Deletion of a proline-rich region and a transmembrane domain in fatty acid amide hydrolase[1]

G. Arreaza, D.G. Deutsch*

Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794, USA

Received 30 March 1999; received in revised form 31 May 1999

Abstract Fatty acid amide hydrolase contains a proline-rich sequence matching a consensus sequence for SH3-binding domains as well as a transmembrane domain. In this study, deletion mutants lacking the proline-rich region and the transmembrane domain were generated. Transfection experiments demonstrated that the proline-rich deleted amidase was enzymatically inactive. While immunostaining of the wild-type was always punctate with strong perinuclear staining characteristic for endoplasmic reticulum, the staining of the mutant was diffuse and distributed throughout the cytoplasm and perinuclear region. These observations along with the loss of activity suggest that the proline-rich region may play a role in the subcellular localization and enzymatic function. The transmembrane domain-deleted mutant was indistinguishable from the wild-type enzyme.

Key words: Anandamide amidase or amidohydrolase; Fatty acid amide hydrolase; Proline-rich domain; SH3-binding domain; Transmembrane domain

1. Introduction

Anandamide amidase, fatty acid amide hydrolase (FAAH), is the enzyme responsible for hydrolysis of neuromodulatory fatty acid amides and esters such as anandamide, 2-arachidonyl glycerol and oleamide [1–3]. This hydrolase contains a proline-rich sequence which may bind to Src homology 3 domain (SH3) of another protein [2]. SH3 domains regulate protein localization, enzymatic activity and may participate in multicomponent signaling complexes [4]. Examples of such interactions have been described for PLCγ [5], the epithelial sodium channel [6], myosin [7] and dynamin [8–10] and the dopamine D4 receptor [11]. It has been shown that removal of the proline-rich region abolishes interactions that prevent assembly of proteins and/or interferes with the normal function of proteins. For example, the proline-rich domain of the GTPase dyanamin has shown to be involved in localization in coated pits and in GTP-binding activity [12]. To understand the relevance of the proline-rich domain in the FAAH function, we constructed a deletion mutant lacking the polyproline region (amino acids 307-PTVPPLPFR-315) and assayed it for enzymatic activity, membrane association and immunolocalization in COS-7-transfected cells. Primary sequence analysis also detected a transmembrane domain from amino acids 9-TLSGVSGVCLACSLLSAAVVL-29 which may function as a site for insertion into the membrane and/or oligomerization of the amidase [2,13]. We constructed a mutant lacking this transmembrane region and characterized it in transfected COS-7 cells for the activity, expression pattern and distribution into membrane and soluble fractions.

2. Materials and methods

2.1. Deletion of the proline-rich domain (ΔPR-FAAH)

The deletion mutant was generated from the cloned rat FAAH cDNA by PCR as follows. The FAAH open reading frame was amplified in two fragments using the following primers. For fragment I, sense primer 5′-GAGAATTCATGGTGCTGAGCGAAG-3′ containing an *Eco*RI site and antisense primer 5′-CCGCTCGAGGTCCAAGGTGAACAAG-3′ containing a *Xho*I site. For fragment II, sense primer 5′-CCGCTCGAGTATAGAAGTTCTAGACCC-3′ containing a *Xho*I site and antisense primer 5′-TAGAATTCCGATGGCTGCTTTTGAG-3′. Two PCR products, 918 bp and 782 bp, were generated, digested with *Xho*I and ligated. The ligation product was subcloned into the *Eco*RI site of pBSKII. The construct was digested with *Hin*dIII/*Xba*I to generate a 390 bp fragment containing the deletion and the fragment was then subcloned into the wild-type construct ΔpcDNA3-FAAH (this construct is the FAAH cDNA cloned into a pcDNA3 vector that has a modified polylinker to facilitate subcloning, see construction below). The ΔPR-FAAH mutant construct was confirmed by DNA sequencing.

