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Original

Transient expression of polymeric immunoglobulin receptor in human adenocarcinoma cell line HT-29

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Abstract: Human polymeric immunoglobulin receptor (pIgR) protein was expressed in the adenocarcinoma cell line HT-29 using a recombinant vaccinia virus transfection method. The pIgR protein was detected as 110- and 120-kDa bands by immunoprecipitation after metabolic labeling. PIgR was released as a free secretory component into the culture supernatant and was detected as a 110-kDa band. PIgR cleavage was investigated by adding the proteinase inhibitor leupeptin or protein kinase C activator PMA. Consistent with previous observations in the Madin Darby canine kidney cell system, cleavage of pIgR was inhibited by leupeptin and enhanced by PMA stimulation, thus indicating that it is regulated by common mechanisms. This experimental system should be very useful for pIgR investigation. (J. Oral Sci. 47, 15-20, 2005)

Keywords: pIgR; HT-29; vaccinia virus; leupeptin; PMA.

Introduction

The mucosal immune system is the first line of defense against a variety of antigens (1-3). The main players in this system are the polymeric immunoglobulins (pIgs). PIgs produced by plasma cells must be transported across epithelial cells in order to exert their protective effects against environmental antigens. This protein transport step is called transcytosis and is mediated by polymeric immunoglobulin receptors (pIgR) (4) expressed on the basolateral surface of glandular epithelial cells.

In a previous study, we reported the establishment of a transient pIgR expression system in baby hamster kidney (BHK) cells, using recombinant vaccinia virus containing T7 RNA polymerase (vTf-7) (5). However, BHK cells are not physiologically appropriate for pIgR protein expression. HT-29 is a well-known cell line derived from human colon adenocarcinoma, which constitutively expresses very low levels of pIgR, and has been widely studied. Generally, the introduction of foreign cDNA into HT-29 cells has been performed by electroporation or regular transfection. With these methods, however, expression of protein was only detectable after several days of culture. vTf-7-based transfection is a well-known transient transfection technique (6). The infectability of HT-29 cells by recombinant vaccinia virus has been reported (7), but the applicability of this transfection method has never been tested.

In the present study, we aimed to express human pIgR in HT-29 cells using vTf-7 infection combined with lipofectamine transfection. Expression of human pIgR protein could be detected only a few hours after transfection. The pIgR expressed in HT-29 cells was cleaved and the extracellular portion was released as a free secretory component (SC). Moreover, some of the mechanisms controlling pIgR cleavage were found to be common to this and other experimental systems.

Materials and Methods

Cell culture

HT-29 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Approximately 5×10^5 cells were

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plated on 35 mm dishes the day before transfection.

Reagents

Leupeptin and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma.

Plasmids

Human polymeric immunoglobulin receptor (pIgR) cDNA and pT7-blue plasmids were as described previously (5).

Transfection and metabolic labeling

Infection with recombinant vaccinia virus and transfection of HT-29 cells was performed as described previously (5). Briefly, before infection, cells were washed once with OPTI-MEM (Invitrogen, CA) and were then infected with vTf-7 (6) at a multiplicity of 10 plaque forming units/cell in 0.5 ml of OPTI-MEM for 30 min with intermittent rocking in a CO_2 incubator at 37°C. The infection medium was removed and the cells were washed with OPTI-MEM twice. Cells were then transfected with pIgR cDNA or pT7-blue vector (mock transfection) using a lipofectamine plus transfection method (Invitrogen, CA) according to the manufacturer's procedure. Briefly, 1 μg of plasmid DNA was mixed with 6 μl of plus reagent in 100 μl of OPTI-MEM, and at the same time, 6 μl of lipofectamine was mixed in 100 μl of OPTI-MEM. After 15 min, the medium containing DNA was mixed with lipofectamine medium and incubated at room temperature for 15 min. This transfection medium was then applied to HT-29 cells and incubated for 5.5 h in a CO_2 incubator at 37°C. After transfection, the cells were washed with labeling medium (Sigma Chemical, MO) and incubated with 1 ml of the same medium for 15 min in order to induce starvation. Cells were then metabolically labeled with 1.1 MBq/ml of Tran-[^{35}S]-label (ICN Biochmicals, CA) for 1 h at 37°C. Immediately after labeling, the cell lysates were collected and subjected to immunoprecipitation. For detecting the secretion of free SC in culture medium, the labeling medium was removed and the cells were washed with 10% FBS-DMEM. Cells were further cultured in the presence or absence of 10 μg/ml of leupeptin or 1 μM of PMA for 16 h. The samples were harvested and immunoprecipitated.

