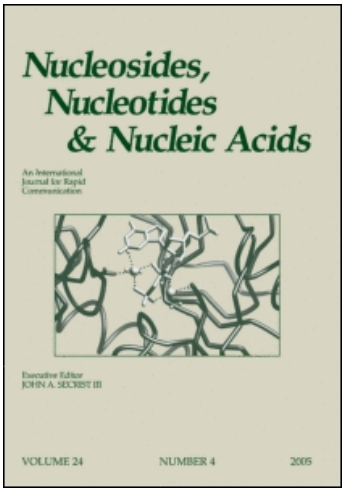

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Endogenously Expressed Truncated P2X7 Receptor Lacking the C-Terminus is Preferentially Upregulated in Epithelial Cancer Cells and Fails to Mediate Ligand-Induced Pore Formation and Apoptosis

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ENDOGENOUSLY EXPRESSED TRUNCATED P2X7 RECEPTOR LACKING THE C-TERMINUS IS PREFERENTIALLY UPREGULATED IN EPITHELIAL CANCER CELLS AND FAILS TO MEDIATE LIGAND-INDUCED PORE FORMATION AND APOPTOSIS

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□ *A truncated naturally occurring variant of the human purinergic receptor P2X7 (P2X7-R) was found in human cancer cervical cells. The novel protein consists of 258 amino acids, and compared to the wild-type P2X7-R it lacks the entire intracellular carboxy terminus, the second transmembrane domain, and the distal third of the extracellular loop. The truncated P2X7-R failed to form pores and mediate apoptosis, and it interacted with the wild-type P2X7-R in a manner suggesting auto-hetero-oligomerization. In contrast to cancer cells the novel truncated P2X7-R was expressed relatively little in normal cervical cells. These data raise the possibility that coexpression of the truncated form could block P2X7 mediated apoptosis and promote uncontrolled growth of cells.*

Keywords Cervix; Epithelium; P2X7; Receptor; Truncated; Variant; Apoptosis

INTRODUCTION

Growth of uterine cervical epithelial cells is regulated by mitogenic stimuli, and controlled by inhibitory mechanisms such as apoptosis. An important apoptotic system in the cervix is the P2X7-receptor (P2X7-R) mechanism.[1] The P2X7-R belongs to the P2X subfamily of P2 nucleotide receptors, which are membrane–bound, ligand–operated channels.[2] The naturally occurring ligand of the P2X7-R is ATP, which is secreted constitutively by cells into the extracellular milieu. In the cervix P2X7-R activation

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is believed to regulate apoptosis via the mitochondrial apoptotic pathway through plasma membrane pore formation and uncontrolled influx of calcium.[1] A recent study described that treatment with the P2X7-R specific agonist 2′,3′-0-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP) can induce apoptosis in both normal and cancer cervical cells. However, the effects were greater in normal than in cancer cells,[1] suggesting that cancer cervical cells have evolved mechanisms that protect them from P2X7-R mediated apoptosis. The present article reports the discovery of a novel variant of the human P2X7-R that is endogenously expressed in human cancer cervical cells but relatively little in normal cervical cells. When expressed exogenously in host cells that lack endogenous expression of the P2X7-R, the variant was deficient in pore formation and failed to mediate apoptosis, suggesting that it could play a role in cervical tumorogenesis.

