Acta Veterinaria Hungarica 50 (4), pp. 481–489 (2002)

THE ENDOSOMAL EPSILON-COATOMER PROTEIN IS INVOLVED IN HUMAN ADENOVIRUS TYPE 5 INTERNALISATION

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(Received December 14, 2001; accepted May 27, 2002)

The effects of bafilomycin A1 and of the reduced level of endosomal epsilon-COP (coatomer protein) on the infectivity of human adenovirus type 5 were investigated in Coxsackie adenovirus receptor- (CAR-) transfected Chinese hamster ovary (CHO) cells. The endosomal proton pump inhibitor bafilomycin A1 was able to cause only partial inhibition. Using ldlF cells (an epsilon-COP thermosensitive mutant CHO cell line) the reduction of epsilon-COP level also had partial inhibitory effect. Based on these results and comparing them to existing models of the adenovirus entry, we propose a refined model in which there are two pathways of adenoviral entry: the first one involves the epsilon-COP as the downstream effector of the acidification and can be blocked by bafilomycin A1 and the second one is a pH-independent pathway.

Key words: Human adenovirus type 5, internalisation, epsilon-COP, bafilomycin A1

Adenoviruses are common pathogens causing diseases in humans and different animals. Human adenoviral vectors are well-characterised transducing agents and they are widely used in human and also in animal models (Mutwiri et al., 2000). They are also used in gene therapy as vectors (Benihoud et al., 1999) and this is based on the powerful penetration mechanism of adenovirus to transduce the target cells. Animal model systems are used to characterise, among the others, the protection of canine myocytes from ischaemia-reperfusion injury by transducing small heat shock protein (sHSP) via adenoviral vectors (Vander Heide, 2002) and the toxicology of adenoviral vectors in porcine model (Morrissey et al., 2002). Recently, adenoviruses of different host animal origin are also engineered into gene delivery systems (Löser et al., 2000; Löser et al., 2002; Nagy and Tuboly, 2000). Despite the widespread use of adenoviral vectors little is known about the infection process and there is a need to obtain a better under-

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0236-6290/2002/$ 5.00

standing of the penetration mechanism. The entry of human adenovirus serotype 5 (hAdv-5) depends on the binding of fiber protein to Coxsackie adenovirus receptor (CAR) (Bergelson et al., 1997) and on interaction of an RGD sequence of the penton base with α integrin receptors of cells (Wickham et al., 1993). This binding promotes receptor-mediated endocytosis, since virus particles bound to receptors are rapidly taken up in clathrin-coated vesicles via coated pits. The normal endosomal pathway progresses via early endosomes, endosomal carrier vesicle (ECV), late endosomes to lysosomes where endocytosed molecules will be degraded. The entire pathway takes 30–40 minutes. Adenoviruses do not follow this pathway to the end. At 2–10 minutes postinfection they appear in the cytosol, suggesting that the early endosomal compartments are the sites of release (Leopold et al., 1998). Ultrastructural studies also support this assumption (Morgan et al., 1969). This process is somehow related to the acidification of the endosome, because chloroquine, an endosomal acidification inhibitor, is able to inhibit the infection of adenovirus (Svensson and Persson, 1984). However, exact biochemical evidence has not yet been published. Examination of the proteins which could be involved both in the endosomal transport and the adenoviral entry process suggested that the epsilon-coatomer protein (COP) is a likely candidate. The epsilon-COP exerts a pH-dependent binding to the endosome membrane, which is indispensable for the action of protein on the endosomal traffic. In the absence of epsilon-COP, the transport between the early endosome and the ECV is abolished (Daro et al., 1997; Gu et al., 1997). These facts underline the possible role of epsilon-COP, which could be a downstream effector of acidification governing the fusion process between the early endosome and the ECV. If this protein is involved in the adenoviral entry, it could shed some light on endosomal processes necessary for the virus entry.

