# Co-localization of P2Y1 receptor and NTPDase1/CD39 within caveolae in human placenta

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Nucleoside triphosphate diphosphohydrolase-1 (NTPDase1/CD39) is the dominant ecto-nucleotidase of vascular and placental trophoblastic tissues and appears to modulate the functional expression of type-2 purinergic (P2) G-protein coupled receptors (GPCRs). Hence, this ectoenzyme could regulate nucleotide-mediated signalling events in placental tissue. This immunohistochemical and immuno-electron microscopic study demonstrates the expression of NTPDase1/CD39, P2Y1 and P2Y2 receptors in different cell types of human placenta. Specifically P2Y1 has an exclusive vascular distribution whereas P2Y2 is localized on trophoblastic villi. Co-localization of P2Y1 and NTPDase1/CD39 are observed in caveolae, membrane microdomains of endothelial cells. The differential localization of these P2 receptors might indicate their unique roles in the regulation of extracellular nucleotide concentrations in human placental tissues and consequent effects on vascular tone and blood fluidity.

Key words: caveolin, caveolae, endothelial cell, G-protein coupled receptor, nucleotide signaling.

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The physiological action of extracellular nucleotides including adenosine 5-triphosphate (ATP), on P2 purinoceptors is controlled by several families of surface-located enzymes (ectonucleotidases) and P2 purinoceptors (Burnstock 1990, Zimmermann 2000). These P2 purinoceptors are both ion channel (P2X) and G-protein coupled receptors (P2Y) and can be expressed by the same cell in association with ectonucleotidases.

Membrane-bound or soluble ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) of the CD39 family are important vascular ectonucleotidases (Zimmermann 1999b). The regulation of P2-signalling systems is related mainly to the combined action of NTPDases and an AMPase termed ecto-5′-nucleotidase (Enjyoji et al. 1999, Zimmermann 1999a, 2000, Zimmermann and Braun 1996). The prototype member of the NTPDase family is the NTPDase1/CD39 (Kaczmarek et al. 1996, Marcus et al. 1997).

The presence of extracellular ATP hydrolyzing enzymes and ATP diphosphohydrolase activity has been reported on syncytiotrophoblast cells of human placenta in several studies to date (Anand et al. 1996, Brunette et al. 1995, Matsubara et al. 1987, Seida et al. 1980, Treinen and Kulkarni 1986a, 1986b, 1987, Valenzuela et al. 1996, Whitsett and Wallick 1980). Makita and co-workers have described human isoforms of ecto-ATP diphosphohydrolase presumably generated by alternative splicing of the pre-mRNA of CD39. Using immunohistochemical analysis, this enzyme was observed in the microvillous membrane of syncytiotrophoblasts and in endothelial cells (EC) (Makita et al. 1998).

ATP is released from endothelial cells by a variety of stimuli, including hypoxia (Dubyak and el-Moatassim 1993, Ralevic and Burnstock 1998) and regulates the level of an important second messenger, intracellular $Ca2^{+}$ via purinergic receptors (Karl et al. 1997). In this way, ATP may be involved in the

modulation of various trophoblastic functions, including hormone secretion and active transport of nutrients. The level of this extracellular nucleotide must be strictly regulated and the presence of NTPDase1/CD39 was anticipated in human placenta (Enjyoji et al. 1999). Indeed, the enzyme was distributed on the surface of endothelial cells and highly concentrated levels of its expression were visible in caveolae that are particularly abundant here. These domains contain cholesterol, glycosphingolipids,glycosylphosphatidylinositol-(GPI)-linked proteins and their main structural proteins, caveolins (Gafencu et al. 1998).

Our studies have revealed targeting of NTPDase1/CD39 to the caveolae of HUVEC (human endothelial umbilical vein endothelial cell culture) cells (Kittel et al. 1999, Koziak et al. 2000). These detergent insoluble, specialized areas at the surface of cells are important in both endocytosis and receptor mediated signaling (Anderson 1993, Thomsen et al. 2002)

Caveolin-1 and caveolin-2, which are marker proteins for caveolae, are expressed at high levels in endothelium of placental capillaries, in endothelial and smooth muscle cells of larger vessels and fibroblasts in areas of the placenta with high connective tissue content (Lyden et al. 2002). Recently caveolin-1 expression has been demonstrated in ultrathin cryosections of terminal villi of the human term placenta (Takizawa and Robinson 2003).