MATERIALS AND METHODS

The experiments utilized human cervical epithelial cancer CaSki, Hela, SiHa and HT3 cells; MDCK (Madin Darby canine kidney cells, strain II, expressing tetracycline regulated repressor); HEK293 (Human Embryonic Kidney 293 cells); and discarded normal human uterine cervical tissues. Total RNA extracted from CaSki cells was used for RT-PCR to amplify the human P2X7-R gene using sense (TTT TTA AGC TTA TGC CGG CCT GCT GCA GCT G) and antisense (TTT TTG CGG CCG CTC AGT AAG GAC TCT TGA AGC C) primers; the amplified P2X7-R genes were subcloned into pcDNA5/FRT vector with Hind III and Not I sites. For inducible expression of P2X7-R in MDCK cells that lack endogenous expression of the receptors, P2X7-R genes were subcloned into pcDNA4/TO vector with Hind III and Not I sites. A Myc tag was attached to the N-terminal with a new Myc-containing sense primer (TTT TAA GCT TAT GGA ACA AAA ACT TAT TTC TGA AGA AGA TCT GCC GGC CTG CTG CAG CTG A) and the same antisense primer. For simultaneous heterologous expression of both the full-length and truncated P2X7-Rs in HEK293 cells, P2X7-R and Myc-P2X7-R (truncated) DNAs were subcloned into pBud 4.1 plasmid with Hind-III and Not-I sites. Transfections of P2X7 plasmid DNAs were performed using GenePORTER Transfection Reagent.

Dynamic confocal laser scanning microscopy was used to determine changes in intracellular ethidium bromide (EB) fluorescence. Cells were seeded at $2–3 \cdot 10^5$ on 35-mm glass bottom petri dishes and allowed to reach confluence. Cultures on glass-bottomed dishes were loaded onto the microscope and imaged at the 488 nm/505 nm exc/emm after adding 5 μM of ethidium bromide to the bathing solutions. Average fluorescence

intensity was quantified from collated images using MetaVue softeware (Fryer Company Inc., Huntley, IL, USA) by subtracting the basal intensity values.

Apoptosis was quantified using Roche Cell-Death Detection Elisa Kit (Roche Applied Science, Penzberg, Germany). Western blots were done as described[3] using rabbit polyclonal anti-P2X7 antibody (APR-008, #AN-01, Alomone Labs, Jerusalem, Israel), or rabbit monoclonal anti-Myc-antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Co immunoprecipitation/immunoblotting assays were described.[3]

RESULTS AND DISCUSSION

RT-PCR assays using CaSki cell lysates revealed two PCR products: the full-length P2X7-R gene (GeneBank Accession number Y09561), and a smaller product that lacks the putative exon 8, except A882. Sequence analysis revealed that retention of the A882 causes a shift of the coding frame, resulting in a variant that encodes a truncated protein consisting of only 258 amino-acids (compared to 595 of the full-length P2X7-R) with 10 altered unique residues at the C-terminus (IRQVLQGKQC). The predicted sequence of this variant lacks the entire carboxy cytoplasmic domain, the second transmembrane domain, and the distal third of the extracellular domain of the full-length P2X7-R. Blast search of available gene database showed that this variant has not been previously reported. (The authors are in the process of submission of the new sequence to the GeneBank). The truncated P2X7-R gene was subcloned and introduced by transfection into MDCK and HEK293 cells, which lack endogenous expression of the P2X7-R. Stable cell lines were successfully generated, showing expression of de-novo 45 KDa–42 KDa protein by Western blots in both types of cells (Figures 1A and 1B).

To determine whether the truncated P2X7-R gene is endogenously translated into protein, lysates of HeLa, SiHa and HT3 cells (Figure 1C) and of CaSki cells (not shown) expressing the truncated P2X7-R mRNA were examined by Western blot with anti-P2X7 antibody. Figure 1C shows two main specific forms at 75 KDa and 45 KDa–42 KDa, that could be blocked by the P2X7 antigen (not shown). The 75 KDa form is most likely the full-length P2X7-R;[2] the 45 KDa–42 KDa is likely the truncated P2X7-R protein since the sizes of exogenously expressed truncated P2X7-R in MDCK cells (Figure 1A) and HEK293 cells (Figure 1B) were identical. Collectively, these data suggest that the truncated P2X7-R (45 KDa–42 KDa cluster) is a naturally occurring P2X7-R variant in cervical cancer cells.

