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Phosphodiesterases 4D and 7A Splice Variants in the Response of HUVEC Cells to TNF-α[1]

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The mRNA accumulation of phosphodiesterases PDE4D and PDE7A was studied by RNA blot analysis in human umbilical vein endothelial cells (HUVEC) incubated with TNFα for different periods. A contrasting behaviour was observed in the mRNA accumulation of the two genes. Further analysis by RT-PCR of the PDE4D and PDE7A splice variants gave different accumulation patterns which may indicate that differential splicing has a role in the regulation of these enzymes. Three previously undescribed PDE4D isoforms, with different accumulation patterns, were also detected. They code for truncated PDE4D isoforms, which could participate in the regulation of PDE4D activity. © 2000 Academic Press

Key Words: **cAMP phosphodiesterase; inflammation; gene expression; tumor necrosis factor; endothelium; alternative splicing.**

cAMP acts as a second messenger of cytokines, growth factors, hormones and neurotransmitters. A fine regulation of the accumulation and intracellular location of cAMP is thus necessary. Its synthesis is mediated by adenylate cyclase (1), while its hydrolysis is catalysed by phosphodiesterases (PDEs). Human PDEs have been classified into 11 families (2, 3). Families 4 and 7 specifically hydrolyse cAMP, the PDE4 family being the largest described, with four genes coding for these proteins (PDE4A, B, C and D) (4). Two members of PDE7 (PDE7A, B) (2, 5) have so far been identified.

One of the reasons for the interest in cAMP-specific PDEs is their involvement in the inflammatory response, as therapeutical benefits have been observed when high cAMP levels are achieved, basically by the use of PDE4 inhibitors. The onset of inflammation is a complex process, with the participation of a large number of cells and different levels of regulation mediated by cytokines, immunoglobulins and other factors. PDE4 has been detected in some of these cells (6). PDE4 inhibitors (mainly rolipram), which are not able to discriminate between the four members of the PDE4 family, were used. This is an obstacle for an accurate physiological interpretation. The inhibitors SB207499 (7), a potent anti-inflammatory agent without the associated side effects of emesis seen with rolipram (8), and RS-33793 (9), are selective for the PDE4D subfamily. Thus, the use of specific inhibitors and the analysis of the regulation of PDE genes may allow to study the functional role of specific PDEs. For these reasons we have chosen to study the PDE4D subfamily. Moreover, the existence of different PDE4D splice variants allows a more detailed analysis of the regulation of this gene.

Less is known about PDE7A because, until very recently (10) there was no inhibitors available for this family. A role for PDE7A has been proposed in T-cell proliferation stimuli (11) indicating a possible function in inflammation. The cellular response during the early steps of inflammation can be mimicked by stimulating human umbilical vein endothelial cells (HUVEC) with TNF-α. We have used this model to study the accumulation of PDE4D and PDE7A mRNA in the early steps of the inflammatory response.

Abbreviations used: PDE, phosphodiesterase; PCR, polymerase chain reaction; RT, reverse transcriptase; UCR, upstream conserved region; HUVEC, human umbilical vein endothelial cells; ICAM-1, intracellular adhesion molecule-1; VCAM, vascular cell adhesion molecule-1; ELAM, endothelial–leukocyte adhesion molecule-1; TNF-α, tumor necrosis factor α; Knt, kilonucleotides.