After transfection, cells were washed with PBS and further incubated with 10% FBS-DMEM for 1, 3 or 5 h. Cell lysates and the culture supernatants were harvested and pIgR was quantified by enzyme-linked immunosorbent assay.

Immunoprecipitation

After 16 h chase, cell lysates and culture supernatants were prepared and subjected to immunoprecipitation. Briefly, cells were lysed with 500 μl of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% TritonX-100). Cell lysates and culture supernatants were obtained by centrifugation (14,000 × *g* for 1 min) and transferred to new tubes. Then, 5 μl of polyclonal rabbit anti-human SC (DAKO Cytomation, Japan) was incubated with samples for 1 h followed by 10 μl of protein G-sepharose (Amersham, NJ) for 1 h at 4°C. After washing the pellets with 500 μl of cell lysis buffer 3 times, pellets were loaded onto 8% SDS-PAGE gels. After electrophoresis, gels were immersed in fluorography solution (125 mM sodium salicylate, 30% methanol) for 30 min at room temperature, dried for 2 h with a gel dryer (Bio-Rad, CA) and exposed to X-ray film (X-OMAT AR, Kodak, Japan) for 18 h. Images were captured by scanner and band intensity was quantified using NIH image analysis.

ELISA

Flat-bottomed 96-well plates were coated with 50 μl of rabbit anti-human SC Ab (× 1,000 diluted with PBS) for 18 h at 4°C. After washing the plates with PBS three times, they were incubated with 200 μl of 1% BSA-PBS for 1 h at 37°C. The 1% BSA-PBS was discarded and 50 μl of cell lysate or culture supernatant was applied to the plates, which were then incubated for 1 h at room temperature. Samples were discarded and plates were washed 3 times with 0.05% Tween 20/PBS. Next, 50 μl of horseradish peroxidase (HRP)-conjugated rabbit anti-human SC Ab (× 1,000 diluted with 1% BSA-PBS) were applied to the plates and incubated for 30 min at room temperature. Plates were extensively washed with 0.05% Tween 20/PBS. Color reaction was performed by incubating the plates with 50 μl of 1 mg/ml *o*-phenylenediamine (Kanto Chemical, Japan) in 0.1 M citrate phosphate buffer (pH 5.0) supplemented with 0.03% H_2O_2 for 20 min at room temperature. The reaction was stopped by adding 25 μl of 2 M H_2SO_4. Absorbance 490 nm was then measured on a microplate reader (model 3550; Bio Rad).

Results

Transient expression of pIgR protein in HT-29 cells

We previously reported the establishment of transient pIgR protein expression in the hamster fibroblastic cell line BHK using the recombinant vaccinia virus vTF-7 (5). Although this virus is known to have a wide host cell range (6), the applicability of this method for human

intestinal adenocarcinoma HT-29 cells and the forced expression of human pIgR in these cells has not been reported. After transfection, cells were incubated with Tran-[^{35}S]-label and the pIgR protein was immunoprecipitated with rabbit anti-human SC Ab. As shown in Fig. 1a, pIgR protein was clearly detected as a 110-kDa band in the pIgR transfectant but not in the mock transfectant (pT7-blue). Although HT-29 cells are known to express endogenous pIgR protein constitutively, the amount of endogenous pIgR protein was undetectable at 1 h after metabolic labeling.

Release of free SC into culture supernatant

PIgR protein expressed in BHK cells was released into the culture supernatant (5). Therefore, pIgR protein release in HT-29 cells was examined. The pIgR-transfected HT-29 cells were metabolically labeled and further cultured for 16 h. The culture supernatants were then harvested and immunoprecipitated. In culture supernatants of the pIgR transfectant, pIgR was cleaved by an unknown proteinase and the extracellular region of pIgR was released as free SC. As shown in Fig. 1b, free SC was detected as a 110-kDa band. On the other hand, in cell lysate, immature (110-kDa) and mature (120-kDa) bands were observed. The amount of free SC in the culture supernatant was estimated by ELISA. After tranfection, the cells were further incubated with 10% FBS-DMEM for the periods indicated

Fig. 1 Expression of pIgR in HT-29 cells and secretion of free SC.
a) After transfection with pT7-blue (mock) or human pIgR cDNA, cells were metabolically labeled with Tran-[^{35}S]-label for 1 h. Cell lysates were immunoprecipitated with rabbit anti-human SC Ab followed by protein G-sepharose. Samples were separated on 8% SDS-PAGE gels, after which gels were immersed in fluorography solution and exposed to X-ray film.
b) Expressed pIgR was labeled and chased for 16 h. Cell lysates (lysate) and culture supernatants (sup) were collected and immunoprecipitated as described above.

