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The Role of Subtilisin-like Proprotein Convertases for Cleavage of the Measles Virus Fusion Glycoprotein in Different Cell Types

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The fusion (F) glycoprotein gene of measles virus (MV) encodes a nonfusogenic precursor protein (F_0) that is activated by cleavage into the F_1 and F_2 subunits during transport to the cell surface. The F protein of both the Edmonston strain and a wild-type MV was found to be cleaved in the *trans*-Golgi cisternae and/or the *trans*-Golgi network (TGN). In HEp-2 cells, B lymphoblastoid cells, and PBMC, the cleavage process required calcium, and calcium deprivation prevented syncytium formation. The calcium dependence indicated the involvement of the pro-protein convertase (PC) endoprotease family. The expression of the presently recognized members of the PC family in human cell types known to be infected during measles was examined by RT–PCR. Among the PCs residing in the TGN, only furin was expressed in all cells. Soluble secreted human furin produced by a recombinant baculovirus cleaved MV F_0 into proteins the exact size of F_1 and F_2 and increased the titer of MV particles released from calcium-deprived or endoprotease defective infected cells. These results strongly indicate that furin is the most important and maybe the only endoprotease involved in activation of the MV F protein.

INTRODUCTION

Measles virus (MV) gains access to the cell cytoplasm through the actions of its two surface glycoproteins, the haemagglutinin (H) protein, which attaches the virus to a receptor on the cell surface, and the fusion (F) protein, which induces fusion of the virus envelope and the cell cytoplasmic membrane (reviewed by Wild and Buckland, 1995). The F and H proteins expressed on the surface of an infected cell also induce fusion of the plasma membrane with the membranes of adjacent cells. This allows the virus to spread from cell to cell without being exposed to the extracellular environment, and it can be seen in cell cultures and patients as syncytia.

The F protein is synthesized as a 60-kDa precursor protein (F_0), which during its intracellular migration to the cell surface is cleaved by one or more host cell proteases into two disulfide linked subunits of 40 kDa (F_1) and 20 kDa (F_2) (Tyrrell and Norrby, 1978; Graves *et al.,* 1978; Fujinami and Oldstone, 1981; Sato *et al.,* 1988). The cleavage is required for the fusion protein to be fusogenic and is often referred to as activation of the F protein. Thus cleavage of F_0 is a prerequisite for the release of infectious virus particles from infected cells and for the spread of virus from cell to cell by cell fusion.

The MV F_0 protein as well as a range of other viral and cellular precursor proteins are cleaved at a polybasic site. Intracellular cleavage at such sites have been attributed to a family of calcium-dependent subtilisin-like serine endoproteases called pro-protein convertases (PCs). Presently, seven different members of the PC family are recognized (furin, PACE4, PC1/PC3, PC2, PC4, PC5/PC6, and PC7/PC8). These PCs differ but overlap in terms of their substrate specificities, tissue distributions, and intracellular localizations. Furthermore, some of the PCs have different isotypes, which also vary for the above properties (for reviews and updates, see Steiner *et al.,* 1992; Klenk and Garten, 1994; Bruzzaniti *et al.*, 1996; De Bie *et al.,* 1996; Mori *et al.,* 1997). In recombinant expression systems, furin and other PCs were capable of cleaving a range of viral precursor glycoproteins, for instance gp160 of HIV-1 (Vollenweider *et al.,* 1996; Hallenberger *et al.,* 1997), haemagglutinin of virulent avian influenza virus (Stieneke-Gröber *et al.,* 1992; Horimoto *et al.,* 1994), and the F proteins of parainfluenza virus type 3 and of virulent Newcastle disease virus (Gotoh *et al.,* 1992; Ortmann *et al.,* 1994).

For MV, Watanabe *et al.* (1995) demonstrated that F_0 was very inefficiently cleaved in a furin deficient cell line and that cleavage of F_0 in cultivated cell lines was blocked by the furin inhibitor α_1-PDX. These findings strongly indicated that furin was responsible for activation of the MV F protein. In recent studies, however, α_1-PDX was found to inhibit not only furin but also other endoproteases capable of cleaving gp160 of HIV-1 (Decroly *et al.*, 1996; Vollenweider *et al.,* 1996). Thus it is possible that other proteases than furin are involved in cleavage of the MV F protein. Furthermore, since MV infects several types of cells in measles patients, the role of individual proteases may vary between different cells.

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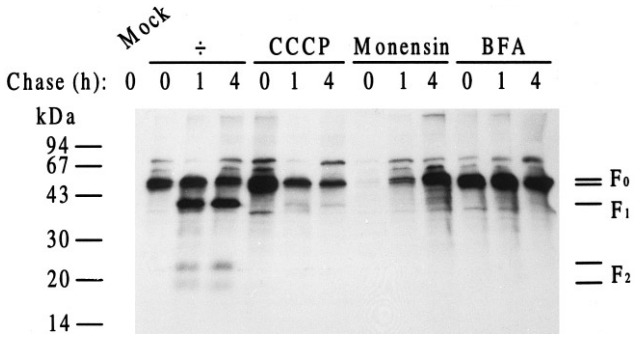

FIG. 1. Pulse–chase analysis of MV-infected HEp-2 cells in normal medium (÷) or treated with inhibitors of the exocytic pathway (CCCP, monensin, BFA). Starved cells were pulsed 5 min with 100 μCi/ml (specific activity = 1000 Ci/mM) [^{35}S]methionine and [^{35}S]cysteine and chased 0, 1, or 4 h prior to lysis. Radiolabeled MV F protein was precipitated from the lysates with anti-MV F Mab. The samples were prepared in parallel and processed under identical conditions.

In the present study, we have investigated the possible role of different PCs for cleavage of the MV F protein in various cells.

RESULTS

The F protein is cleaved in the *trans*-Golgi cisternae or the *trans*-Golgi network of MV-infected HEp-2 cells

Initially, we tried to determine the subcellular location of the endoprotease(s) that activate(s) the MV F protein. The synthesis and cleavage of the F protein in HEp-2 cells infected with the Edmonston strain of MV and treated with inhibitors of the exocytotic pathway was followed by pulse–chase and radio immunoprecipitation assay (RIPA) using anti-MV F monoclonal antibody (Mab) (Fig. 1).