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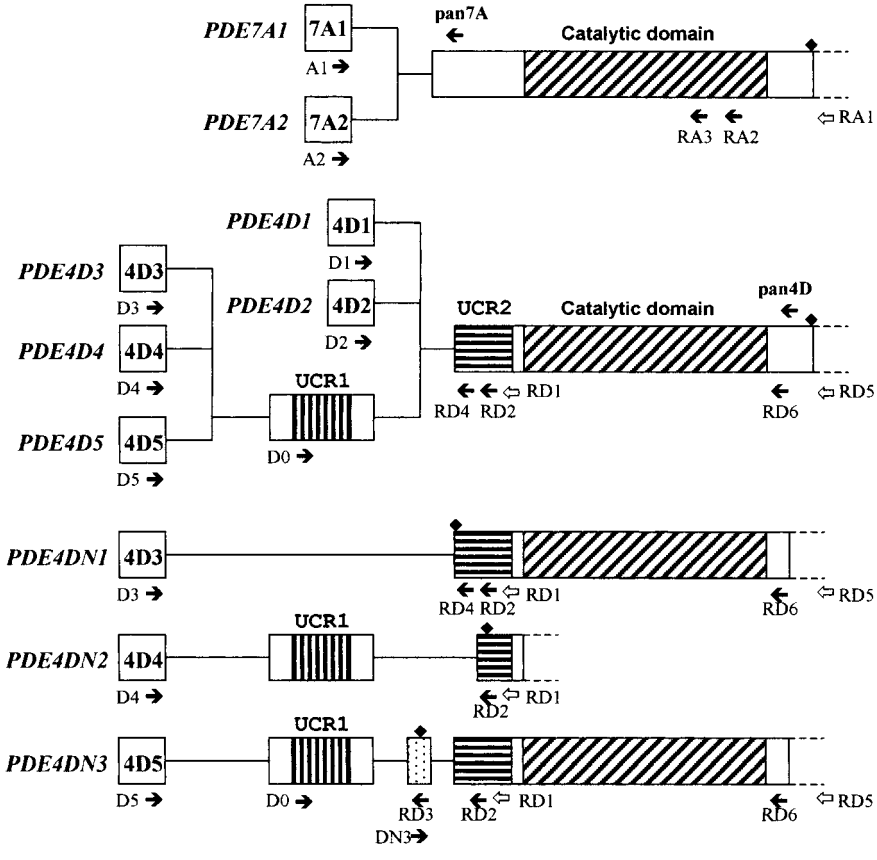

FIG. 1. PDE7A and PDE4D isoforms. Schematic representation of the previously described PDE7A and PDE4D isoforms, together with the new PDE4DN1, PDE4DN2, and PDE4DN3 splice forms. Regions of the strongest sequence conservation are shown by hatched regions (UCR1, straight lines; UCR2, horizontal lines; catalytic domain, sloping lines). The isoform-specific N′-extremes are distinguished by names inside the boxes. They are not to scale. The deletion present in PDE4DN2 is shown by a smaller UCR2 box. The new exon in PDE4DN3 is shown with a dotted box. The position of termination codons is marked by a dark rhombus. The white arrows indicate the oligonucleotides used for the reverse transcriptase reaction. Dark arrows indicate the oligonucleotides used for the PCR reaction study. Pan7A and pan4D were used for Northern analysis.

MATERIALS AND METHODS

Cell culture and TNFα treatment. Human Umbilical Vein Endothelial Cells (HUVEC) were isolated as previously described (12). Briefly, the umbilical vein was cannulated, flushed, and then incubated with 1% collagenase for 15 min at 37°C. Following removal of collagenase, detached cells were established as primary cultures in medium 199 (Bio-Whittaker, Walkersville, MA) containing 20% FCS. HUVEC were serially passaged (at 1:3 split ratio) and maintained using medium 199 supplemented with 20% FCS, 50 μg/ml endothelial growth factor and 100 μg/ml heparin in 175 cm^2 tissue culture flasks precoated with 0.5% gelatine. Cells were incubated in a 5% CO_2 air atmosphere at 37°C and used within the second and fifth passages. HUVEC were grown to 100% of confluence. At this time, for TNF-α activation, medium was replaced by serum-free medium, and TNF-α was added to a final concentration of 25 ng/ml and the cells were incubated for a period of time of 4 or 24 h (in this last case, 1% FCS-medium was used). Cells were detached using Trypsin/EDTA (GibcoBRL, Life Technologies, Paisley, Scotland), washed with PBS and frozen at −80°C until RNA isolation. An aliquot of these cells was analysed by cytofluorometry to observe the expression of adhesion molecules. Cells were washed with cold PBS, spinning 5 min at 1200 rpm and incubated at 4°C for 30 min in the presence of the first antibody. After washing and labelling for a further 15 min at 4°C in the presence of a second antibody, an FITC-conjugated rabbit F(ab)′2 anti-mouse IgG (Dakopatts, Glostrup, Denmark), samples were analyse in a FACScan cytometer (Becton Dickinson). Data were expressed as mean fluorescence intensity.