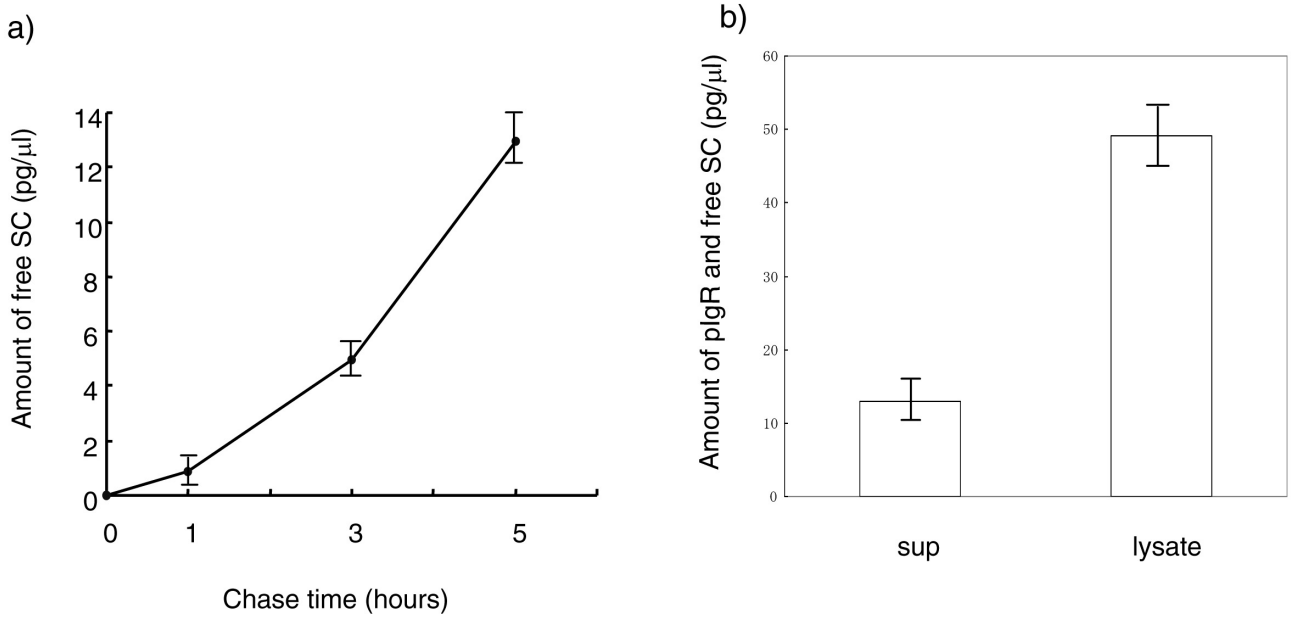

Fig. 2 Time-dependent secretion of SC from pIgR-transfected HT-29 cells.
a) HT-29 cells were transfected with pIgR and chased for the indicated times. The culture supernatants were harvested and the amount of free SC was estimated by ELISA.
b) HT-29 cells were transfected with pIgR and chased for 5 h. The culture supernatants (sup) and the cell lysates (lysate) were collected and subjected to ELISA.

in Fig. 2a. The amount of free SC increased in a time-dependent manner, and after 5 h chase, free SC concentration reached 12.9 pg/μl. Total cellular pIgR was 49.2 pg/μl at this point, and thus the released free SC represented approximately 20% of the total amount (Fig. 2b).

Free SC release is accelerated by activation of protein kinase C (PKC)

The release of free SC is reportedly inhibited by the proteinase inhibitor leupeptin (8,9). On the other hand, activation of PKC has been shown to enhance the release of free SC (10). The influence of these two reagents on the release of free SC was therefore examined.