The presence of several purinoceptors has also been described in trophoblast. Relatively high levels of P2Y6 receptors have been demonstrated by Somers and colleagues and a role suggested in trophoblastic development, differentiation and neoplasia (Somers et al. 1999). Transcripts of two types of human P2Y1 cDNA clones have been also found (Ayyanathan et al. 1996, Leon et al. 1996). The expression of P2X receptors have been described on chorionic surface arteries (Dobronyi et al. 1997). Recent results suggested that P2X receptors participate in the humoral regulation of placental blood flow (Valdecantos et al. 2003).

Although functional relationships between purinoceptors and ectonucleotidases have been proposed, co-localization has not been demonstrated to date. Our goal was to demonstrate the presence of NTPDase1/CD39 in human placenta and to investigate possible co-localization of this ectonucleotidase with P2Y1 or P2Y2 receptors.

## Materials and Methods

### *Reagents*

All reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified. CD39 monoclonal antibody was purchased from Accurate (Accurate, CA, USA), polyclonal anti-caveolin-1 from Santa Cruz (Santa Cruz Biotechnology, CA, USA), P2Y1 and P2Y2 polyclonal antibodies against P2Y1 and P2Y2 receptors were obtained from Alomone (Alomone Labs, Jerusalem, Israel).

Human placenta tissue samples at term were obtained from healthy pregnancies after vaginal delivery or post-cesarean section. Other material was obtained from legal terminations of pregnancy in collaboration with the 1st Department of Obstetrics and Gynecology, Semmelweis Medical School (Hungary). Proper consent forms were obtained and ethical regulations were strictly observed.

### *Light microscopic demonstration of P2Y1 and P2Y2 receptors in human placenta*

After delivery, small tissue blocks of 3mm thickness from human placenta at various gestational ages were embedded in OCT (Miles Inc. Diagnostic Division, Elkhart, IN) and immediately frozen in liquid nitrogen. Sections were cut at a thickness of 4 µm and mounted on polyionic slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). After fixation with PBS-buffered acetone (at -4°C, for 15 min) and several rinses in PBS, tissue sections were blocked with 7% NGS in PBS for 30 min and incubated overnight with the antibodies to P2Y1 or P2Y2 (in 1:50 dilution) on a shaking plate at 4°C. After draining the antibody solution and washing with PBS, endogenous peroxidase activity was blocked by 0.15% H2O2/PBS for 10 min. Biotinylated anti-rabbit IgG was used in 1:200 dilution as the secondary antibody. The ABC method was used with DAB as chromogen (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturers' instruction. In control experiments 7% NGS/PBS was used instead of primary antibody.

### *Ultracryoimmunogold electron microscopy for the demonstration of CD39, caveolin-1, P2Y1 and P2Y2 receptors*

After fixation in 3% paraformaldehyde/PBS for 1 hour and washing in PBS for 1 hour, 1 mm3 tissue blocks were infiltrated in 2.3 M sucrose for 1h and ultracryo-sections were cut at -105°C under liquid