HeLa cells were grown at 37°C under 5% CO_2 in Dulbecco's Modified Eagle's Medium with 10% foetal bovine serum and 2 mM glutamine. The cells (between 80 and 90% confluency) were harvested with a plastic scraper in cold PBS and collected by centrifugation, washing them with cold PBS three times. Jurkat cells were cultured, harvested and resuspended as previously described (13). The cells were stored as pellets at −20°C until use.

RNA blot analysis. Total RNA from HeLa, Jurkat and control and TNF-α treated HUVEC cells was isolated as reported (14). Oligodeoxyribonucleotides were used as probes for PDE4D and PDE7A. They were either complementary to the base sequence encoding the PDE4D carboxy terminus (pan4D) or to an amino region present in both PDE7A1 and PDE7A2 isoforms (pan7A) (see Fig. 1 and Table 1). The oligonucleotides were labelled and the Northern analysis done as previously described (15, 16). These results were confirmed in two independent experiments carried out independently and quantified by densitometric analysis of the autoradiograph films with the Quantity One program (Bio-Rad, Hercules, CA), using ribosomal genes as reference, and a one-way analysis of variance ($P < 0.05$) was carried out.

TABLE 1

Oligonucleotides

	Oligonucleotide	Bases	Accession no.
PDE7A	A1	32–53	L12052
	A2	168–190	U67932
	RA1	1535–1514	L12052
	RA2	1415–1395	L12052
	RA3	1161–1141	L12052
	pan7A	289–245	L12052
PDE4D	D0	228–242	U50159
	D1	120–137	U50157
	D2	7–24	U50158
	D3	57–74	U50159
	D4	739–756	L20969
	D5	118–135	AF012073
	Dn3	626–643	AJ250854
	RD1	654–637	U50159
	RD2	628–611	U50159
	RD3	643–626	AJ250854
	RD4	587–570	U50159
	RD5	2099–2083	U50159
	RD6	1839–1824	U50159
	pan4D	1960–1916	U50159

Note. The list of the oligonucleotides used, corresponding or complementary to the bases of the sequence marked by their accession number.

Reverse transcriptase-PCR amplifications. HeLa, control or TNF-α-treated HUVEC cells total RNA, previously treated with RNase-free DNase, were reverse-transcribed into cDNA using standard protocols with RD1 (PDE4D) and RA1 (PDE7A) oligonucleotides as described in Table 1. The PCR amplification with PDE4D-specific oligonucleotides were performed with RD2 and the specific oligonucleotides for PDE4D1 (D1), PDE4D2 (D2), PDE4D3 (D3), PDE4D4 (D4), or PDE4D5 (D5). RD3, DN3 (PDE4DN3-specific oligonucleotides), RD4 and RD6 were also employed. In the case of PDE7A-specific oligonucleotides, the PCR amplifications were with the A1 and RA2 oligonucleotides for PDE7A1, and A2 and RA3 oligonucleotides for PDE7A2. These results were confirmed in 3 independent experiments. To confirm the identity of all PCR amplified bands, they were blotted onto nylon membrane and hybridised as described above with PDE4D, PDE7A or UCR1-specific ^{32}P-labelled oligonucleotide probes (data not shown).

RESULTS

Phosphodiesterase PDE4D and PDE7A mRNA Accumulation after Activation of HUVEC Cells with TNF-α

Treatment of endothelial cells with TNF-α induces the expression of cell adhesion molecules and mimics the early steps of the inflammatory response. To confirm that in our experimental conditions the HUVEC have acquired the proinflammatory phenotype, confluent endothelial cells were treated with the inflammatory cytokine TNFα for a period of time of 4 or 24 h and afterwards the surface expression of the adhesion molecules ELAM, VCAM-1 and ICAM-1 was analysed. Maximal expression of ELAM was observed at 4 h after the stimulation and reach basal levels at 24 h. ICAM-1 and VCAM-1 showed a weak expression at 4 h with maximal induction after 24 h (Fig. 2). These data are in agreement with those previously reported by several authors (17).