Aim of the study: Considering that CAR-transfected CHO cells are fully permissive for adenovirus infection, a new, advantageous animal model has been established. Using recombinant human adenovirus serotype 5 in a viral transducing assay we have assessed the effect of the withdrawal of functional epsilon-COP protein on the efficiency of infection in this model. To further clarify the role of epsilon-COP in the adenoviral entry process we have studied the possible synergistic effect between bafilomycin A1, an endosomal proton pump inhibitor, and the epsilon-COP withdrawal.

Materials and methods

Cells, viruses, materials

HeLa cells were grown and maintained in high glucose Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 10% FBS (Sigma) and antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin). Wild-type Chinese Hamster Ovary [(wt) CHO] and mutant ldlF cell lines (generous gift of Dr M. Krieger)

were grown and maintained in F12 medium (Sigma) with 10% FBS and antibiotics. Recombinant E1A deficient human adenovirus type 5 containing a SV40 promoter driven luciferase construct (Ad5-Luc3, a gift obtained from Dr F. L. Graham) (Mittal et al., 1993) was propagated in 293 cells, banded on CsCl and the collected virus was stored at –80 °C. The virus titres were determined by TID_{50} titration on 293 cells using the extrapolation method of Reed and Muench.

Bafilomycin-A1, chloroquine and luciferin were obtained from Sigma. The human CAR expressing plasmid (phCAR) was described by Bergelson et al. (1997) and generously provided by Dr J. Bergelson.

Experiments

Adenovirus infectivity assay

The adenovirus infectivity assay was performed on CHO cells. Wt CHO and ldlF cells were grown on 6 cm dishes (8×10^5 cells/dish in F12 with 10% FBS) for one day. Cells were transfected with 3 μg/dish of plasmid DNA using LipofectAmine 6 μl/dish (Gibco) according to the manufacturer's instructions. Twenty h post-transfection cells were collected and plated on 96-well cell culture plates (3×10^4 cells/well in F12 with 10% FBS) and grown overnight. Plates were incubated at permissive temperature (34 °C) or at restrictive temperature (40 °C) for 6 h before infection. During this period the cells were treated with bafilomycin A1 (1 μM) or chloroquine (0.5 mM) where applicable. Cells were infected at 5000 multiplicity of infection (MOI). Plates were extensively washed at 30 min postinfection three times with PBS containing 1% BSA and once with F12 with 10% FBS and the luciferase activity was measured 20 h postinfection. Briefly, the cells were harvested by the addition of 100 μl luciferase buffer (50 mM glycine, 1 mM Tris, 5 mM $MgSO_4$, 0.5 mM EDTA, 0.1% BSA and 0.1% sodium azide) containing 1% Triton X-100. This was added directly to the cell culture medium. One hundred μl of lysate was mixed with 100 μl luciferase buffer containing 0.15 mM luciferin 0.1 μM ATP in a Berthold LB8010 tube luminometer by the built-in injection system and the generated light was measured.

Proliferation assay

For the proliferation assay (wt) CHO, ldlF and HeLa cells were plated onto 96-well plates and grown overnight. Plates were incubated at permissive or at restrictive temperature for the same time as the incubation time used in the experiments. Cells were treated with bafilomycin A1 according to the experimental protocol. After treatment the cells were further incubated for an appropriate time (usually for one day) and measured with a MTT protocol: the cells were incubated in the presence of 0.5 mg/ml MTT (Sigma) for 3 h and at the end of

the incubation period the medium was removed. The converted dye was solubilised with acidic isopropanol (0.1 N HCl in absolute isopropanol) and the signal was measured at 570 nm.

Assay for the acidification of endosomes

To detect the acidification of endosomes the cells were grown on cover slips, in sealed chambers and were incubated in the presence of 33 μM acridine orange for 1 min. The neutralisation effect was visualised with a fluorescent microscope and compared to untreated controls.