Before chase, only F_0, which was present as a double band of ~58 and 61 kDa, could be seen. During chase, labeled F_0 protein in untreated cells was cleaved into F_1, seen as a band of ~42 kDa, and F_2, seen as two bands of ~20 and 25 kDa (Fig. 1), as has previously been reported (Sato *et al.,* 1988; Alkhatib *et al.*, 1994; Watanabe *et al.,* 1995). From autoradiographs exposed for shorter periods than the one shown in Fig. 1, it can be seen that the intensities of the F_1 and F_2 bands increased between 1- and 4-h chase and that the majority of the labeled F_0 protein was cleaved after 4-h chase (not shown). When the chase was extended beyond 4 h, cleavage of the remaining radiolabeled F_0 protein took place at a much slower pace and after a 24-h chase, a weak but distinct F_0 band was still present (not shown). After a 16-h chase, the intensities of the F_1 and F_2 bands had diminished compared to their intensities after a 4-h chase (not shown), indicating that at this point, activation of the labeled F protein took place at a slower pace than the turnover of the protein.

The inhibitors carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), monensin, and brefeldin A (BFA) all prevented or seriously diminished cleavage of F_0 during the entire 4-h chase (Fig. 1). CCCP blocks the transfer of glycoproteins from the endoplasmic reticulum (ER) to the Golgi complex (Tartakoff and Vasali, 1977), whereas monensin blocks the transport from the *medial*- to the *trans*-Golgi cisternae (Griffiths *et al.*, 1983). It thus appears that cleavage of F_0 takes place in a cellular compartment distal to the *medial*-Golgi cisternae. BFA prevents transport from the ER to the Golgi complex and induces a redistribution to the ER of proteins normally resident in the *cis*-, *medial*-, and *trans*-Golgi cisternae. In the ER, redistributed Golgi enzymes may carry out their processing activity on the retained glycoproteins (Misumi *et al.,* 1986; Doms *et al.*, 1989). Proteins from the *trans*-Golgi network (TGN) are not redistributed to the ER (Chege and Pfeffer, 1990). Since F_0 was not cleaved by the redistributed Golgi enzymes in BFA-treated cells, the cleaving enzymes must either belong to a compartment distal to the *trans*-Golgi cisternae or be nonfunctional in the ER environment.

Besides the bands representing F_0, F_1, and F_2, bands of ~39, 70, and 76 kDa were coprecipitated by the anti-F Mab (Fig. 1). We have previously shown that these bands probably represent the MV matrix, nucleocapsid, and H proteins, respectively (unpublished data). Immediately after pulse, the amount of radiolabeled F_0 precipitated from CCCP-treated cells was increased compared to untreated cells. During chase of the CCCP-treated cells, the intensity of the F_0 band decreased although cleavage into F_1 and F_2 did not take place (Fig. 1). The same pattern was found in repeated experiments, but the reasons still remain to be determined. It is possible that the degradation of the F_0 protein was enhanced due to its retention in the ER. In contrast, the F_0 band precipitated from monensin treated cells immediately after pulse was almost invisible. During chase, the amount of radiolabeled F_0 precipitated from the monensin-treated cells increased significantly (Fig. 1). This pattern was also a consistent finding in repeated experiments. One possible explanation is that monensin, besides blocking the exocytic pathway, also delayed early steps in the processing of the F protein required for its recognition in the immunoprecipitation assay.

To further determine the location of F protein activation, F protein was precipitated from lysates of cells incubated with radiolabeled mannose or fucose. The mannose residues of N-linked oligosaccharides are added to glycoproteins in the ER (Kornfeld and Kornfeld, 1985). In the present experiment, radiolabeled mannose was present in both F_0 and F_2 protein (Fig. 2), which is in agreement with the above finding that F_0 are not cleaved in the ER. The mannose-labeled F_0 stained more intense than F_2. F_1 was not labeled, since it does not contain glycosylation sites (Richardson *et al.,* 1986; Buckland *et al.*, 1987). Upon labeling with fucose, F_0 gave a very weak

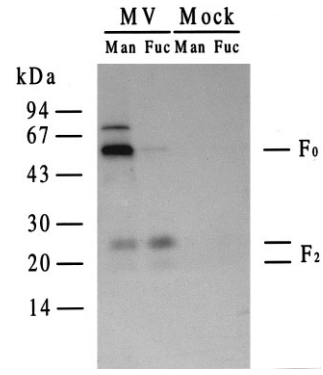

FIG. 2. Lysates of [^{3}H]mannose (Man) or [^{3}H]fucose (Fuc) labeled MV- or mock-infected HEp-2 cells precipitated with anti MV-F Mab.

band, whereas F_2 stained much stronger (Fig. 2). The most obvious interpretation of this pattern is that F_0 was cleaved shortly after fucose incorporation, which takes place in the *medial-* or *trans*-Golgi cisternae (Kornfeld and Kornfeld, 1985; Rios-Martin *et al.*, 1993). Combined with the above finding that cleavage is located distal to the *medial*-Golgi cisternae, it appears that cleavage of F_0 takes place in the *trans*-Golgi cisternae and/or the TGN of HEp-2 cells.

Cleavage of the F protein in MV-infected HEp-2 cells requires calcium

The members of the PC family are calcium dependent. Therefore we investigated the role of calcium ions for activation of the MV F_0 protein. HEp-2 cells infected with the Edmonston strain of MV were depleted for calcium by incubation in calcium-deficient medium with the calcium-binding ionophore A23187 (Tartakoff and Vassalli, 1977).

During a 4-h chase, the majority of the labeled F_0 was cleaved in cells incubated in normal medium with or without A23187 and in cells incubated in calcium deficient medium without A23187 (Fig. 3). When increasing concentrations of A23187 were added to cells chased in calcium-deficient medium, the proportion of cleaved F protein diminished in a dose-dependent manner (Fig. 3). This demonstrates that calcium ions are required for the intracellular cleavage of MV F_0. Morphological changes were noticed in cells incubated with concentrations of A23187 >4 μM (not shown).

MV-infected cells incubated in calcium-deficient medium with 0.25 μM A23187 for 36 h exhibited no or very little syncytium formation (Fig. 4A) compared with similar cells in normal medium without A23187 (Fig. 4C). Incubation of MV-infected cells in normal medium with A23187 did not appear to influence syncytium formation (Fig. 4E). These findings indicate that in calcium-deprived MV-infected cells, the amount of activated F protein expressed at the cell surface was not sufficient to induce syncytia.