Interestingly, expression of the truncated P2X7-R protein (the 45 KDa–42 KDa immunoreactive forms) was detected mainly in cancer cervical cells but not in 3 histologically normal ectocervical tissues (Figure 1C).

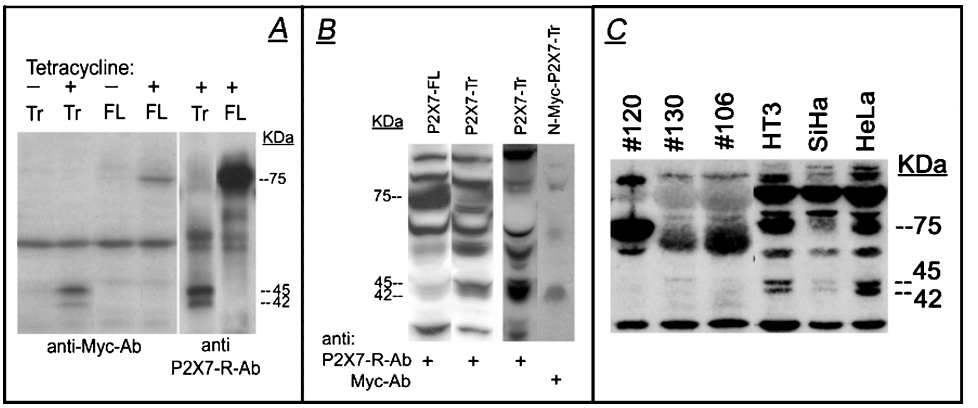

FIGURE 1 Exogenous (**A**, **B**) and endogenous (**C**) expression of the human full-length P2X7-R (FL) and truncated P2X7-R (Tr). **A**. MDCK cells expressing tetracycline regulated repressor were transfected with N-Myc-P2X7-R-FL or N-Myc-P2X7-R-Tr cDNAs. Expression of P2X7-R specific proteins was induced by treatment with tetracycline. **B**. HEK293 cells were transfected with P2X7-R-FL, P2X7-R-Tr, or with N-Myc-P2X7-R-Tr cDNAs. Western blots were done using the indicated antibodies. Note that transfections with P2X7-R-FL cDNAs resulted in expression of the full-length 75 KDa P2X7-R protein, and transfections with P2X7-R-Tr cDNAs resulted in expression of a 45 KDa–42 KDa cluster. **C**. Endogenous expression of the full-length P2X7-R (75 KDa) and truncated P2X7-R (45 KDa–42 KDa) proteins by human cervical cells. Western blots utilized the anti-P2X7-R antibody. Immunoblots are of lysates of the cancer cervical cells HeLa, SiHa, and HT3 cells (similar results, not shown, were obtained using CaSki cells); and of normal discarded ectocervical tissues of patients #106, 130, and 120. Note the abundance of expression of the full-length P2X7-R (75 KDa) in normal tissues, and the abundance of expression of the truncated P2X7-R (45 KDa–42 KDa) in lysates of cancer cervical cells.

In contrast, expression of the full-length 75 KDa immunoreactive form was abundant in the normal tissues compared to the cancer cells (Figure 1C). These results suggest that normal cervical epithelial cells express mainly the full-length P2X7-R, while cancer cervical epithelial cells express both the full-length P2X7-R and the truncated P2X7-R variant.

To determine the functional capacity of the truncated P2X7-R, apoptosis assays were performed in HEK293 cells. Expression of the full-length P2X7-R resulted in increased baseline apoptosis (Figure 2A), possibly induced by ATP secreted by the cells to the extracellular milieu and mediated in an autocrine-paracrine mechanism by the full-length P2X7-R system.[1] This conclusion is supported by the finding that expression of the full-length P2X7-R also predisposed cells to augmented apoptosis in response to treatment with BzATP (Figure 2A). Expression of the truncated P2X7-R, on the other hand, did not enhance apoptosis (Figure 2A), suggesting that the variant truncated P2X7-R is nonfunctional in mediating agonist-induced apoptosis.