2.2. Construction of the transmembrane domain deletion (ΔHDI-FAAH)

A mutant lacking the N-terminal hydrophobic domain (amino acids 9–29) was constructed by PCR as follows. The plasmid ΔpcDNA3-FAAH was amplified in two fragments using the following primers. For fragment I, sense primer 5′-CGAAATTAATACGACTCAC-3′ and antisense primer 5′-CTTGGATCCGGTCCACACTTCG-3′, the antisense primer contained a *Bam*HI site. For fragment II, the sense primer was 5′-GAGGATCCCGATGGACCGGG-3′ and antisense primer 5′-TAGAATTCCGATGGCTGCTTTTGAG-3′ and the sense primer contained a *Bam*HI site. Two PCR products were generated, digested with *Bam*HI and ligated. The ligation product was subcloned into the *Eco*RI site of pBSKII. The construct was digested with *Eco*RI/*Hin*dIII to generate a 644 bp fragment containing the deletion. This mutant fragment was subcloned into the wild-type construct ΔpcDNA3-FAAH. The construct was verified by DNA sequencing.

2.3. Construction of ΔpcDNA3 expression vector

To facilitate subcloning of mutant fragments, the expression vector pcDNA3 (Invitrogen) was double-digested with *Hin*dIII/*Xba*I to remove all but one *Eco*RI cloning site. The digestion product was filled-in with Klenow and the blunt-end plasmid was religated and transformed into XL-1 blue competent cells. The plasmid was digested with *Apa*I, dephosphorylated and ligated to phosphorylated *Apa*I/*Eco*RI adapters. The resulting plasmid is a pcDNA3 with a unique *Eco*RI cloning site. The construct was verified by restriction analysis. FAAH cDNA was cloned as an *Eco*RI fragment into this vector (ΔpcDNA3-FAAH).

*Corresponding author. Fax: (1) (516) 632 8575.
E-mail: ddeutsch@notes.sunysb.edu

[1] A preliminary report of these results has been presented at the International Cannabinoid Research Society, La Grande Motte, France in July 1998 and at the Society of Neuroscience, Los Angeles, CA, USA in November 1998.

PII: S0014-5793(99)00774-7

2.4. Cell culture and transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and L-glutamine (complete DMEM) at 37°C and 5% CO_2 atmosphere to 70% confluence. Cells were transiently transfected with wild-type and mutant constructs using lipofectamine (Gibco BRL) in serum-free DMEM. After 5 h incubation, complete medium was added and cells were further incubated for 36 h prior to analysis.

2.5. Preparation of cellular membranes

Cells were rinsed once in phosphate-buffered saline (PBS, 150 mM NaCl, 20 mM sodium phosphate, pH 7.4) and then lysed in 20 mM Tris, pH 9.0, 1 mM EDTA. Lysates were homogenized in the presence of protease inhibitors as described [14]. The particulate fraction was collected by centrifugation of the lysates at $100\,000 \times g$ for 30 min. Pellets were resuspended and homogenized in lysis buffer. Total protein from supernatant and pellet fractions was determined using the Bradford method (Bio-Rad).

2.6. Anandamide amidase assay

Enzymatic activity was measured as previously described [15] with some modifications. Briefly, samples were incubated with 30 μM anandamide containing 0.01 μCi of arachidonoyl ethanolamide [1,2-^{14}C]ethanolamine (120 mCi/mmol) in 0.1 M Tris, pH 9.0, 2.5 mg/ml defatted BSA, for 30 min at 37°C. Reactions were terminated by addition of two volumes of methanol:chloroform (1:1) and radioactivity was measured by scintillation counting of the aqueous phase.

2.7. Polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting

SDS-PAGE was performed according to Laemmli after dissolving samples in 5×loading buffer. Proteins were separated on 10% polyacrylamide gels using the Mini-Protean II system (Bio-Rad). Proteins were transferred to a PVDF membrane (Millipore) using a mini Trans-Blot apparatus (Bio-Rad) under conditions recommended by the manufacturer. Blots were blocked in PBS containing 0.1% Tween 20 and 5% non-fat dried milk for 1 h. Blots were incubated with rabbit anti-FAAH antibodies, a gift of Benjamin Cravatt, and then with horseradish peroxidase-conjugated goat anti-rabbit IgG. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham).