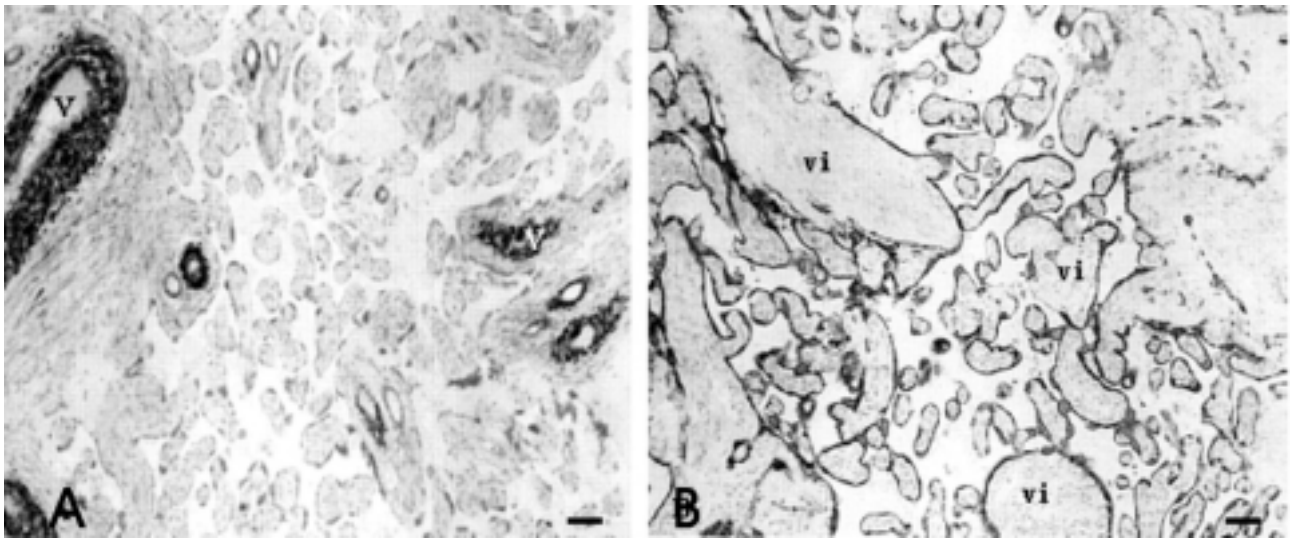


**Figure 1. Expression of P2Y1 and P2Y2 receptors on cryosections of human placenta. A. Diamino-benzidine (DAB) staining shows the localization of P2Y1 receptor within full-term placenta. Precipitates are exclusively in the vasculature (v). B. ABC-DAB staining for P2Y2 receptor within full-term placenta. Precipitates are on the surface of trophoblastic villi (vi), indicative of syncytiotrophoblast cells. Bar 15 μm.**

nitrogen, according to the Tokuyasu method (Tokuyasu 1986). Immunostaining was performed on ultrathin, 80 nm sections on drops of different solutions. After 3 washing steps with PBS (2 min each) and blocking with 1% BSA in PBS for 10 min, sections were incubated with the antibodies in 1% BSA/PBS (CD39 antibody in 1:500, caveolin in 1:300, P2Y1, P2Y2 1:50 dilution) at room temperature for 30 min. Sections were washed in PBS four times altogether for 15 min. Protein-A coupled immunogold (15 or 10 nm in size) in BSA/PBS was applied as the secondary antibody for 20 min, followed by four repeated washes for 15 min.

For double labeling the procedure was repeated with the other antibody, after 1 min fixation with 1% glutaraldehyde (for 5 min) and 4 changes in 15 min of 0.2 M glycine. After washing (four times in PBS and six times in water), the sections were counterstained in 4% uranyl acetate in water for 5 min and embedded in 2% methyl cellulose/4% uranyl acetate 9:1 mixture for 5 min on ice. In control experiments primary antibody was omitted from the incubation medium. Sections were investigated under Jeol 1200EX electron microscope.

### *Pre-embedding immunostaining and demonstration of CD39, caveolin-1, P2Y1 and P2Y2 receptors by use of the ABC-DAB method in 10- week-old human placenta*