In some studies, A23187-induced calcium deprivation appeared to inhibit the exocytic pathway (Tartakoff and Vassalli, 1977; Johnson and Schlesinger, 1980), although this effect was not found in other studies (Klenk *et al.*, 1984; Oda, 1992; Moulard *et al.,* 1994). Therefore we examined the expression of MV glycoproteins on the cell surface by flow cytometry. Unfortunately, only weak signals were obtained from the MV F protein on infected cells, although several different anti-F Mabs were tested. Instead we compared the cell surface expression of the MV H protein. In MV-infected cells incubated in calcium-deficient medium with 0.25 μM A23187, the H protein surface expression was significantly reduced compared to that of cells incubated in normal medium, but considerable amounts of H protein had reached the cell surface (Fig. 5). For comparison, BFA, which is known to block the exocytic pathway, completely prevented surface expression of the H protein (Fig. 5). Below we show that virus particles with uncleaved F protein were released from MV-infected calcium-deprived HEp-2 cells. This further demonstrates that MV glycoproteins were transported to the cell surface. Thus in the present study, the inhibition of MV F protein activation by calcium deprivation was primarily caused by inhibition of the actual cleavage process and not by blocking the transport of glycoproteins to the site of cleavage. Nevertheless we can not exclude the possibility that calcium deprivation also inhibited the exocytic pathway to a certain extent.

Since calcium dependence is one of the hallmarks of the PC endoprotease family, the present data strongly indicate that one or more members of this family are crucial for the cleavage activation of the MV F protein in HEp-2 cells.

The F protein is cleaved in the *trans*-Golgi cisternae or the TGN by (a) calcium-dependent endoprotease(s) also at lower than core body temperatures, in human PBMC and in cells infected with wild-type MV

In measles patients, the infection is initiated in the respiratory mucosa (reviewed by Griffin and Bellini,

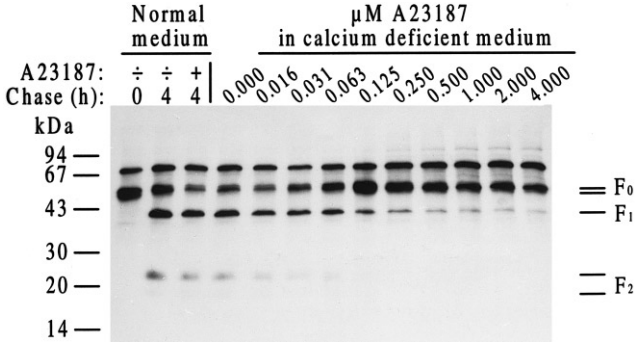

FIG. 3. Lysates of MV-infected HEp-2 cells chased in normal medium with or without 4 μM A23187 or in calcium deficient medium with increasing concentrations of A23187 and precipitated with anti MV-F Mab.

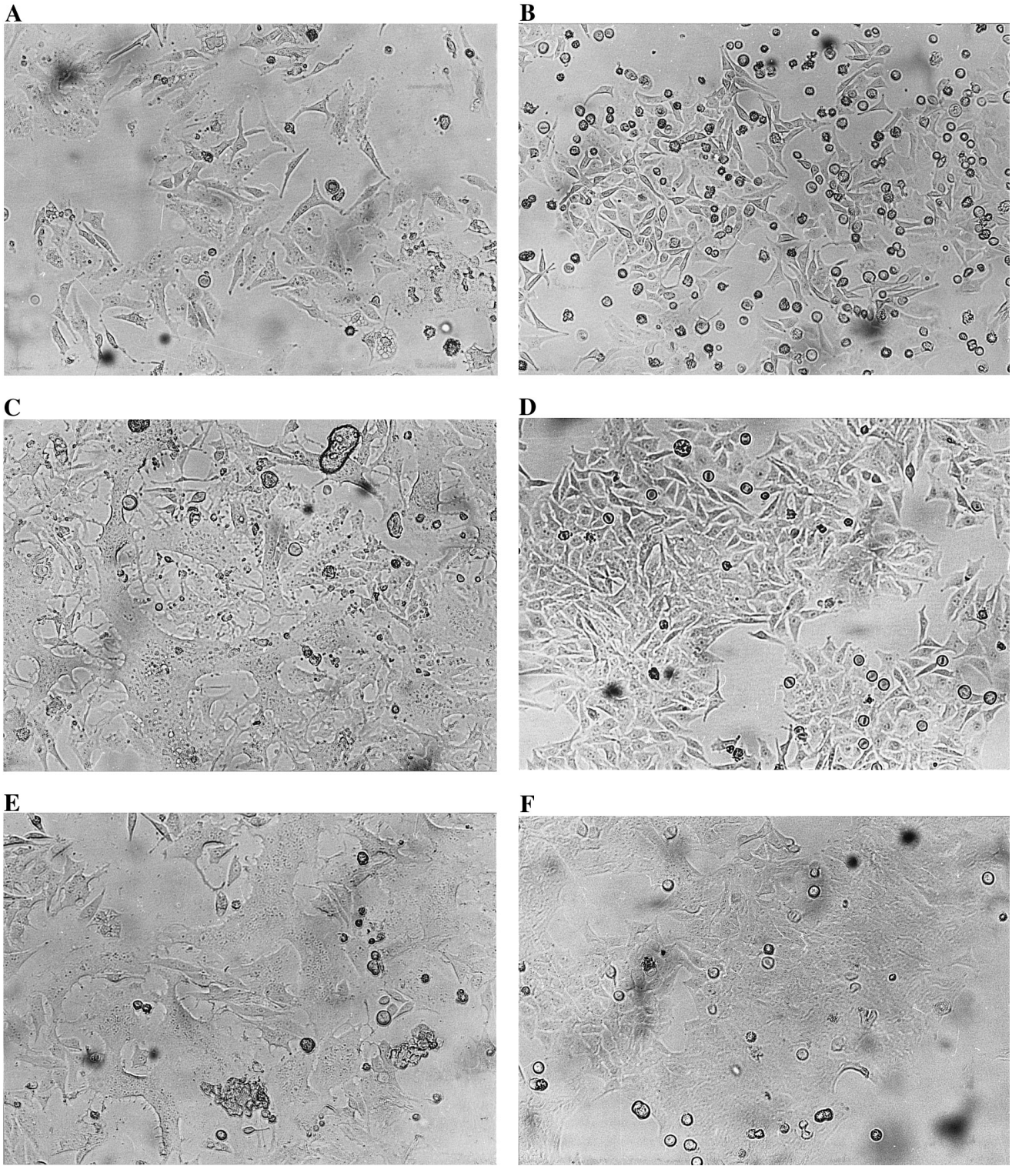

FIG. 4. Cultures of Hep-2 cells 36 h after infection with MV at a m.o.i. of 3. The cells were exposed to MV for 15 h, thoroughly washed, and incubated in the below media without serum. (A) MV-infected cells incubated in calcium-deficient medium with 0.25 μM A23187. (B) Uninfected cells incubated as in (A). (C) MV-infected cells incubated in normal medium without A23187. (D) Uninfected cells incubated as in (C). (E) MV-infected cells incubated in normal medium with 0.25 μM A23187. (F) Uninfected cells incubated as in (E).