2.8. Immunofluorescence

COS-7 cells were grown on polylysine-coated coverslips and were transfected as described above. Cells were fixed in PBS containing 3% paraformaldehyde for 30 min at room temperature, permeabilized with 1% Triton X-100 in PBS and blocked with PBS containing 10 mM glycine and 1% bovine serum albumin. Cells were incubated with polyclonal anti-FAAH antibodies and then with fluorescein-conjugated goat anti-rabbit IgG, each for 30 min at room temperature. Coverslips were mounted using a Slow Fade Anti Fade kit (Molecular Probes) before visualization by fluorescence microscopy. Anti-calnexin amino-terminal polyclonal antibody was obtained from StressGen Biotechnologies (Victoria, B.C., Canada).

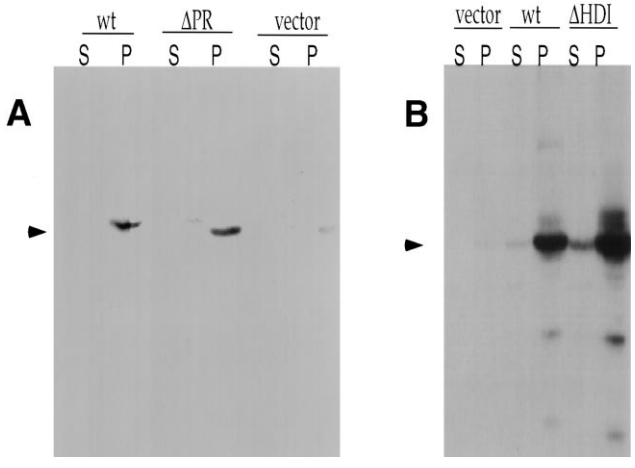

Fig. 1. Western blot analysis of soluble (S) and (P) particulate fractions from COS-7 cells transfected with (A) ΔpcDNA3-FAAH (WT), the mutant lacking the proline-rich region (ΔPR) and ΔpcDNA3 (vector) and (B) vector, wild-type FAAH and the ΔHDI mutant. The arrow denotes the position of the 64 kDa molecular weight marker.

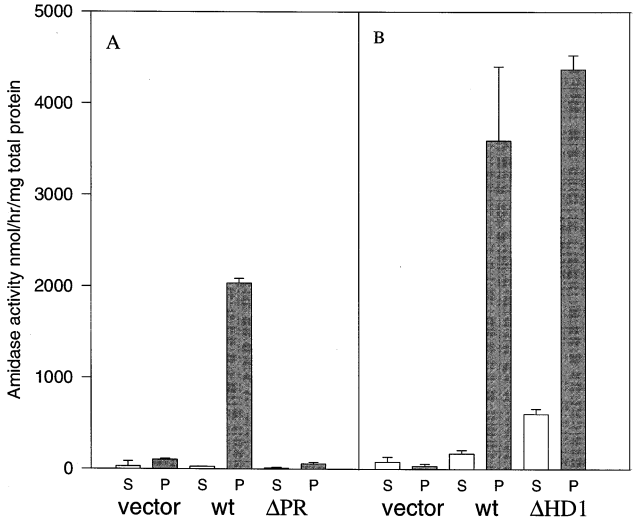

Fig. 2. Enzymatic activity of soluble (S) and (P) particulate fractions from COS-7 cells transfected with (A) vector, wild-type FAAH (WT) and the ΔPR-FAAH mutant (ΔPR) and (B) vector, wild-type FAAH (WT) and the ΔHDI-FAAH mutant (ΔHDI).

3. Results and discussion

A deletion mutant lacking amino acids 307–316 encoding the consensus class II SH3-binding domain sequence [2,16] was generated by PCR as described. A *Hin*dIII/*Xba*I fragment containing the deletion was subcloned into the expression construct ΔpcDNA3-FAAH. As a control, the *Hin*dIII/*Xba*I wild-type fragment was subjected to the same manipulations as the mutant. All constructs were verified by DNA sequencing. When expressed in COS-7 cells, both the wild-type, and proline deletion mutant, ΔPR, migrated at an apparent molecular weight of approximately 64 kDa (Fig. 1A). Although the vector had some bands which cross-reacted with the antibody near this region, they were light staining and were equally detectable in the soluble and particulate fractions (data not shown). However, when the membranes are probed with pre-absorbed antibody against non-transfected cell lysate, only the band near 64 kDa is observed in the particulate fraction (Fig. 1A). As with the wild-type, the ΔPR mutant always associated with the particulate fraction from homogenates of transiently transfected COS-7 cells (Fig. 1A). Therefore, the proline-rich region does not affect the association of FAAH with soluble or membrane fractions. This is not true in the case of the synaptojanins, for example, where the nature of the proline-rich region determines whether the protein is localized to particulate or soluble fractions [17]. As shown on the SDS-PAGE Western blot in Fig. 1B, deletion of a transmembrane region (amino acids 9–29) did not result in the release of significant soluble form of the enzyme in transfected COS-7 cells either. Like the wild-type, the hydrophobic deletion mutant (ΔHD1) was detected mainly in the particulate fraction. Anandamide amidase is a membrane-associated en-