Fixation of the tissue was performed in 3% paraformaldehyde in PBS as above. After washing three times in PBS, 100 nm sections were processed and endogenous peroxidase activity was blocked by 0.15% H2O2/PBS for 10 min. Sections were thoroughly washed in PBS and incubated in blocking solution (7% NGS-7% NHS in PBS) for 30 min and then incubated with the respective primary antibodies on a shaking plate at 4°C, overnight. Control sections were incubated with fresh blocking solution. Biotinylated anti-rabbit or anti-mouse IgG was applied in 1:200 dilution for 1 hour after washing and the ABC method with DAB as chromogen (Vector Laboratories, Inc., Burlingame, CA, USA) was used. Postfixation of the tissue sections was carried out with 1% osmium tetroxide for 30 min. After washing in 30% and 50% ethanol, sections were incubated for 30 min with 2% uranyl acetate in 70% ethanol in the dark. Following further dehydration with 90% ethanol and twice with 100% ethanol, sections were transferred for 30 min at 37°C to a 1:1 (v/v) absolute ethanol/Taab 812 resin mixture. This was followed by 30 min infiltrations with pure Taab 812 at 60°C and overnight embedding at 60°C. Ultrathin sections were cut and examined with a Hitachi 7100 transmission electron microscope.

## Results

### *Bright-field microscopy distribution of P2Y1 and P2Y2 receptors in human placenta*

Immunostaining performed on cryosections of full-

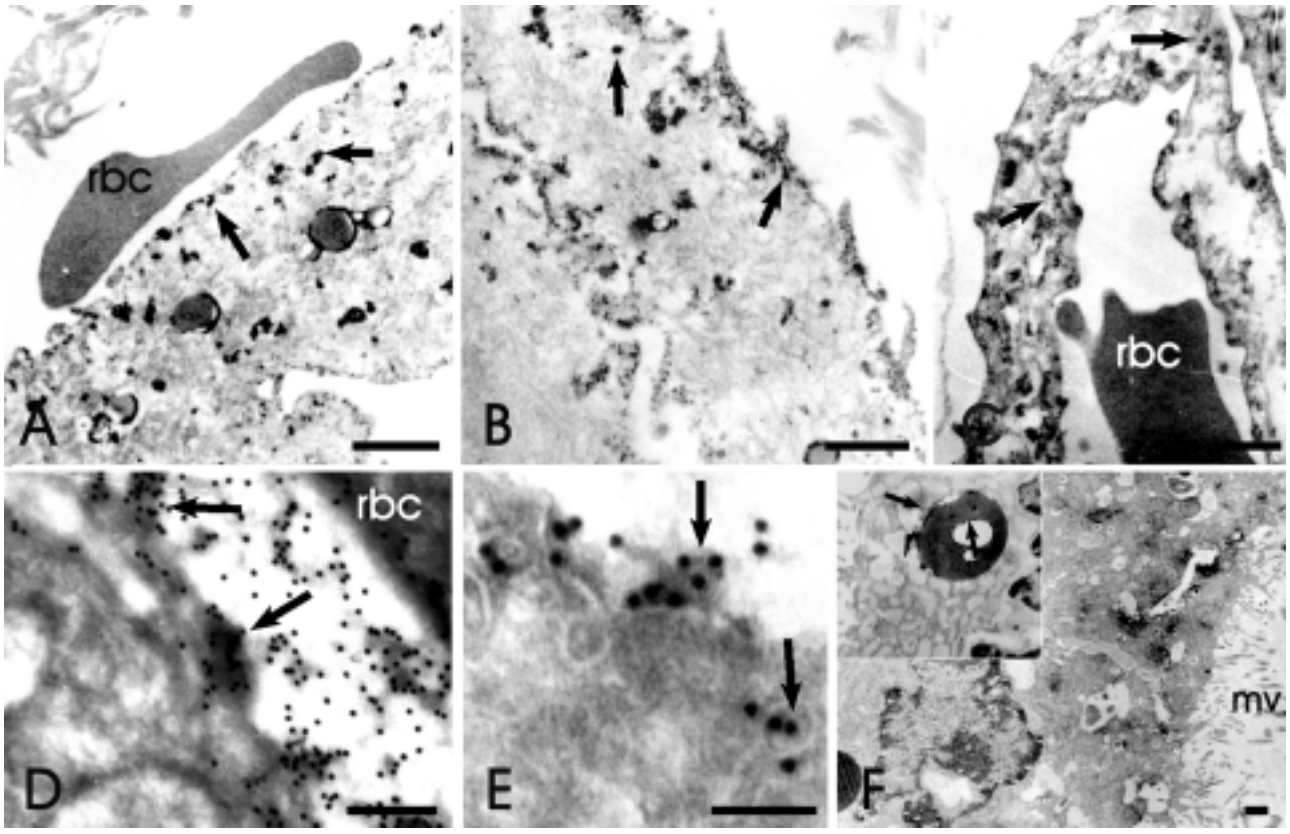


**Figure 2. Expression of caveolin-1, P2Y1 receptor and CD39 in human placenta. A. Caveolin-1 staining of 10-week-old human placenta. DAB precipitates are observed in the small caveolae-like structures on endothelial cells (arrows). Red blood cells (rbc) are free of staining. B. P2Y1 staining in 10-week-old placenta (arrows). The localization pattern is similar to the caveolin staining above. C. ABC-DAB staining for CD39 in 10-week-old human placenta, confirming staining in the caveolae of an endothelial cell (arrows). Red blood cells (rbc) are not stained. D. Double immunogold labeling on ultracryo sections of full-term placenta. 