P2X7-R–mediated apoptosis in cervical cells involves formation of pores.[1] To determine whether the truncated P2X7-R can mediate pore formation, ethidium bromide (EB) influx assays were done in HEK293 cells. In cells expressing the full-length P2X7-R treatment with BzATP in

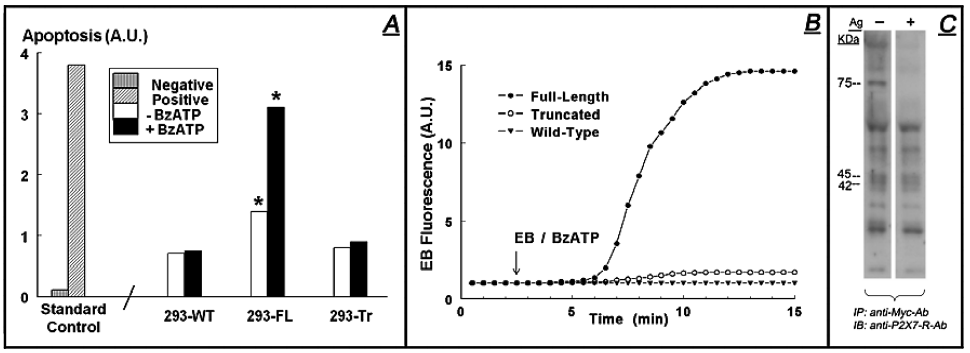

FIGURE 2 Functional capacities of the truncated P2X7-R. **A** and **B**. Experiments utilized either wild-type HEK293 cells (WT) or HEK293 cells transfected with the full-length P2X7-R (FL) or truncated P2X7-R (Tr). In **A**, cells were shifted to serum-free medium for 6 hours in the absence or presence of added BzATP (100 μM), and apoptosis was determined as described in Methods. *—$p < 0.01$. In **B**, cells attached on glass-bottomed dishes were shifted to medium containing 5 μM ethidium bromide (EB) and treated with 100 μM BzATP. A.U.—arbitrary units. **C**. Lysates of P2X7-R-FL—HEK293 cells co transfected with N-Myc-P2X7-R-TR cDNA were immunoprecipitated with the anti-Myc antibody, and immunoblotted with the anti-P2X7-R antibody in the absence or presence of the P2X7-R antigen.

the presence of EB resulted in time-related increase in EB fluorescence (Figure 2B), indicating EB influx through BzATP-induced pore formation. Expression of the truncated P2X7-R failed to mediate a significant change in EB fluorescence in response to BzATP, compared to wild-type cells (Figure 2B). These data suggest that failure of HEK293 cells expressing the truncated P2X7-R to undergo apoptosis in response to BzATP is the result of inability of the variant receptor to mediate pore formation.

Functional P2X7-Rs operate as homotrimers.[3] A possible mechanism by which the truncated P2X7-R could block P2X7-R–mediated apoptosis in cells expressing both the full-length and truncated receptors, is oligomerization of the variant isoform with the full-length receptor, thereby blocking P2X7-R action. The experiment in Figure 2C tested this hypothesis, and it shows that in lysates of HEK293 cells expressing the full length receptor and co transfected with N-Myc-P2X7-R-Tr cDNA the 75 KDa wild type P2X7-R can be specifically co immunoprecipitated with the 45 KDa–42 KDa variant using anti-Myc antibodies. These data suggest that the truncated P2X7-R protein can interact with the full length receptor.

In summary, the present data identify a new P2X7-R variant with apoptosis-inhibitory characteristics, and suggest a novel distinct regulatory property for a truncated variant receptor to antagonize its wild type isoform through auto-hetero-oligomerization. This may represent a novel general paradigm for regulation of a protein function by its variant. In the case of cervical cells, co expression of the truncated variant could block apoptosis and contribute to tumorogenesis.

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