zyme while the other amidases in this family, with which it shares homology, are water soluble enzymes [13,18,19]. This membrane association is perhaps essential considering the hydrophobicity of its substrates. From the above experiments, we conclude that neither the proline-rich region or transmembrane region are in themselves responsible for this enzyme's strong association with the membrane. It is likely that deletion of the transmembrane region is not sufficient to 'solubilize' the amidase because of other regions which also interact with the membrane.

The enzymatic activity of the wild-type enzyme is also strongly associated with the membrane with only background detected in the soluble fraction as shown in Fig. 2A. Interestingly, deletion of the proline-rich region completely abolished the enzymatic activity from the particulate fraction. This is not due to lower levels of ΔPR-FAAH, in transfected COS-7 cells, since the expression of the ΔPR-FAAH mutant was as robust as that observed for the wild-type FAAH (Fig. 1A). Unlike the situation with the proline-rich region, deletion of the transmembrane domain had no effect upon the activity in the particulate fraction (Fig. 2B). Only a small amount of enzymatic activity was observed in the soluble fraction of ΔHDI, suggesting little release of the enzyme from the membrane. From these studies, we conclude that the proline-rich domain is essential for FAAH activity while the amino acids 9–29 in the hydrophobic region are not important for catalytic activity. These results are in agreement with those reported where the N-terminal 29 amino acids were truncated and the purified enzyme had identical enzymatic properties to the native enzyme except for loss of the ability of the mutated enzyme to self-hybridize [13]. Under the concentrations used for our experiments, we did not observe a significant amount of dimers or other higher molecular weight species in SDS-PAGE gels, especially if the antibody was pre-absorbed with untransfected cell lysate proteins.

To investigate whether the proline-rich region and transmembrane region have a role in FAAH subcellular localization, we examined expression of wild-type (WT-) FAAH, ΔPR-FAAH and ΔHD1-FAAH in COS-7 cells by indirect immunofluorescence analysis (Fig. 3). The pattern of expression of WT-FAAH and ΔHDI-FAAH were indistinguishable. However, cells transiently transfected with the ΔPR-FAAH mutant were quite different. Staining of WT-FAAH was always punctate with strong perinuclear staining characteristic for the endoplasmic reticulum. The similarity of the staining we observed to that reported for an endoplasmic reticular pattern of expression was verified by immunostaining of the FAAH-transfected COS-7 cells with anti-calnexin (data not shown). This antibody is specific for calnexin which is an endoplasmic reticulum transmembrane protein [20]. Our immunofluorescence results for the WT-FAAH appear to be very much like those recently reported for COS-7 cells expressing the rat FAAH [21]. In contrast, staining of ΔPR-FAAH was not punctate in character, but rather diffuse in the cytoplasm and the perinuclear region. The vector-transfected COS-7 cells exhibited only very light staining (Fig. 3). These observations along with the loss of activity suggest that the proline-rich region may play a role in subcellular localization and might be important for the enzymatic function.