Experimental condition and control experiments

The ldlF cell line is a mutant CHO derivative expressing a thermosensitive mutant epsilon-COP with enhanced degradation of epsilon-COP at non-permissive temperature (Daro et al., 1997). Incubation of ldlF cells at 40 °C (non-permissive temperature) will result in a low level of epsilon-COP blocking the transport between the early endosome and the transport vesicles (Gu et al., 1997). Bafilomycin A1 is a potent and specific inhibitor of the vesicular proton pump responsible for the acidification of early endosome and in the experiments a concentration of 1 μM was used, which has been shown to neutralize the endosomal pH in one hour (Yoshimori et al., 1991).

To examine the effects of epsilon-COP and bafilomycin A1 on the entry of adenovirus, a viral transducing assay was used. CHO and ldlF cells were transfected with phCAR and infected with Ad5-Luc3; the expressed luciferase activity was measured by luminometry. The E1A deficiency ensured that the luciferase activity was independent of the effects of virus replication. To exclude the possibilities that (wt) CHO cells and the ldlF cells have different responses to temperature, a series of assays were set up specifically to measure the effects of temperature. The effect of temperature was determined by measuring the temperature-induced decrease of proliferation on CHO cells (MTT assay). The level of expressed CAR protein was compared by FACS on the cell surface of HeLa cells (HeLa naturally expresses the CAR protein) at both temperatures to assess the differences in the cell surface expression. In order to assess the translational differences caused by the change of temperature, a strong viral promoter driven luciferase construct was measured in response to temperature on transfected CHO cells.

Results and discussion

Assessing the effect of temperature, no differences were found between the cell lines at a given temperature. However, in response to non-permissive temperature, the plasmid-borne luciferase activity decreased, while the MMT

signal increased in a parallel manner in both cell lines. To eliminate the effects of temperature shift and also the differences in transfection efficiency between transfected cells, we used both temperature and untreated transfected controls and the data were normalised and expressed as a percentage of the control.

Parallel results were found in the series of assays with both cell lines. Therefore the usage of (wt) CHO temperature control, to exclude the pure effect of temperature, was validated and could be used to normalise the results of the ldlF cell line at non-permissive temperature.

The performance of the infectivity assay was also validated in a test experiment where the well-studied inhibition of adenoviral entry by chloroquine treatment was reproduced (Fig. 1A) on HeLa cells (Seth et al., 1984).

Adenovirus infectivity was also detected in non-transfected CHO cells. Employing very high amounts of virus (approx. 10,000 MOI) a low level of luciferase signal could be produced in non-transfected (wt) CHO and ldlF cells but the resulting signal was 1000-fold lower than in CAR-transfected parallels. It is important to exclude the effect of the untransfected cells on the assay, because the infection in untransfected cells could follow a different pathway and cause misunderstandings in the interpretation; however, the low level of adenoviral transduction on (wt) CHO cells excluded this possibility. A high multiplicity of infection was used throughout the experiments. This high MOI probably saturates the expressed CAR proteins at the binding step but saturation of the endosomal mechanisms is not likely because endosomal traffic will filter out the excess bound virus. No significant antiproliferative effect caused by bafilomycin A1 has been found with the MMT proliferation assay on CHO cells. The acidification inhibition resulting from bafilomycin A1 treatment was detected and confirmed by acridine orange accumulation test in our system.

Investigating the effect of bafilomycin A1, Perez and Carrasco (1994) concluded that, despite the chloroquine behaviour, bafilomycin A1 could not to be used to inhibit the entry of adenovirus. In the quantitative assay on CAR-transfected (wt) CHO cells at the permissive temperature, after 1 h bafilomycin A1 pretreatment, we did find an inhibition of 50% compared to the control (Fig. 1B). The difference may be explained by that our assay is probably more sensitive compared to that of Perez and Carrasco. The inhibition cannot be increased by prolonged pretreatment by bafilomycin A1 (up to 6 h), proving that the possible maximal inhibition was achieved. At the same time, the acridine orange accumulation assay showed complete neutralisation of endosomes after 1-h pretreatment. A similar effect was detected on HeLa cells. In contrast, chloroquine pretreatment with 40-min incubation time exerts almost full inhibition in HeLa cells (Fig. 1A). It is proposed that the chloroquine effect is to terminate the endosome acidification and it is also proved that the only proton pump, which is responsible for the endosome acidification, is blocked by bafilomycin A1 (Yoshimori et al., 1991). With this in mind, the incomplete inhibition of adenoviral en-