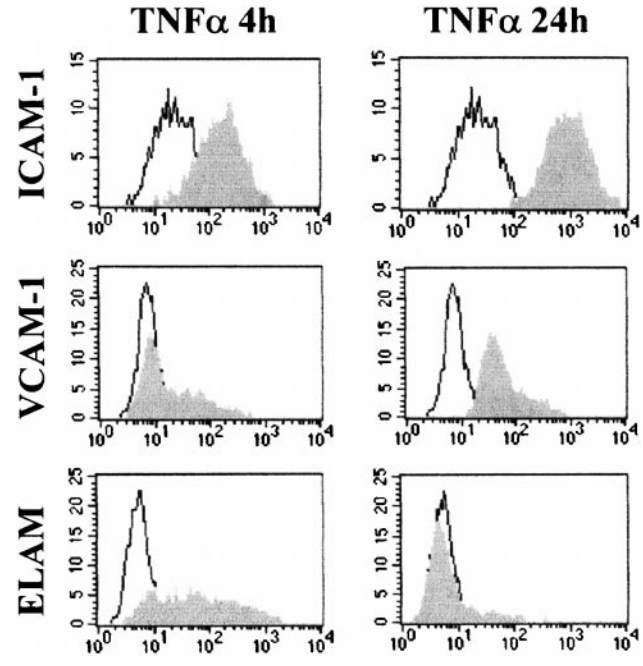

FIG. 2. Flow-cytometry analysis of ICAM-1, VCAM-1 and ELAM expression. HUVEC were untreated (control) or incubated for 4 or 24 h with TNF-α (25 ng/ml). At the end of the treatment cells were collected and subjected to FACS analysis. The *y*-axis indicates the cell number. Untreated cells are represented as a thick line and treated are represented as a dark profiles. TNF-α activation for 4 h is represented in the left column and, on the right, TNF-α activation for 24 h.

mRNA accumulation of PDE4D and PDE7A was measured during the activation of HUVEC by TNF-α at 4 and 24 h by RNA blot hybridisation with specific oligonucleotides (described in the Materials and Methods section). mRNAs from HeLa and Jurkat T-cells were used as a control. Figure 3 shows that PDE4D

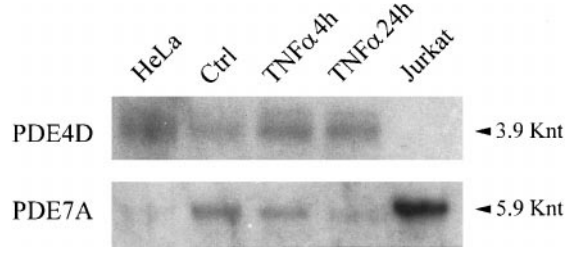

FIG. 3. RNA blot analysis of PDE4D and PDE7A in untreated and TNF-α incubated HUVEC. Using HeLa and Jurkat cells total RNA as controls, the mRNA accumulation of PDE4D and PDE7A was analysed in untreated (Ctrl) and after 4 (TNFα 4 h) or 24 (TNFα 24 h) h incubation with TNFα as described under Materials and Methods, using the oligonucleotide pan4D for PDE4D and pan7A for PDE7A.

mRNA, which is already present in untreated HUVEC, is further induced after a 4 h treatment with TNF-α with a significant increase (30%), and decreases near the control level (7%) after 24 h. PDE4D mRNA is present in HeLa cells, but it is undetectable in Jurkat T-cells. Thus, PDE4D is induced by TNF-α treatment in this *in vitro* system.

In contrast, our results show that PDE7A mRNA levels are diminished by the treatment. The steady state level of PDE7A mRNA, which is relatively high in untreated HUVEC (Fig. 3), significantly diminishes after a 4 h TNF-α treatment (40%), and after 24 h (54%). PDE7A was detected in both HeLa and Jurkat T-cells, with a high level of expression in the latter, as expected (18).