1996), where the temperature can be lower than the core body temperature. Therefore the cleavage of radiolabeled F protein in Edmonston strain-infected HEp-2 cells chased at 33°C was examined. In untreated cells, the majority of the labeled F_0 was cleaved into F_1 and F_2 after a 4-h chase period (not shown), as was the case after

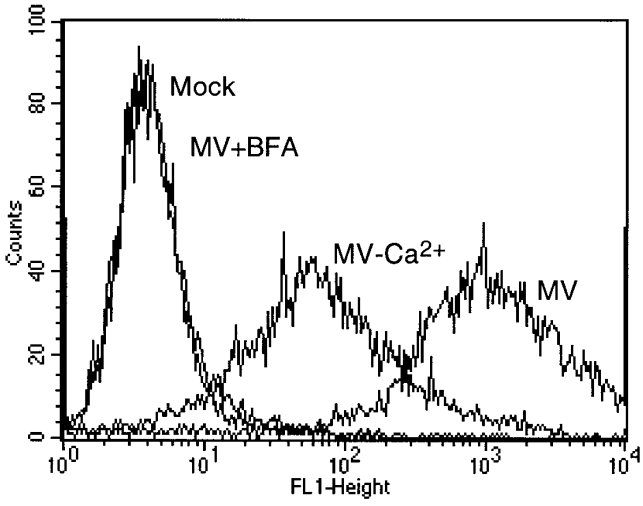

FIG. 5. Flow cytometric analysis of MV H protein surface expression in Hep-2 cells 36 h after infection with MV at a m.o.i. of 3. The cells were exposed to MV for 1.5 h, thoroughly washed, and incubated in normal medium (MV), calcium-deficient medium with 0.25 μM A23187 (MV-Ca^{2+}), or normal medium with 5 μg/ml BFA (MV+BFA).

chase at 37°C (Fig. 1). CCCP, monensin, and BFA, which block the exocytic pathway at different steps, all significantly inhibited cleavage of radiolabeled F_0 (not shown) as they did at 37°C (Fig. 1). Immunoprecipitation of F protein after incorporation of radiolabeled mannose and fucose at 33°C (not shown) also gave the same pattern as that seen after incorporation at 37°C (Fig. 2). Finally, cleavage of F_0 in HEp-2 cells chased at 33°C in calcium-deficient medium with 0.25 μM A23187 (not shown) was inhibited to same extent as at 37°C (Fig. 3). It thus appears that the activation of the MV F protein at lower than core body temperatures is identical to the activation at 37°C.

For HIV-1, a calcium-independent endoprotease capable of cleaving gp160 to gp120 and gp41 has been found in human $CD4^+$ T lymphocytes and other cells, and it appears that this protease as well as several members of the PC family can activate gp160 (Kido *et al.,* 1993; Kamoshita *et al.,* 1995; Vollenweider *et al.,* 1996; Hallenberger *et al.*, 1997). The above results on MV were obtained *in vitro* with the HEp-2 cell line. In light of the cited data obtained for HIV-1, it was relevant to examine whether the present findings also applied for cells known to be involved in *in vivo* MV infections. In measles patients, several types of cells become infected with MV. Leucocytes belong to the major targets cells of MV, and they also play an important role for the dissemination of MV in the body through cellular viremia (reviewed by Griffin and Bellini, 1996). We therefore investigated F protein cleavage in phytohemagglutinin-L (PHA-L)-stimulated human peripheral blood mononuclear cells (PBMC).

Recent studies have shown that the passage history of MV strains is important for their biological properties. Wild-type isolates of MV, which has only been passaged on lymphoid cells, remain more pathogenic to monkeys than attenuated vaccine and laboratory strains of MV and appear to use another host cell receptor than CD46 (Kobune *et al.,* 1990; van Binnendijk *et al.,* 1994; Buckland and Wild, 1997; Hsu *et al.,* 1998; Bartz *et al.*, 1998). The above experiments of the present study were carried out with the attenuated Edmonston strain. Therefore, we also examined the activation of the F protein from a wild-type MV (DK96A1) in JP cells, which is an Epstein–Barr virus (EBV)-transformed human B lymphoblastoid cell line (B-LCL).

CCCP, monensin, and BFA all significantly inhibited cleavage of radiolabeled F_0 in PBMC infected with the Edmonston strain (Fig. 6) and in JP cells infected either with wild-type DK96A1 (not shown) or with the Edmonston strain (not shown), as they did in HEp-2 cells infected with the Edmonston strain (Fig. 1). Calcium deprivation by chase in calcium-deficient medium with 0.25 μM A23187 also inhibited cleavage of F_0 in both the PBMC infected with the Edmonston strain (Fig. 6) and in JP cells infected either with wild-type DK96A1 (not shown) or with the Edmonston strain (not shown), as was the case in HEp-2 cells infected with the Edmonston strain (Fig. 3). Calcium deprivation also prevented syncytium formation in JP cells infected either with wild-type DK96A1 (Fig. 7) or with the Edmonston strain (not shown).

Thus it appears that also in human PBMC, the F protein of MV is activated in the *trans*-Golgi cisternae and/or the TGN by one or more calcium-dependent endoproteases. This also seems to apply both for the attenuated Edmonston strain and for the DK96A1 wild-type isolate.

The expression pattern of the individual PC endoproteases in different MV-susceptible cells indicate a key role for furin in MV F protein activation

Having substantiated that members of the PC family are important for the cleavage of the MV F protein, we examined the expression of the individual PCs in differ-

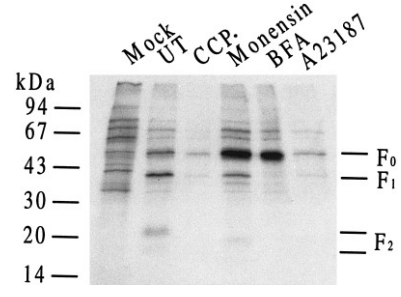

FIG. 6. Pulse–chase analysis of the MV F protein in PHA-stimulated human PBMC. The F protein was chased in normal medium (UT) with inhibitors of the exocytic pathway (CCCP, monensin, BFA) or calcium-deprived by chase in calcium-deficient medium with 0.25 μM A23187.

