel) although with less efficacy (≈50%).

Phosphorylation experiments showed that a 70–80 kDa labeled band was immunoprecipitated with the antibody suggesting that the human α_{1D}-AR is a phosphoprotein (Fig. 4, lower panel). Such phosphorylation was increased twofold by noradrenaline or TPA. The effect of TPA was blocked by 300 nM staurosporine indicating that the effect is mediated through PKC. The data are consistent with what has been observed for other α_1-ARs and suggest that activation of PKC modulates the action of human α_{1D}-ARs through receptor phosphorylation.

The hamster α_{1B}-AR is the only receptor of this subfamily in which the phosphorylation sites have been studied [6]. No information is yet available regarding the other isoforms or on any of the human α_1-ARs. Phosphorylation sites in G protein-coupled receptors mainly exist at the third intracellular loop and at the carboxyl tail. The PKC phosphorylation sites present in the hamster α_{1B}-ARs were defined using receptors mutants and have been located at the carboxyl terminus and correspond to S^{394} and S^{400} [6]. Human (572 amino acids) and rat α_{1D}-ARs (561 amino acids) only have an 83.8% sequence identity and this is further decreased to 71% at the carboxyl tail. The third intracellular loop of these receptors is of identical length (72 amino acids) but the carboxyl tail of the human α_{1D}-AR is longer than that of the rat counterpart (166 versus 161 amino acids). Sequence analysis of these domains (http://scansite.mit.edu/) of the human α_{1D}-AR suggests the existence or two putative PKC phosphorylation sites at the third intracellular loop (^{287}S and ^{328}T) and two more at the carboxyl tail (^{287}S and ^{516}S). Identification of the actual phosphorylation sites in these receptors remains an important scientific goal. The three human receptor isoforms (α_{1A}-, α_{1B}-, and α_{1D}-ARs) are subjected to modulation by PKC (Refs. [7,9] and this work).

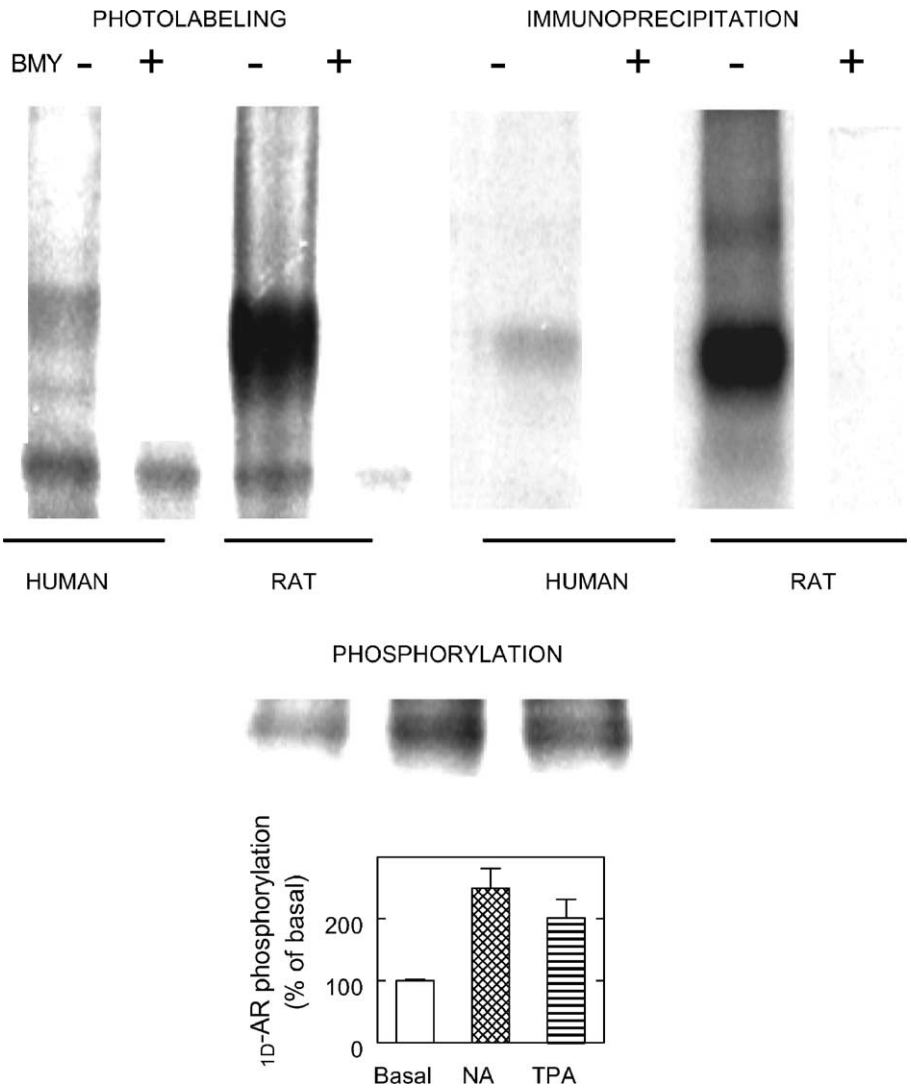

Fig. 4. Photolabeling, immunoprecipitation, and phosphorylation of α_{1D}-ARs. Upper panels: representative autoradiographs of α_{1D}-AR photolabeling and immunoprecipitation. Symbols indicate the absence (−) or presence (+) of 10 μM BMY 7378 during photolabeling. A parallel experiment using rat α_{1D}-ARs is presented for comparison. Lower panel: effects of 10 μM noradrenaline (NA) and 1 μM tetradecanoyl phorbol acetate (TPA) on human α_{1D}-AR phosphorylation. Plotted are the means and vertical lines represent the S.E.M. of five experiments using different cell preparations. A representative autoradiograph is presented.

α_{1D}-ARs seem to play key roles in the regulation of the vascular tone responsible of the maintenance of blood pressure. There is a large amount of evidence that suggest that they participate in the pathogenesis of hypertension in different rodent models (reviewed in Ref. [3]). In fact, data from α_{1D}-AR-knockout mice are entirely consistent with the key role of these adrenoceptors in maintenance of blood pressure through vasoconstriction [22]. The participation of α_{1D}-ARs in the pathogenesis of hypertension in human beings is not yet known and remains a challenging avenue for research.

Acknowledgments

This research was partly supported by grants from Dirección General de Asuntos del Personal Académico (IN206302) and Consejo Nacional de Ciencia y Tecnología (36230-N). The authors thank Dr. Rosa Navarro for her help with the sequence analysis.

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