try by bafilomycin A1 raises the possibility of the existence of two different pathways, both of which have an effect on the entry process. The first is acidification dependent, whereas the other is acidification independent. The first is blocked by bafilomycin A1, although chloroquine can block both of them by terminating the acidification and by an additional, unknown mechanism.

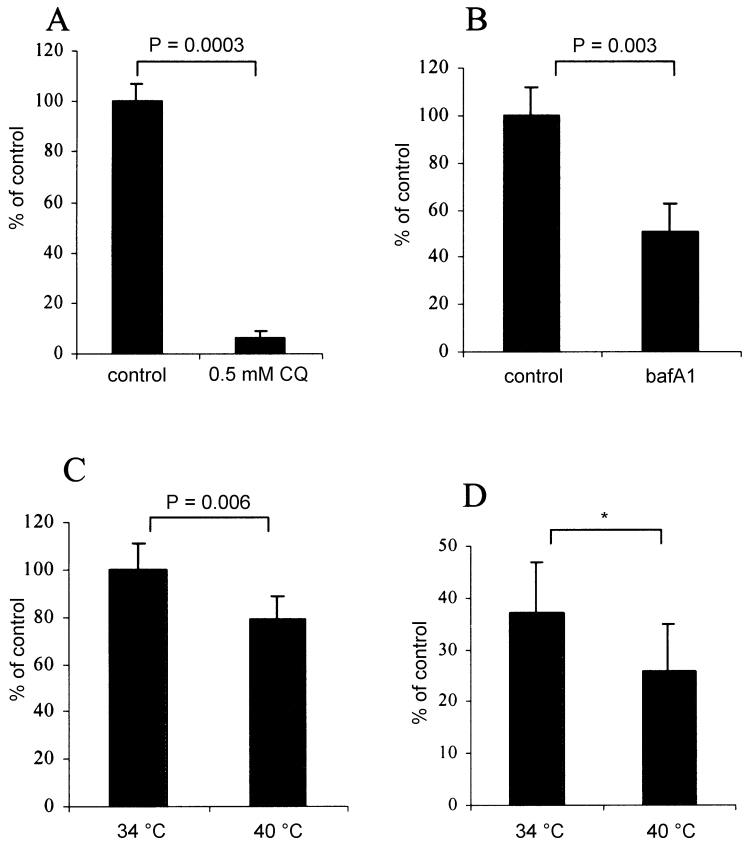

Fig. 1. Ad5 Luc3 induced luciferase expression under different conditions. **A.** HeLa cells treated with 0.5 mM chloroquine (CQ) at 30 min postinfection (control) and 20 min preinfection showed marked inhibition of adenoviral entry. **B.** (wt) CHO cells were untreated (control) or treated with 1 μM bafilomycin A1 (bafA1). Bafilomycin A1 could not achieve complete inhibition, but significantly reduced the level of entry indicating that the acidification of endosome is involved in the entry process. **C.** ldlF cells were incubated at permissive (34 °C) and at non-permissive (40 °C) temperature. The absence of epsilon-COP was also able to reduce the adenoviral entry, showing its role in the entry process. **D.** ldlF cells were treated with 1 μM bafilomycin A1 and incubated at permissive (34 °C) and at non-permissive (40 °C) temperature. The level of inhibition was not significantly increased by incubation of the bafilomycin A1 treated cells at non-permissive temperature, indicating that the pH-dependent steps of the entry process and the epsilon-COP are elements of the same pathway. The used infectivity assay was described under the Materials and methods section. The asterisk indicates non-significant differences