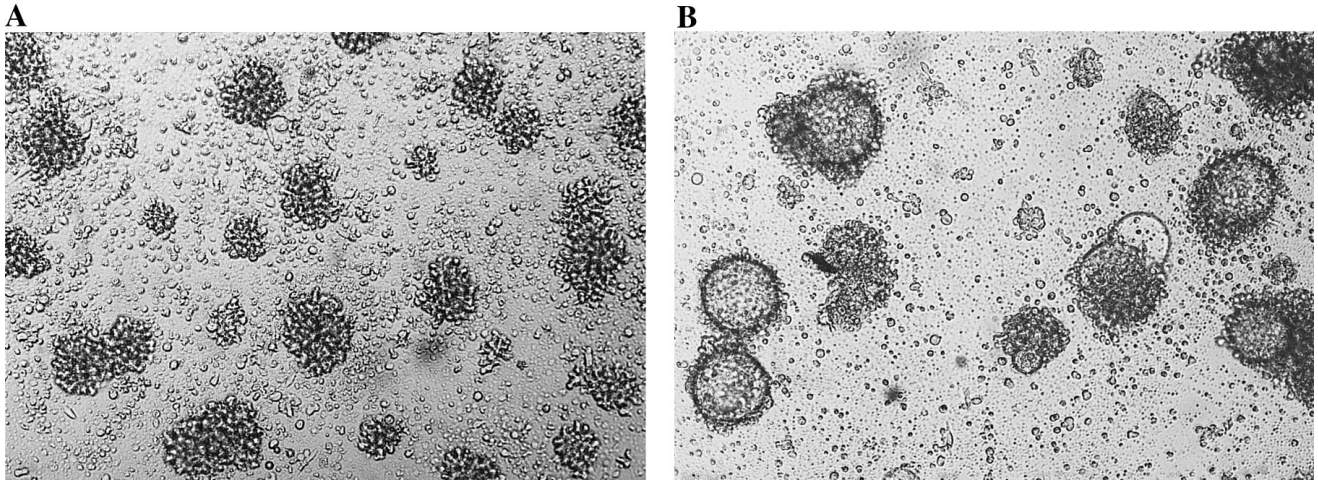

FIG. 7. Cultures of the B-LCL JP 72 h after infection with the MV wild-type isolate DK96A1 The cells were exposed to MV for 24 h, thoroughly washed, and incubated in calcium-deficient medium with 0.25 μM A23187 (A) or normal medium without A23187 (B).

ent cells susceptible to MV by reverse transcription and polymerase chain reaction (RT–PCR). To avoid amplification of genomic DNA, the RNA extraction procedure included DNase digestion. As a control for the absence of genomic DNA, amplification from the RNA extractions after RNase A digestion was also attempted (not shown). The authenticity of the amplification products was verified by restriction analysis (not shown) as described by Decroly *et al.* (1996).

The assays included human PBMC incubated with and without PHA. Among the PBMC of children with measles, monocytes appeared to be the primary target cell of MV (Esolen *et al.,* 1993). Therefore we also assayed the PC expression of monocytes selected from PBMC by adherence. In the tissues of MV-infected rhesus monkeys, B lymphocytes was the most commonly infected cell type (McChesney *et al.,* 1997), and three human B lymphoblastoid cell lines (B-LCLs) (AO, GR, and JP) were thus included in the study. Finally, the PC expression of the HEp-2 and LoVo cells lines were also assayed. LoVo cells produce a mutant furin molecule (Takahashi *et al.,* 1993) and are very inefficient in cleavage of the MV F protein (Watanabe *et al.,* 1995). RNA extractions from uninfected and MV Edmonston strain-infected cells were assayed for mRNA of the presently known PCs except PC4, which has only been detected in testicular germ cells (Nakayma *et al.,* 1992). Furthermore the RNA extractions were assayed for mRNA of ribosomal protein L27 (low expression level housekeeping gene) and RNA of the MV H gene.

PC2 mRNA was not detected in any of the assayed cells (not shown). This did not appear to be a failure of the assay, since the correct PC2 PCR product was amplified from a plasmid with human PC2 cDNA (not shown). The distribution of the remaining PC endoproteases varied among the different cells (Fig. 8). MV infection generally did not appear to influence the PC expression of the cells qualitatively. B-LCLs infected with MV wild-type DK96A1 expressed the same PCs as B-LCLs infected with the Edmonston strain (not shown). The tumor cell lines HEp-2 and LoVo expressed a much broader range of PCs than the remaining cell types (Fig. 8). There is evidence that the expression of PCs is increased in certain tumor cells (Cheng *et al.,* 1997 and references herein). This underlines the need to interpret data on cleavage activation obtained in tumor cell lines with caution. For the interpretation of the present data, one must also keep in mind that the expression of a gene does not always mean that functional proteins are produced.

Among the assayed PCs, furin, PC5/PC6-B, and PC7/PC8 are known to reside in the TGN (Molloy *et al.,* 1994; De Bie *et al.,* 1996; Decroly *et al.,* 1996), which we in the present study have found to be a potential site for activation of the MV F protein. Furin was expressed in all cells tested (Fig. 8), in agreement with its proposed role as an ubiquitous processing endoprotease in the constitutive exocytic pathway (Steiner *et al.,* 1992; Klenk and Garten, 1994). PHA stimulation seemed to induce the expression of PACE4, PC5/PC6, and PC7/PC8 in PBMC, but this is probably not relevant for the pathogenesis of measles, since the block of MV multiplication in non-stimulated PBMC appears to be earlier in the infectious cycle than protein processing (reviewed by Borrow and Oldstone, 1995). Besides furin, adherent PBMC only expressed PACE4 and PC5/PC6, whereas the B-LCLs only expressed PC7/PC8. Combined, these data suggest that furin is sufficient and the most important endoprotease for activation of the MV F protein.