10 nm immunogold particles indicate the localization of caveolin-1 and 15 nm gold particles the localization of P2Y1 receptor on the surface of the endothelial cell. Concentrated gold particles in the caveolae-like structures (arrows). Background staining on precipitated plasma proteins is observed. E. Immunogold staining for caveolin-1. 15 nm particles in the caveolae (arrows) of an endothelial cell from full-term human placenta (Ultracyo section). F. There is no staining for P2Y1 receptor on the surface of syncytiotrophoblast cells. The microvillous membrane (mv) is free of DAB precipitates Insert: ABC-DAB staining for P2Y1 receptor in 10-week-old human placenta. The surface of microvillious membrane of syncytiotrophoblast is free of staining, but some precipitates are found in the lysosomes of the cell (arrows). Bars indicate 0.5 µm.**

term human placenta showed the localization of P2Y1 exclusively with blood vessels (Figure 1A) whereas strong staining for P2Y2 receptor was observed on syncytiotrophoblasts (Figure 1B). The vessel endothelium was free of P2Y2 staining.

### *Localization of caveolin-1, P2Y1 receptor and CD39 in human placenta at the electron microscopic level*

Strong caveolin-1 staining was found in the endothelial cells of 10-week-old human placenta (Figure 2A). The number of caveolae was few and their size was small (max. 50 nm). A similar distribution pattern was observed for P2Y1 staining (Figure 2B) and CD39 (Figure 2C).

Double immunogold labeling was performed on ultracryo sections of full-term placenta. Both caveolin-1 (10 nm immunogold particles) and P2Y1 receptor (15nm immunogold particles) were localized on the surface of endothelial cells. The gold particles accumulated in the caveolae (Figure 2D). 15 nm immunogold particles demonstrate caveolin-1 in the caveolae of an endothelial cell (Figure 2E). ABC-DAB staining was performed for P2Y1 receptor. Staining was negative for syncytiotrophoblast in full-term placenta (Figure 2F). However, some DAB precipitates were found in the lysosomes of syncytiotrophoblast cell of 10-week-old placenta, but no immunoreactivity for P2Y1 receptor was present on the surface of microvilli (Figure 2F insert).

### *Localization of CD39 and P2Y2 receptors in full-term placenta*

Immunogold staining (10 nm gold particles) established the presence of CD39 on the microvilli of a syncytiotrophoblast cell (Figure 3A). Figure 3B