To confirm this new finding we examined another possible element of the acidification-dependent route, the effect of epsilon-COP on the entry. This protein has a pH-dependent function on the early endosome-ECV material transport, making this protein a good candidate for further characterisation of this route of entry. To study the possible involvement of epsilon-COP in the adenoviral entry, we used the same viral transducing assay on CAR-transfected (wt) CHO and ldlF cell lines incubated at permissive and non-permissive temperatures. At the non-permissive temperature a partial inhibition was found in ldlF cells, indicating the involvement of epsilon-COP in the adenoviral entry (Fig. 1C). This partial inhibition can be interpreted in two ways. Firstly, at non-permissive temperature the epsilon-COP is only decreased in ldlF cells and not totally eliminated; this low level of functional protein can support only part of the viral entry. The other possibility is the two-pathway model. The epsilon-COP is involved only in one pathway so the other pathway could support the viral entry but at a reduced level. Concerning the bafilomycin A1 action, which should be placed upstream of epsilon-COP (the pH regulates the epsilon-COP function), the picture favours the latter possibility. Additionally, it is unlikely that the remnant function of epsilon-COP could support more than 70% of adenoviral entry because the endosomal material traffic is almost abolished under these conditions (Gu et al., 1997). Recently new findings also support the possibility of the existence of a second pathway. Rauma et al. (1999) showed the involvement of rab5 protein in the adenoviral entry, and in their experimental system only partial inhibition was achieved by overexpression of the rab5 dominant negative mutant (DNrab5) by plasmid transfection. In a manner similar to the epsilon-COP action, the DNrab5 also inhibits the early endosome ECV material transport step. This partial inhibition of DNrab5 reflects the effect of the reduced level of epsilon-COP and, considering their common point of action on the endosomal system, it is possible that they are elements of the same pathway. However, this should be investigated further.

If the inhibition caused by bafilomycin A1 cannot be increased by incubating the ldlF cells at non-permissive temperature, this could provide evidence that acidification and epsilon-COP are the elements of the same pathway. As shown in Fig. 1D, this is really the case: in ldlF cells at permissive temperature, bafilomycin A1 caused the same level of inhibition as the same treatment produced in ldlF cells at non-permissive temperature. We initiated a search to find a DN epsilon COP mutant, which could be used to investigate these effects further.

The action of chloroquine on the adenoviral entry process was considered to be pH dependent and only one mode of action to neutralise the acidification, i.e. a buffering mechanism, was proposed (Seth et al., 1984). Although conflicting results can be found in the literature (Perez and Carrasco, 1994), indicating that the picture is more complicated, no one attempted to revise the theory. Our more sensitive, quantitative re-examination of the originally qualitative data

raises the possibility that at least two different inhibitory pathways may exist and only one of them is pH dependent.

We propose a new model pointing out that acidification could not be directly coupled to the adenoviral entry but it is important for normal endosomal function, being a prerequisite or consequence of actions of the endosomal proteins. We propose that different acidification blocking drugs could inhibit different sets of endosomal proteins having different or only partially overlapping actions, as have been shown for bafilomycin A1 and chloroquine (Prasmickaite et al., 2000). In case of adenovirus entry our results and the literature also support the effect of a diverse set of endosomal proteins. Recently it has been shown for wortmannin, a specific inhibitor of IP3 kinase, that active IP3 kinase is necessary for adenoviral entry (Li et al., 1998) and it has been shown for rab5 that the kinase regulates its action (Kurosu and Katada, 2001).

It is proposed that the endosomal material traffic involves fusion or pore formation between compartments, which is highly regulated, and several proteins and pathways participate in the process (Palfrey and Artalejo, 1998). The mechanism of the so-called adenovirus 'endosome-disrupting activity', which seems to be fundamental in the entry process, is not known, and the involvement of epsilon-COP and rab5 in the adenoviral entry raises the possibility that the disruption of the endosome is somehow related to the fusion process.

Acknowledgement

This work was supported by grants No. 17052 and 23619 of the Hungarian Scientific Research Fund (OTKA) and the Hungarian Academy of Sciences (TKI).

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