Recombinant human furin cleaves and activates MV F protein *in vitro*

To test whether furin can actually cleave the MV F_0 protein, furin was produced by infecting insect cells with the recombinant baculovirus bac:fur-595. Bac:fur-595 en-

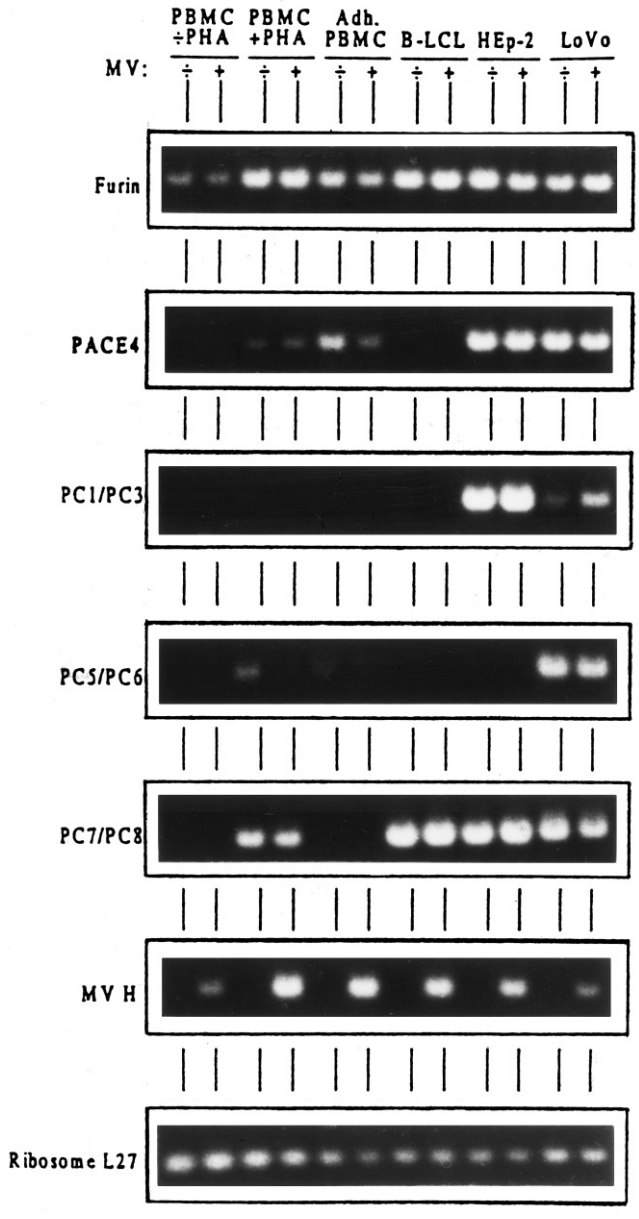

FIG. 8. RT–PCR analysis of the expression of PC endoproteases in human PBMC incubated without or with PHA, adherence selected PBMC (Adh.), EBV-transformed B lymphoblastoid cell lines (B-LCLs), the HEp-2 cell line, and the LoVo cell line. Identical results were obtained for PBMC of three donors and for three B-LCLs. The weak PC5/PC6 bands obtained for PHA-stimulated PBMC and adherent PBMC were authentic, as they were cleaved into fragments of the correct size in restriction analysis (not shown).

codes soluble secreted human furin, which is released to the cell medium (Bravo *et al.*, 1994).

Radiolabeled immunoprecipitated F protein from cells MV-infected cells was incubated at 37°C with medium from bac:fur-595-infected insect cells or medium from similar cells infected with an irrelevant baculovirus (Fig. 9). Radiolabeled F protein preparations consisting only or mainly of uncleaved F_0 protein were obtained from HEp-2 cells lysed immediately after pulse (0-h chase), from HEp-2 cells chased with CCCP, from HEp-2 cells chased under calcium deprivation, or from LoVo cells. A proportion of the F_0 protein from these cells was cleaved into F_1 and F_2 by the recombinant human furin (Fig. 9). Similar results were obtained when the precipitated F proteins were incubated with the insect cell media at 33°C (not shown), demonstrating that furin is also capable of cleaving the MV F protein at lower than core body temperatures. Radiolabeled F_0 protein from JP cells infected with MV wild-type DK96A1 and chased under calcium deprivation or with CCCP was also cleaved into F_1 and F_2 by the recombinant human furin (not shown).

The extent of cleavage of the radiolabeled F_0 proteins varied between the different preparations but the cleavage was never complete. For some preparations, only a minor fraction of the radiolabeled F_0 was cleaved (Fig. 9). Cleavage was not enhanced by increased incubation times or increased amounts of furin (not shown). The reason for these observations still remains to be determined. Several explanation can be hypothesized. It is possible that the access of furin to the cleavage site is influenced by steps in the posttranslational processing of the F protein taking place before or after the F protein passes the cellular compartment where cleavage is supposed to take place. It is also possible that the cleavability of the F protein can be reduced or destroyed by the immunoprecipitation procedure.

To examine whether the F protein cleavage performed by the recombinant furin resulted in functional fusiogenic F protein, we tested the ability of the recombinant furin to activate virus particles released to the medium of MV-infected cells. Incubation of medium from Edmonston-strain-infected LoVo cells or medium from calcium-deprived Edmonston-strain-infected HEp-2 cells with recombinant furin induced a very significant rise in the MV

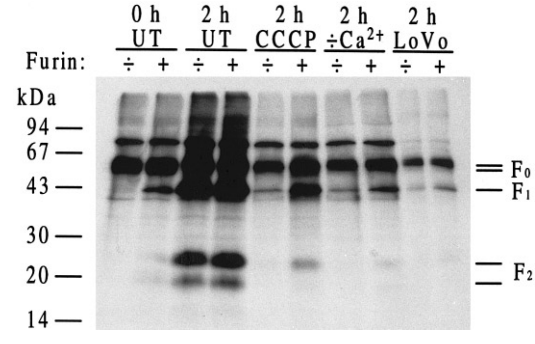

FIG. 9. *In vitro* digestion of immunoprecipitated MV F protein with medium from insects cells infected with the recombinant baculovirus bac:fur-595 producing secreted soluble furin (furin+) or medium from insects cells infected with an irrelevant baculovirus (furin÷). The F proteins were precipitated from MV-infected HEp-2 cells lysed immediately after pulse (0 h, UT), lysed after 2 h chase in normal medium (2 h, UT), lysed after 2 h chase with CCCP (2 h, CCCP), lysed after 2 h chase in calcium-deficient medium with 0.25 μM A23187 (2 h, ÷Ca^{2+}), or from MV-infected LoVo cells chased 2 h in normal medium (2 h, LoVo).