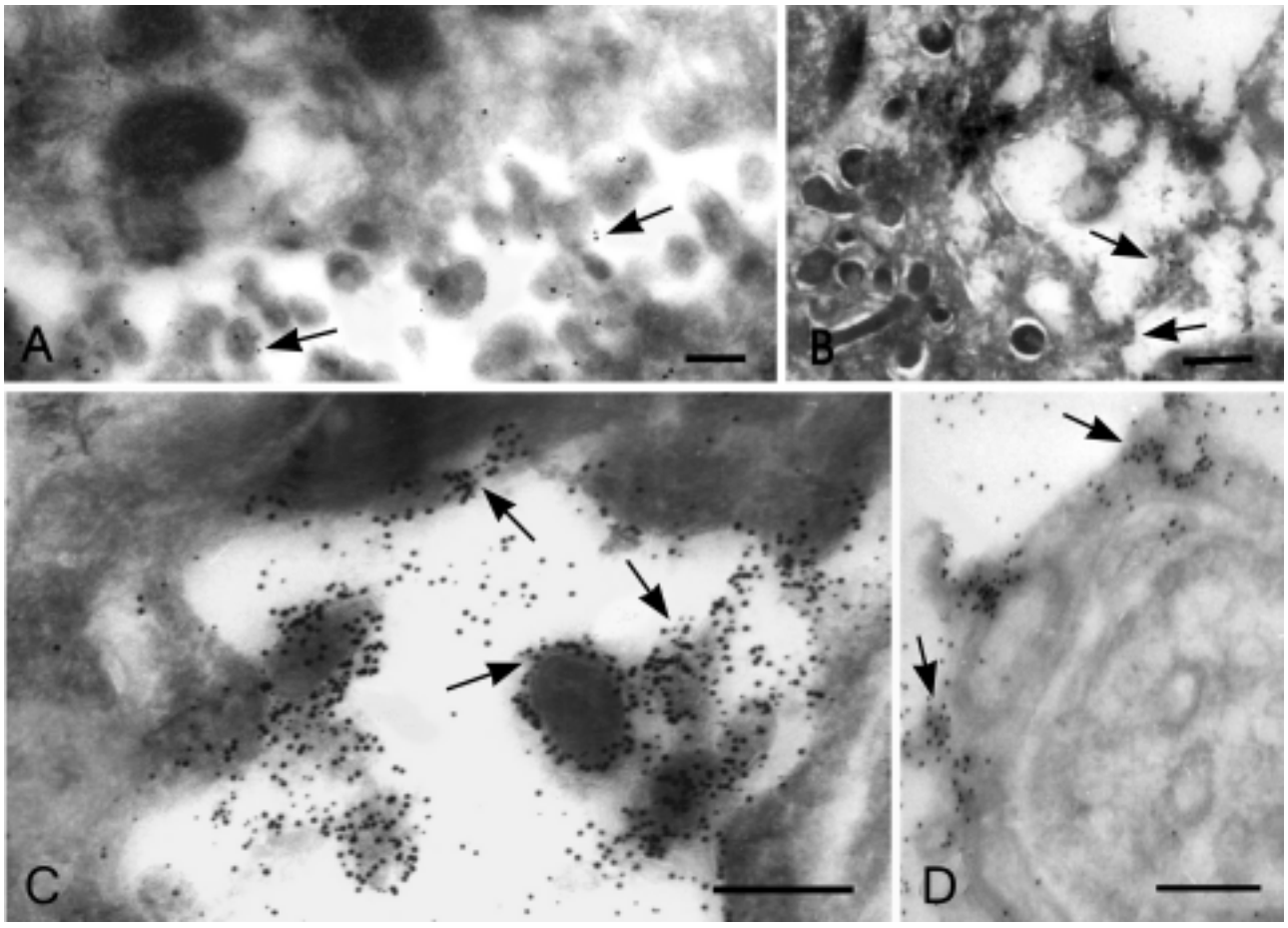


**Figure 3. Expression of CD39 and P2Y2 receptor on ultracryo sections of trophoblast cells. A. CD39 immunoreactivity is demonstrated by 10 nm immunogold particle labeling of the microvillous membrane of syncytiotrophoblast (arrows). B. P2Y2 staining of syncytiotrophoblasts. Labeling with 10 nm immunogold particles (arrows) confirms localization of P2Y2 receptor on the microvillous membrane of the syncytiotrophoblast cells. C. Double immunogold staining for CD39 (10 nm) and P2Y2 (15 nm). Both sizes of immunogold particles are observable on the surface of the syncytiotrophoblast cells (arrows). D. Double labeling for CD39 and P2Y1 receptor. The cytotrophoblast cell is labeled only for CD39 (arrows). No P2Y1–staining could be demonstrated in these sections. Bars indicate 0.4 μm**