SH3 domains bind to target proteins by associating with proline-rich motifs and mediate protein-protein interactions to regulate subcellular localization. Gout et al. [8] showed that dynamin, a GTPase implicated in synaptic vesicle endocytosis and recycling, binds to and is activated by several SH3 domain-containing proteins. The observation that SH3 domains are able to stimulate the rate of GTP hydrolysis on

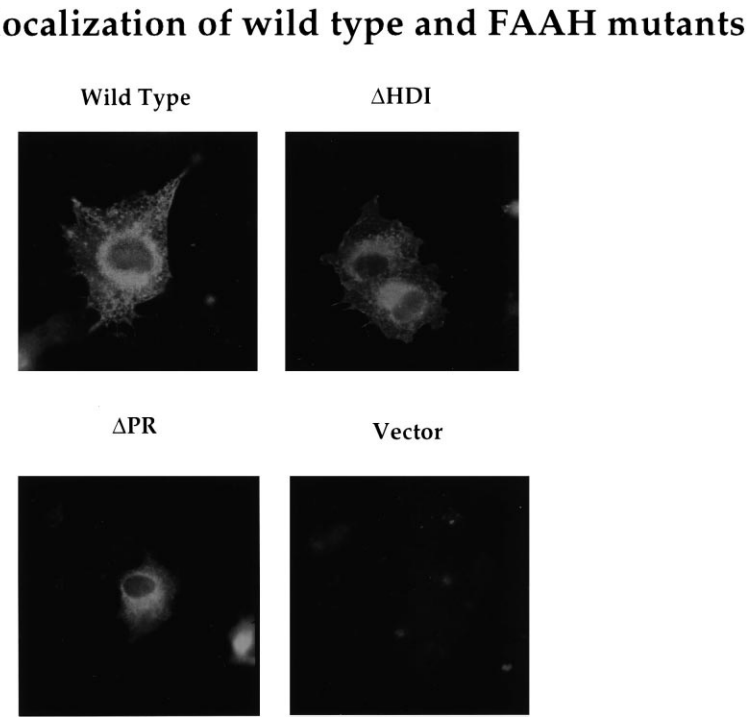

Fig. 3. Immunofluorescence of COS-7 cells transfected with WT-FAAH, ΔPR-FAAH, ΔHDI-FAAH and vector stained with anti-FAAH antibody. An insignificant amount of staining was observed in untransfected cells. Magnification, 630-fold. These data are representatives of at least three separate transfections for each expression construct and observations of approximately 100 cells from each.

dynamin directly was the first indication that these domains are not only involved in protein-protein interaction but they can also regulate the function of associated molecules. Furthermore, recently, it has been demonstrated that amphiphysin, a binding partner for dynamin I in nerve terminals, binds with high affinity to a unique site in its proline-rich domain and that the SH3-binding motif of dynamin is required for its targeting to clathrin-coated pits [8,9,22]. Amphiphysin, which interacts with the clathrin adapter AP2, may play a role in the recruitment of dynamin I at clathrin-coated pits involved in synaptic vesicle formation. It has also been shown that the proline-rich domain of dynamin I undergoes phosphorylation/dephosphorylation [23]. It is possible that phosphorylation may serve to regulate the interactions of dynamin with the SH3 domain of amphiphysin.

Our results suggest further experiments to study the possible binding partners for FAAH and a possible role for phosphorylation of a specific threonine residue present in its proline-rich motif. To better understand the role of FAAH in the metabolism of the neuromodulator anandamide, it is important to determine if proline-rich domain-mediated interactions are required for localization of the enzyme at the site of action and/or if these interactions are somehow involved in regulation of the enzyme function. It is possible that the proline-rich region may play an essential role in the correct folding of the amidase and its deletion results in loss of enzymatic activity and a modified intracellular localization. However, it is interesting to note that a sequence similar to PTVPPLPFR is not found in the other non-mammalian members of the amidase family, but does occur in many other unrelated proteins. Also, we cannot rule out that overexpression of FAAH in COS-7 may saturate normal compartments. It is important to see if the same observations can be extended to cells naturally expressing the enzyme. The localization of the enzyme to the endoplasmic reticulum is consistent with the observation that the enzyme has been reported to be mainly microsomal (microsomes are pinched off endoplasmic reticular membranes) in nature [1,24,25]. The study of FAAH-binding partners and analysis of its post-translational modifications may help to elucidate its role in the regulation of the endocannabinoids.

Acknowledgements: This work was supported by DA 09374. We thank Ben Cravatt for providing the cloned enzyme and the anti-FAAH antibody.