TABLE 1

Titer of MV Edmonston Strain (p.f.u./ml) or MV Wild-Type (TCID/ml) in Cell Culture Supernatants (Average of Triplicate Assays)

		Insect cell medium	
	Untreated	Control virus	Bac:fur-595
Edmonston-strain-infected LoVo cells in normal medium	0.8×10^2	1.3×10^2	4.9×10^3
Edmonston-strain-infected HEp-2 cells in calcium-deficient medium with A23187	2.0×10^1	2.0×10^1	2.5×10^2
Edmonston-strain-infected HEp-2 cells in normal medium	0.9×10^4	1.1×10^4	2.0×10^4
Wild-type DK96A1-infected JP cells in normal medium	0.6×10^4	0.7×10^4	1.4×10^4

titer of the medium (Table 1). Infectious virus could not be detected in the medium from calcium-deprived JP cells infected with wild-type DK96A1 neither before nor after incubation with recombinant furin. The recombinant furin did, however, induce a modest rise in the titer of medium from normally treated HEp-2 cells infected with the Edmonston strain and of medium from normally treated JP cells infected with wild-type DK96A1 (Table 1).

These findings demonstrate that human furin is capable of activating the MV F protein of both Edmonston strain and wild-type MV by cleaving F_0 into functional F_1 and F_2.

DISCUSSION

In the present study, we have investigated the cleavage activation of the MV F protein.

In the HEp-2 cell line, in EBV-transformed human B lymphocytes, and in human PBMC, cleavage was found to be a calcium-dependent process that took place in the *trans*-Golgi cisternae or the TGN. The calcium dependence strongly indicate that the involved endoprotease(s) belong(s) to the subtilisin-like PC family. This is in agreement with the results of Watanabe *et al.* (1995), who found that cleavage of the MV F protein was inhibited by α_1-PDX, an inhibitor of furin and other members of the PC family (Anderson *et al.*, 1993; Decroly *et al.,* 1996; Vollenweider *et al.,* 1996).

The cleavage activation of the F proteins of virulent Newcastle disease virus (NDV) and of respiratory syncytial virus have also been located to the *trans*-Golgi cisternae or the TGN (Morrison *et al.*, 1985; Collins and Mottet, 1991). Activation of the NDV F protein and of the haemagglutinin protein of virulent avian influenza virus was inhibited by calcium deprivation with concentrations of A23187 similar to those found to inhibit cleavage of the MV F protein in the present study (Klenk *et al.,* 1984; Kawahara *et al.,* 1992).

Since MV F_0 is cleaved in the *trans*-Golgi cisternae and/or the TGN, the activating protease(s) must reside in one or both of these compartments. Among the presently known PCs, none has been found in the *trans*-Golgi cisternae, whereas three (furin, PC5/6-B, and PC7/PC8) reside in the TGN (Molloy *et al.,* 1994; De Bie *et al.*, 1996; Decroly *et al.,* 1996). Of these three PCs, only furin was expressed in all MV-susceptible cells tested in the present study. PC5/PC6 and PC7/PC8 mRNA were detected in the furin-deficient LoVo cell line, which, in the study of Watanabe et al. (1995) and in the present study, was very inefficient in activating the MV F protein. Although the synthesis of functional PC5/PC6-B and PC7/PC8 in LoVo cells still remains to be demonstrated, these findings strongly indicate that PC5/PC6-B and PC7/PC8 do not play an important role for activation of the MV F protein. Thus furin appears to be the only good candidate for the MV F protein activating endoprotease. Recombinant furin cleaved radiolabeled MV F_0 into proteins the exact size of F_1 and F_2 and increased the titer of viral particles released from MV-infected LoVo cells and MV-infected calcium-deprived HEp-2 cells, demonstrating that furin is indeed capable of activating the MV F protein. Collectively, these data strongly indicate that furin is by far the most important and perhaps the only endoprotease involved in activation of the MV F protein. Nevertheless, we can not exclude the possibility that in certain cells, other endoproteases than furin may contribute to the cleavage. Since furin also activates a wide range of cellular proteins, use of protease inhibitors as therapeutic agents against measles does not seem realistic, especially as measles is a systemic viral infection.

We did not find any indications of differences in the activation process of the F protein between the Edmonston MV strain and the wild-type MV isolate DK96A1, which has only been passaged on human B lymphoid cells. Thus furin is probably the most important endoprotease for activation of the F proteins from both attenuated and wild-type MVs. Although sequence analyses comparing the F protein genes of MV wild-types with those of MV vaccine strains are not yet available, the similarity in F protein activation was not unexpected. The F proteins of morbilliviruses are well conserved (Rota *et al.,* 1992; Bolt *et al.*, 1994), and since furin is ubiquitous, there is probably no or very little evolutionary pressure on the F protein cleavage site during adaptation of MV from one type of cell to another. We have previously shown that the cleavage site and flanking regions of the dolphin morbillivirus F protein was unaffected by isola-

tion of the virus from dolphin lung tissue to primary canine epithelial kidney cells and its subsequent adaptation to Vero cells (Bolt *et al.,* 1994).

The temperature in the respiratory mucosa, which is an important target in the pathogenesis of measles (reviewed by Griffin and Bellini, 1996), may be a few degrees below that of the core body. In the present study, activation of the MV F protein in cells incubated at 33°C appeared to be identical to the activation in cells incubated at 37°C, and recombinant human furin cleaved MV F_0 protein at 33°C. These findings indicate that furin is also the most important endoprotease for activation of the MV F protein at lower than core body temperatures.

In the present and previous (Fujinami and Oldstone, 1981; Watanabe *et al.*, 1995) studies, noninfectious MV particles with uncleaved F proteins were released from cells in which F protein cleavage did not take place or was significantly reduced. Thus using reverse genetic systems (Radecke *et al.,* 1995), it is theoretical possible to produce measles viruses with engineered F protein cleavage sites that can be activated *in vitro* and then only perform a single infectious cycle or only multiply in a certain subset of cells. Such viruses could be useful as vaccines for immunosuppressed individuals.