Accumulation of PDE4D and PDE7A Splicing Forms

A pool of different splice variants of PDE4D or PDE7A mRNAs were detected in RNA blots (Fig. 3). We decided to analyse the accumulation of the five different splice forms of PDE4D and the two of PDE7A, already described. Figure 4 shows RT-PCR analysis performed with oligonucleotides corresponding to the PDE4D and PDE7A splice forms. PDE7A1 and PDE7A2 mRNAs are present in the control untreated HUVEC and their steady state level decreases during treatment with TNF-α (see Fig. 4A), similarly to that found for total PDE7A mRNA (see Fig. 3). In contrast, the different spliced forms of PDE4D have different accumulation patterns upon treatment of HUVEC with TNF-α. The level of the short forms, PDE4D1 and PDE4D2, remains unaltered during treatment of the cells (see Fig. 4B) as well as for the long form, PDE4D3, (see Fig. 4C). However, PDE4D4, which is not detected in the control untreated cells, accumulates slightly after 4 h of treatment increasing after 24 h (see fig.4C). Moreover, PDE4D4 was the only PDE4D spliced form not detected in HeLa cells. A third pattern of expression is shown by PDE4D5 which is only transiently induced after 4 h of TNF-α treatment, being inhibited and becoming undetectable after a 24 h treatment (see Fig. 4C).

Identification of New Spliced Forms of PDE4D

During the amplification of the PDE4D differentially-spliced forms from activated HUVEC RNA, additional unexpected bands were amplified. The cloning and sequencing of these bands revealed previously undescribed cDNAs that may correspond to new spliced variants of PDE4D. Figure 4C shows an additional smaller band (PDE4DN1) obtained when amplifying PDE4D3. The cloning and sequencing of this band revealed a new combination of previously described PDE4D exons where the UCR1 domain was deleted (see Fig. 1). As shown in Fig. 4C the amplification of PDE4D4 from induced HUVEC RNA also produced two

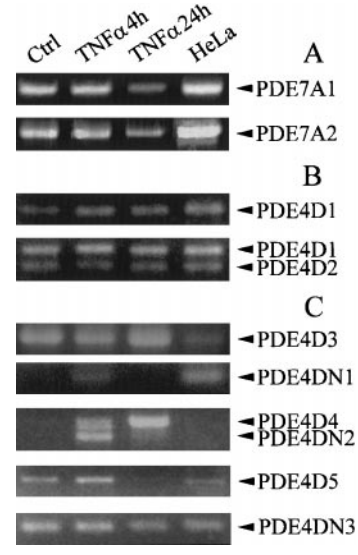

FIG. 4. RT-PCR analysis of the expression of PDE7A and PDE4D isoforms in untreated and TNF-α incubated cells. HeLa and untreated HUVEC total RNAs were used as controls. (A) Using the RA1 oligonucleotide in the RT reaction, PDE7A isoform expression was analysed using the A1-RA2 oligonucleotides for PDE7A1 and A2-RA3 oligonucleotides for PDE7A2. (B) Using the RD1 oligonucleotide in the RT reaction, the expression of PDE4D short forms was detected with the D1-RD2 oligonucleotides for PDE4D1, and the D2-RD2 oligonucleotides for both PDE4D1 and PDE4D2. (C) Expression of the PDE4D long forms was studied using the RD1 oligonucleotide in the RT reaction, and also RD5 when RD6 oligonucleotide is used in the PCR amplification. Unexpected bands were amplified. PDE4D3 was detected with the D3-RD4 oligonucleotides, and a smaller band PDE4DN1 was also amplified in HeLa and 4 h TNF-α incubated HUVEC. With the oligonucleotides D4-RD2, some PDE4D4 was detected after 4 h and a stronger band after 24 h TNF-α incubation of HUVEC. Again, a smaller band PDE4DN2 was detected in 4 h TNF-α-treated HUVEC. Both PDE4D4 and PDE4DN2 were undetected in HeLa cells. Here we show the PCR amplification with the DN3-RD2 oligonucleotides.