shows the result of P2Y2 staining, with 10 nm immunogold particles located on the microvilli of syncytiotrophoblasts. Double immunogold staining demonstrated the presence of CD39 and P2Y2 on the surface of syncytiotrophoblast cells (Figure 3C). Cytotrophoblast cells were also strongly labeled for CD39 but double staining was negative for P2Y1 receptor (Figure 3D).

## Discussion

Nucleotide-mediated signaling may be important in the regulation of human placental circulation. The identification and pharmacological characterization of several P2X receptor populations in the smooth muscle of superficial vessels (Valdecantos et al. 2003) suggest participation of P2X receptors in the humoral regulation of placental blood flow. The activation of P2Y receptors preferentially expressed on endothelial cells could further modulate blood flow within the placenta.

Caveolins are known to bind to several types of signaling molecules such as GTP-binding proteins (G-proteins), receptors and effectors (Anderson 1998, Sargiacomo et al. 1993). Targeting of NTPDase1/CD39 to these specialized plasmalemmal domains suggested integration of cellular activation events, also known from our previous work (Kittel and Bácsy 1994, Kittel et al. 1999, Koziak et al. 2000). The covalent lipid modification of the N-terminal region of CD39, that undergoes palmitoylation in a constitutive manner, appears to be important both in plasma membrane association and in targeting CD39 to caveolae (Koziak et al. 2000).

We have observed differences in both the distribution and size of caveolae depending on the age of

gestation. The observed differences in the size and localization pattern of caveolae might be connected with the maturation process of placenta, including changes in the structure of caveolae. The number and type of signal molecules concentrated in these invaginations may also change during the maturation of human placenta, as previously shown for ATPase activity in thymocytes and other cells (Beguin et al. 1998, Hehl et al. 1985).

Our demonstration of the localization of P2Y1 receptors within caveolae is a novel finding. P2Y1 immunoreactivity was found in the membrane as well as in these invaginations of endothelial cells. Cytotrophoblasts and syncytiotrophoblasts, irrespectively of the gestational age of the placenta, were free of reaction product.

Syncytiotrophoblasts of full-term human placenta were positive for P2Y2 while endothelial cells did not show any staining. However, we could not find any P2Y2 staining on the microvilli of syncytiotrophoblasts in 10-week-old placenta. This may also suggest maturation-dependent expression of this receptor in a manner comparable to P2Y6 receptor that is suggested to play a role in trophoblastic development and differentiation (Somers et al. 1999). Expression of P2 (P2Y1 and P2Y11) receptors in human placenta has been reported (Ayyanathan et al. 1996, Communi et al. 1997). The endothelial P2Y1 purinoceptor was cloned from human placenta (Leon et al. 1996). This receptor has been suggested to play a crucial role in platelet aggregation, in the mediation of nucleotide-induced release of nitric oxide, and in the modulation of neuronal signal transmission.

In the light of our present knowledge about the role of caveolae in signal transduction and the suggested connection between ectonucleotidases and purinoceptors, the co-expression of NTPDase1/CD39 and P2Y1 within the caveolae may have a special significance in human placenta under both physiological and pathophysiological conditions. The co-localization of the receptor and one of its main regulators would serve to fine-tune the activity of extracellular nucleotides.

In conclusion, we have studied patterns of NTPDase1/CD39 localization in endothelial and syncytiotrophoblast cells in human placenta and observed expression within the endothelial caveolae. Cavolae in endothelial cells also contained P2Y1 receptors. P2Y2 receptors were found abundantly in syncytiotrophoblasts of full-term but not 10-week-old placenta. Although the physiological functions remain unknown, different mediators trigger these P2 receptors Hence, the differential distribution of two P2Y receptors may indicate a previously unrecognized mechanism for regulation of nucleotide signaling in trophoblastic tissue.

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