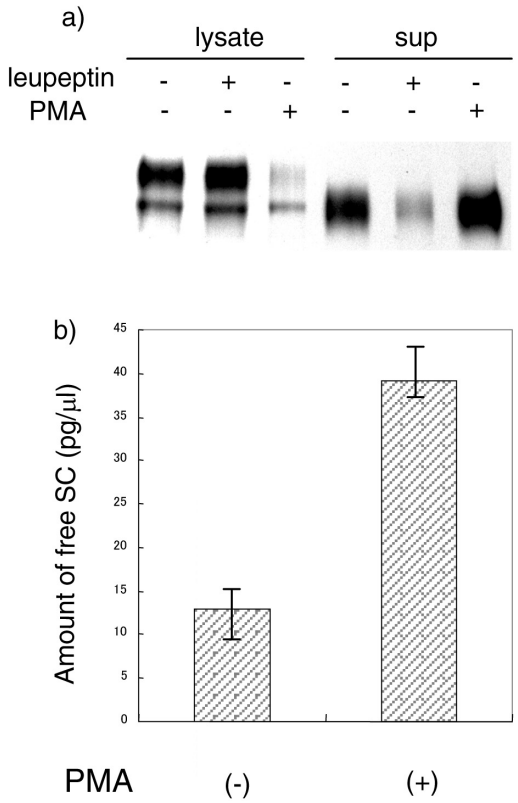

Fig. 3 Free SC secretion was inhibited by leupeptin but increased by PMA.
a) HT-29 cells were transfected with pIgR and chased with 10% FBS-DMEM in the presence or absence of 10 μg/ml of leupeptin or 1 μM of PMA and chased for 16 h. Cell lysates (lysate) and culture supernatants (sup) were collected and immunoprecipitated.
b) PIgR transfected HT-29 cells were cultured in the presence or absence of 1 μM of PMA for 5 h. The amount of free SC secreted into the culture supernatants was estimated as described for Fig. 2. After 5 h of culture in the presence of PMA, the amount of free SC increased approximately 3-fold when compared to controls without PMA.

Transfectants were metabolically labeled for 15 min and were chased for 16 h in the presence or absence of 10 μg/ml of leupeptin or 1 μM of PMA. Cell lysates and supernatants were collected and subjected to immunoprecipitation. The amounts of pIgR and free SC were calculated as described in Materials and Methods. In the absence of these two reagents, free SC released into the culture supernatant was 30.8% of the total labeled protein. In the presence of leupeptin, the amount of free SC decreased dramatically to only 10.7% of the total. On the other hand, after PMA stimulation, the released free SC reached 74.1% of the total, while the amount of pIgR in the cell lysates decreased drastically (Fig. 3a).

Free SC released into the culture supernatant after PMA stimulation was also estimated by ELISA (Fig. 3b). After 5 h chase, the amount of free SC released into the culture medium was 39.2 pg/μl in the presence of PMA. This value corresponded to a 3-fold increase when compared to controls without PMA.

Discussion

The majority of information concerning pIgR transcytosis has been acquired using the Madin Darby Canine Kidney (MDCK) cell experimental system (11). MDCK cells are widely used for studies on cell polarity and membrane traffic, and the system is based on the fact that these cells, when stably transfected with rabbit pIgR cDNA, can form well polarized monolayers with tight junctions (11). Using this system, it has been demonstrated that transcytosis of rabbit or rat pIgR can be stimulated by binding of its ligand, polymeric IgA (pIgA) (12-14). In contrast, in transfectants of human pIgR cDNA in MDCK cells, it was found that human pIgA binding does not stimulate transcytosis of the receptor (15,16). Although the intracellular signal evoked by pIgA binding was equivalent in rabbit and human pIgR, stimulation of human pIgR transcytosis was not observed (15). These discrepancies raised questions regarding species differences as there are several known pIgR differences between species, as follows: 1) alternative splicing gives rise to the smaller functional pIgR in rabbit but not in other species (17); 2) in rat and rabbit (18-20), pIgR was not able to bind to IgM as efficiently as human pIgR (21); and 3) PIgR is expressed in rat and rabbit hepatocytes, but not in human hepatocytes (22). These questions might be resolved when human pIgR can be force expressed in its original host cells which normally express pIgR. In this study, human pIgR was expressed in HT-29 cells using a recombinant vaccinia virus system.

Protein expression in HT-29 cells was detected immediately after transfection and the properties of pIgR

described in MDCK cells were found to be conserved in HT-29 cells. In the MDCK cell system, it has been demonstrated that activation of PKC by PMA enhanced transcytosis of the pIgR molecule, thus increasing the amount of free SC in the culture medium (10). In addition, the release of free SC was inhibited by the proteinase inhibitor leupeptin (8,9). This was tested in the present experimental system and was also demonstrated in HT-29 cells. Unlike MDCK, this experiment did not utilize a polarized culture system. If HT-29 cells were polarized on a filter support, as is possible with MDCK cells, it might be a very useful experimental tool to investigate pIgR transcytosis.

It has been demonstrated that PKC α and ε are recruited to the plasma membrane by PMA activation (10). Although the major phosphorylation sites of pIgR are Ser^{664} and Ser^{726}, transcytosis of substitution mutants was not affected by PMA stimulation, indicating that these two residues are not the substrates of PKC. The system used in this study might contribute to further elucidation of the actual PKC substrates.

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