References

[1] Deutsch, D.G. and Chin, S.A. (1993) Biochem. Pharmacol. 46, 791–796.
[2] Cravatt, B.F., Giang, D.K., Mayfield, S.P., Boger, D.L., Lerner, R.A. and Gilula, N.B. (1996) Nature 384, 83–87.
[3] Goparaju, S.K., Uueda, N., Yamaguchi, H. and Yamamoto, S. (1998) FEBS Lett. 422, 69–73.
[4] Sudol, M. (1998) Oncogene 17, 1469–1474.
[5] Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. and Schlessinger, J. (1993) Cell 74, 83–91.
[6] Rotin, D., Bar-Sagi, D., O'Brodovich, H., Merilainen, J., Lehto, V.P., Canessa, C.M., Rossier, B.C. and Downey, G.P. (1994) EMBO J. 13, 4440–4450.
[7] Anderson, B.L., Boldogh, I., Evangelista, M., Boone, C., Greene, L.A. and Pon, L.A., J. Cell Biol. 141, pp. 1357–1370.
[8] Gout, I. et al. (1993) Cell 75, 25–36.
[9] Grabs, D., Slepnev, V.I., Songyang, Z., David, C., Lynch, M., Cantley, L.C. and De Camilli, P. (1997) J. Biol. Chem. 272, 13419–13425.
[10] Volchuk, A., Narine, S., Foster, L.J., Grabs, D., De Camilli, P. and Klip, A. (1998) J. Biol. Chem. 273, 8169–8176.
[11] Oldenhof, J., Vickery, R., Anafi, M., Oak, J., Ray, A., Schoots, O., Pawson, T., von Zastrow, M. and Van Tol, H.H. (1998) Biochemistry 37, 15726–15736.
[12] Okamoto, P.M., Herskovits, J.S. and Vallee, R.B. (1997) J. Biol. Chem. 272, 11629–11635.
[13] Patricelli, M.P., Lashuel, H.A., Giang, D.K., Kelly, J.W. and Cravatt, B.F. (1998) Biochemistry 37, 15177–15187.
[14] Arreaza, G., Devane, W.A., Omeir, R.L., Sajnani, G., Kunz, J., Cravatt, B.F. and Deutsch, D.G. (1997) Neurosci. Lett. 234, 59–62.
[15] Omeir, R.L., Chin, S., Hong, Y., Ahern, D.G. and Deutsch, D.G. (1995) Life Sci. 56, 1999–2005.
[16] Feng, S., Chen, J.K., Yu, H., Simon, J.A. and Schreiber, S.L. (1994) Science 266, 1241–1247.
[17] Nemoto, Y., Arribas, M., Haffner, C. and DeCamilli, P. (1997) J. Biol. Chem. 272, 30817–30821.
[18] Mayaux, J.F., Cerbelaud, E., Soubrier, F., Yeh, P., Blanche, F. and Petre, D. (1991) J. Bacteriol. 173, 6694–6704.
[19] Kobayashi, M., Fujiwara, Y., Goda, M., Komeda, M. and Shimizu, S. (1997) Proc. Natl. Acad. Sci. USA 94, 11986–11991.
[20] Wada, I., Rindress, D., Cameron, P.H., Ou, W.J., Doherty, J.J.D., Louvard, D., Bell, A.W., Dignard, D., Thomas, D.Y. and Bergeron, J.J. (1991) J. Biol. Chem. 266, 19599–19610.
[21] Giang, D.K. and Cravatt, B.F. (1997) Proc. Natl. Acad. Sci. USA 94, 2238–2242.
[22] Shpetner, H.S., Herskovits, J.S. and Vallee, R.B. (1996) J. Biol. Chem. 271, 13–16.
[23] Robinson, P.J., Sontag, J.M., Liu, J.P., Fykse, E.M., Slaughter, C., McMahon, H. and Sudhof, T.C. (1993) Nature 365, 163–166.
[24] Hillard, C.J., Wilkison, D.M., Edgemond, W.S. and Campbell, W.B. (1995) Biochim. Biophys. Acta 1257, 249–256.
[25] Ueda, N., Kurahashi, Y., Yamamoto, S. and Tokunaga, T. (1995) J. Biol. Chem. 270, 23823–23827.