different bands. The cloning and sequencing of these bands revealed that the smaller one (PDE4DN2) corresponds to a new variant where the UCR2 domain is partially deleted (see Fig. 1). Although the human genomic sequence of the PDE4D gene remains unknown, we are able to compare this 113 bp deletion with the published human PDE4A (19) and rat PDE4D (20) genomic sequences. In both cases, UCR2 is formed by two complete exons and the beginning of a third one. The UCR2 fragment deleted in PDE4DN2 has the same size and it is in an equivalent position to, the first UCR2 exon, that corresponds to exon 6 in the human PDE4A gene (19) and the exon located between the introns A and B in the rat PDE4D gene (20).

RT-PCR analyses (Fig. 4C) showed that amplification of PDE4D5 produces no additional bands. However, in a parallel attempt to amplify UCR1, UCR2 and the catalytic domains of PDE4D, a band larger than expected was obtained (PDE4DN3). The sequence of this band revealed an insertion of 44 nucleotides

within a previously described PDE4D splicing point. To elucidate the putative 5′ end of PDE4DN3, a PDE4DN3-specific oligonucleotide was designed and RT-PCR assays on HUVEC RNA were done with combinations of this RNA and oligonucleotides corresponding to the 5′ specific regions of PDE4D3, PDE4D4 and PDE4D5. A band with the predicted size hybridising to a UCR1 specific oligonucleotide probe was amplified only when using a PDE4D5 specific oligonucleotide, suggesting that PDE4DN3 is actually an amplified mRNA fragment present only in PDE4D5-specific mRNAs (see Fig. 1).

In order to rule out a possible artifactual origin for PDE4DN1, PDE4DN2 and PDE4DN3, RT-PCRs were performed with RNA obtained from HeLa cells. The bands obtained were hybridised with specific oligonucleotide probes, cloned and sequenced. The sequences obtained were 100% identical to those obtained from HUVEC RNA, confirming the existence of RNAs with this structure in both HUVEC and HeLa cells. PDE4DN2, as well as PDE4D4, was not detected in HeLa cells (see Fig. 4C). Surprisingly in all these cases the new sequences generate stop codons that produce prematurely truncated proteins containing neither the conserved catalytic site of PDE4Ds nor the UCR2 domain. We propose a new name for these non-catalytic PDE variants adding N and a number after the letter of the gene, therefore these forms have been named PDE4DN1, PDE4DN2 and PDE4DN3.

The steady state level of PDE4DN3 mRNA is relatively high in control HUVEC and it is hardly affected by treatment with TNF-α. In contrast, PDE4DN1 mRNA is undetectable in HUVEC but its accumulation is slightly induced after a 4 h treatment of the cells with TNF-α. This induction is transient, and the mRNA becomes undetectable after a prolonged treatment of the cells with TNF-α (24 h) (see Fig. 4C), as found for PDE4D5 although this was present in untreated cells. PDE4DN2 was detected at a level similar to PDE4D4 after a 4 h treatment, both being undetectable in control HUVEC cells. However, while PDE4D4 was clearly enriched after 24 h, PDE4DN2 was undetectable (see Fig. 4C).

DISCUSSION

In this report we have analysed the expression of the cAMP-specific phosphodiesterases PDE4D and PDE7A in endothelial cells treated with TNF-α. We found that, while the mRNA corresponding to PDE4D increases along with the induction of several adhesion molecules after activation of HUVEC by TNF-α, the accumulation of the mRNA corresponding to PDE7A is inhibited during this treatment. The induction of PDE4D suggests that at least part of the PDE activity that has been shown to be involved in inflammatory processes can be attributed to PDE4D. The results obtained for PDE7A mRNA, however are somewhat surprising. Little is known about the physiological role of PDE7A. Here we show that PDE7A mRNA is present at high levels in untreated endothelial cells and that the activation of these cells by TNF-α correlates with a decrease of the steady state level of PDE7A mRNA.