MATERIALS AND METHODS

Cell culture

HEp-2 (human epidermoid carcinoma) cells and LoVo (human adenocarcinoma) cells were cultivated in minimum essential medium with Earle's salts (MEM) and Ham's F12 medium, respectively. The human B-LCLs AO, GR and JP (van Binnendijk *et al.*, 1989, 1990) and the marmoset B-LCL B95–8 were grown in RPMI 1640 with 10^{-5} M 2-mercaptoethanol. Penicillin, streptomycin, and 10% foetal calf serum (FCS) were added to all media. PBMC were isolated from stabilized blood samples of three healthy donors by density gradient centrifugation on Lymphoprep (Nycomed Pharma). For cultivation of nonselected PBMC, 1×10^6 cells/well were seeded in 24-well flat-bottom microwell plates with or without 2.5 μg/ml PHA-L (Sigma) in the same medium as the B-LCLs. For selection of monocytes, 3×10^6 PBMC/well were seeded without PHA-L, and the nonadherent cells were washed away. Hi5 (*Trichoplusia ni*) insect cells were cultivated as previously described (Christensen *et al.*, 1993).

MV infection

The Edmonston strain of MV was obtained from American Type Culture Collection and passaged in HEp-2 cells. Cells were infected by the Edmonston strain with a m.o.i. of 3–5. Nonselected PBMC were infected 16–20 h after isolation and seeding with or without PHA-L. Adherent PBMC were infected 7 days after isolation. Monolayers of HEp-2 and LoVo cells were infected when 70–90% confluent. The wild-type MV isolate DK96A1 was obtained from a measles patient and solely passaged on human B-LCLs. JP cells were infected by wild-type DK96A1 with a m.o.i. of 0.1.

Metabolic labelling and RIPA

For pulse–chase assay, the medium was replaced with methionine and cysteine deficient MEM without FCS 22 h p.i. of HEp-2 cells, LoVo cells, and B-LCLs and 48 h p.i. of PBMC. The cells were starved for 1 h. [^{35}S]methionine and [^{35}S]cysteine (PRO-MIX; Amersham) (100 μCi/ml) were added to the deficient medium, and the cells were pulsed for 5 min. The deficient medium was then replaced with normal MEM with 10% FCS, and the cells were chased for various intervals. Different chemicals were added at various times during the pulse–chase and then maintained in the media until harvest of the cells. Monensin (20 μM, Sigma) and 5 μg/ml BFA (Sigma) were added 45 min before the beginning of pulse. CCCP (50 μg/ml, Sigma) was added at the end of pulse, and A23187 (Calbiochem) (concentrations indicated in the text) was added immediately at the beginning of chase. Chase with A23187 was carried out in calcium-deficient MEM without FCS. All steps were carried out at 37°C unless stated otherwise.

For mannose and fucose incorporation, 100 μCi/ml [^{3}H]mannose or [^{3}H]fucose (Amersham) was added to infected HEp-2 cells 22 h p.i. The cells were labeled for 2 h and then lysed.

All radiolabeled cells were lysed in cold RIPA buffer consisting of 10 mM Tris–HCl pH 7.8, 150 mM NaCl, 600 mM KCl, 5 mM EDTA, 2% Triton X-100, and protease inhibitors (Complete; Boehringer Mannheim). The lysates were centrifuged 20,000 *g* for 5 min, and the supernatants stored at −80°C. MV F protein was immunoprecipitated from the lysates with anti-MV F Mab 7–21-17–8 (de Vries *et al.* 1988) and Protein A-conjugated Sepharose beads (Pharmacia) by a slightly modified version of the method described by Sheshberadaran *et al.* (1983) and analysed by reducing SDS–PAGE and fluorography.

In vitro furin digestion

Hi5 cells were infected at a high m.o.i. with a recombinant baculovirus (bac:fur-595) expressing secreted soluble human furin (Bravo *et al.,* 1994) and maintained in serum-free medium. At 41 h p.i. the medium was harvested, cleared by centrifugation, and snap frozen in liquid nitrogen. Control medium from Hi5 cells infected with an irrelevant recombinant baculovirus was produced in parallel.

For furin digestion of radiolabeled MV F protein, the protein was immunoprecipitated and bound to Sepharose beads as described above. The beads were washed two times in RIPA buffer and four times in a

buffer of 100 mM Tris–HCl pH 7.5, 1 mM $CaCl_2$, 0.01% Triton X-100 (Bravo *et al.*, 1994), divided in two parts, suspended in bac:fur-595 medium or control medium diluted 1:3 in the above buffer, and rotated for 1.5 h at 37 or 33°C prior to analysis by SDS–PAGE and fluorography.

For furin digestion of MV particles, MV-infected HEp-2 or LoVo cells were maintained in serum-free medium for 48 h, whereas MV-infected JP cells were maintained in the same medium for 72 h. The medium was harvested, cleared by centrifugation 10,000 *g* for 5 min, divided in three parts, and rotated for 1 h at 37°C with a third volume of bac:fur-595 medium, control medium or no medium. 10% FCS was added, and the samples were frozen at −80°C until plaque assay.

Quantification of viral titer

The titer of supernatants from cells infected with the Edmonston strain was determined by plaque assay. HEp-2 cells 70–90% confluent were incubated for 90 min at 37°C with virus sample diluted in MEM with 5% FCS. The cells were covered with 1% agarose (SeaPlaque; FMC Bioproducts) in MEM with 2% FCS and incubated for 7 days at 37°C. The overlay was removed, and the cells were stained for 15 min with 1% crystal violet in 10% Formalin.

The titer of supernatants from cells infected with wild-type DK96A1 was determined by titration on B95–8 cells, which were then inspected for cytopathic effects.

Flow cytometry

Cells were washed, incubated 30 min on ice with anti-MV H Mab I41 (Sheshberadaran et al. 1983), washed, incubated 30 min on ice with FITC-conjugated goat anti-mouse immunoglobulin (DAKO), washed and analysed in a FACSCalibur flow cytometer (Becton Dickinson). All washes and incubations were carried out in PBS with 1% FCS and 0.1% NAN_3.

RT–PCR assay for PC expression

RNA was extracted from MV- and mock-infected cells 36 h p.i. (HEp-2, LoVo, B-LCLs) or 72 h p.i. (PBMC) using the SV Total RNA Isolation System (Promega). The RNA extractions were analysed by RT–PCR for mRNA of furin, PACE4, PC1/3, PC2, PC5/6, PC7/8, and ribosomal protein L27 using the Superscript One-Step RT–PCR System (Life Technologies) and the primers described by Decroly *et al.* (1996). MV RNA was detected by amplification of nucleotides 1189–1555 of the MV H gene with the primers 5′-TCCAAGCACTCTGCGAG-3′ and 5′-GAACTGAGTTTGACATC-3′.

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