Analyses of the accumulation of the PDE4D and PDE7A splice forms reveals another level of complexity of the regulation of phosphodiesterase activity during the TNF-α response. Although PDE7A1 and PDE7A2 variants have the same pattern of accumulation in activated HUVEC as the complete PDE7A mRNA as analysed by RNA blotting, PDE4D isoforms have different patterns of expression. PDE4D5 is induced transiently after 4 h of TNF-α treatment, coinciding with the maximal expression of the ELAM adhesion molecule (21), while PDE4D4 is induced after a 4 h treatment but it increases after 24 h, as usually found for ICAM-1 and VCAM-1 (22, 23). Thus the pattern of expression of different PDE4D variants accompanies those of well characterised molecules expressed during the inflammatory response suggesting a functional significance of this differential regulation. It has been shown that PDE4D isoforms are targeted to discrete subcellular compartments and that they can be induced by hormones in a temporal and spatial dependent manner (24). In addition, a yeast two-hybrid screen has recently shown that PDE4D5 can interact with RACK1, a signalling scaffold protein (25), while RACK1 does not interact with other PDE4D isoforms or with PDE4A, PDE4B, and PDE4C proteins. Otherwise, of the five PDE4D isoenzymes only PDE4D4 is able to bind to SH3 domains, what would allow for association with certain SH3 domain-containing proteins (26). It is thus essential to be able to discriminate between the different variants when analysing the expression of PDE4D mRNAs.

We also detected three previously undescribed PDE4D spliced variants. Independent cloning and sequencing from both HUVEC and HeLa cells rules out a possible artifactual origin. Sequencing of these PDE4D forms showed that they generate stop codons (see Fig. 1) to produce prematurely truncated proteins without UCR2 and the conserved catalytic site of PDE4Ds. Although this is the first time that such defective spliced forms are described for the PDE4D gene, similar structures have been described in the PDE4A gene. Two different insertions have been described within the PDE4A splice variant TM3 (27). The first one disrupts the UCR1 domain generating a premature stop codon, while the second is truncated just before the UCR2 domain, as predicted for the PDE4DN3 described here. Another PDE4A splice variant called 2EL has been isolated from a human T-cell Jurkat cDNA library (28). It has a 34 bp insertion within the PDE4A catalytic domain, between exons E and F (19), being catalytically inactive.

Defective forms generated by differential RNA splicing modulating the activity of proteins have been described for example in the control of sexual development in *Drosophila melanogaster*, where the male flies produce non-functional splice variants of the gene *Sex-lethal* (29). In other cases alternative splicing can lead to the separate expression of single domains of a protein. A noncatalytic domain of the focal adhesion kinase $pp125^{FAK}$ is autonomously expressed as a separate protein and acts as an inhibitor of the kinase (30). A variant of FosB generated by alternative splicing (ΔFosB) inhibits Fos/Jun activity presumably due to competition with FosB on heterodimer formation with Jun (31). α_{2i}, a variant form of the α_2 subunit of soluble guanylyl cyclase contains an in-frame insert of 31 amino acids within the catalytic domain, and interacts with the β1 subunit to form an inactive enzyme (32).

Here we describe three new spliced forms that could give rise to the separate expression of the N-terminal region of PDE4D3 (PDE4DN1), PDE4D4 (PDE4DN2) and PDE4D5 (PDE4DN3), or to the UCR1 domain (PDE4DN2, PDE4DN3). N-terminal domains could interact either with other proteins that participate in the same pathway or with functional PDE4s modulating their activity. It is also known that the UCR1 domain can interact with the UCR2 domain (4). This interaction, proposed to be intramolecular, could block the inhibitory activity of the UCR2 domain of PDE. The expression of a single UCR1 regulatory domain could lead to intermolecular blocking of UCR2, resulting in an increased activity of that PDE4. This mechanism might also modulate the activity of the short forms (PDE4D1 and PDE4D2), that lack the UCR1 domain. Then, although these short forms remain unaltered on TNF-α treatment, their activity could be modulated by the truncated isoforms, whose expression is clearly affected by the activation of HUVEC with TNF-α.

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