The Human Poliovirus Receptor Alpha Is a Serine Phosphoprotein

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The human receptors for poliovirus (hPVR) are members of the immunoglobulin superfamily. Whereas the two membrane-bound isoforms, hPVR α and hPVR δ , share identical three-domain extracellular portions, their C-terminal cytoplasmic parts differ considerably. This feature is well conserved in the corresponding monkey proteins AGM α 1, AGM δ 1, and AGM α 2. The cellular function of these proteins is presently unknown. In this short communication we report that hPVR α and possibly also AGM α 1 and AGM α 2, but not the δ isoforms, are phosphoproteins. The phosphorylation occurs at a serine in the cytoplasmic tails of these receptors. We further present evidence suggesting that the kinase responsible for the phosphorylation is calcium/calmodulin kinase II.

The poliovirus receptor (PVR) has been determined to be the cell surface entity which mediates poliovirus binding and confers susceptibility to poliovirus infection upon cells in which it is expressed (18, 19). Other viral receptors are rapidly being identified as cell surface proteins, and a number of these are known to play roles in cell adhesion, cell-cell interaction, and cell-extracellular matrix interactions. Some, like PVR, are members of the immunoglobulin superfamily (for recent reviews listing viral receptors see references 10 and 27). Unlike those of most of these viral receptors, the normal cellular function of PVR is unknown. The study of this protein originated with the discovery of its role in the initiation of viral infection.

PVR is not a single molecular species but is a family of homologous isoforms. Four different mRNA splice variants of the human form of PVR (hPVR) encode two class Ia membrane-bound isoforms (hPVR α and hPVR δ) and two secreted isoforms (hPVR β and hPVR γ) (16, 19), all of which possess three immunoglobulinlike domains and originate from a single gene (16). The two membrane-bound isoforms are complex branched-chain N-linked glycoproteins which occur as broad 80-kDa protein bands upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Treatment with *N*-Glycanase resolves these two isoforms into a 46-kDa species and a 43-kDa species, the ratio of which varies in a cell type-specific fashion (1). hPVR α and hPVR δ have identical amino acid sequences throughout their extracellular and membrane-spanning regions, with eight potential N-glycosylation sites. They differ only in the lengths and amino acid compositions of their cytoplasmic portions, with these portions of hPVR α and hPVR δ being 50 and 25 amino acid residues long, respectively.

Three membrane-bound isoforms of the monkey PVR (mPVR) originate from two separate genetic loci (17). Two splice variants, AGM α 1 and AGM δ 1, originating from one locus encode isoforms homologous to hPVR α and hPVR δ ,

respectively, with seven potential N-glycosylation sites. The cytoplasmic domains of AGM α 1 and AGM δ 1 are 50 and 25 amino acids long, respectively. A single isoform, AGM α 2, originates from a second genetic locus. AGM α 2 is homologous with hPVR α and AGM α 1 but differs significantly in that it has a different signal sequence (2), only five potential N-glycosylation sites, and a cytoplasmic domain of 38 amino acids (17).

Both human membrane-bound isoforms, hPVR α and hPVR δ , are capable of mediating viral attachment and infection, since cells expressing only one form or the other are susceptible to infection with poliovirus (19). Poliovirus-receptor interactions are primarily mediated by the N-terminal extracellular immunoglobulinlike domain (domain 1) of membrane-bound PVR, since mutated forms lacking this domain no longer mediate viral infection (16, 23). In fact, domain 1 can be fused to other cell surface proteins, namely, ICAM-1, the receptor for the major group of rhinoviruses (25), and CD4, the receptor for human immunodeficiency virus (7, 15), and the resulting chimeric molecules mediate poliovirus infection (23, 24). The theory that the virus is not opportunistically dependent upon the unknown cellular function of the receptor is further confirmed by the demonstration that complete deletion of the cytoplasmic domain of PVR does not prohibit susceptibility to infection (16).

To determine if PVR is a phosphoprotein, we initially labeled JA-1 cells (Fig. 1, lane 2), a previously described cell line derived by stable transformation of mouse L cells with a cosmid containing the hPVR gene (1), HeLa cells (Fig. 1, lane 3), derived from a cervical epithelial carcinoma, and Hep-2 cells (Fig. 1, lane 4), derived from a laryngeal carcinoma, with ³²P_i. hPVR-negative mouse L cells were also labeled as a control. After immunoprecipitation of cell lysates a broad hPVR-specific 80-kDa band, shown in Fig. 1, could be observed upon analysis by SDS-PAGE for all the cell types except L cells. In other experiments this band was found to comigrate with the [³⁵S]methionine-labeled immunoprecipitated hPVR, which has previously been described (1, 3) (data not shown). A minor nonspecific band of about 93 kDa occurred in all immunoprecipitations of ³²P_i-labeled material.

To determine if phosphorylation of PVR occurs in other cell lines, we performed the same experiment with a wider panel of cells, which in addition to the cells described above included

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FIG. 1. Immunoprecipitation of hPVR from the lysates of cells labeled with $^{32}\text{P}_i$. Cells (5×10^6) of the types mentioned in the text and indicated at the tops of the lanes were washed and labeled for 4 h in DMEM without phosphate (Life Technologies) with 0.5 mCi of $^{32}\text{P}_i$ (Amersham) per ml. Cells were lysed and immunoprecipitated with monoclonal antibody P44. Sample processing and SDS-PAGE were performed as previously described (1, 3) except that the lysis and immunoprecipitation buffer contained 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.5), 100 mM NaCl, 1 mM EDTA, 10 mM NaF, 30 mM sodium PP_i , 1 mM sodium orthovanadate, and 0.5% Nonidet P-40. Cells were lysed in 250 μl of this buffer, and unincorporated $^{32}\text{P}_i$ was removed by Sephadex G-50 NICK columns (Pharmacia) prior to immunoprecipitations. The 15% Laemmli gel was dried prior to autoradiography. Lane 5 contained ^{14}C -labeled protein molecular mass standards (Pharmacia).

COS-7 cells, a monkey kidney cell line; DiFi cells, a colorectal cancer cell line; Henle cells, which are intestine 407 embryonic cells; RD cells, a cell line derived from a rhabdomyosarcoma; and SK-N-SH cells, which were derived from a neuroblastoma. The results (data not shown) were the same as those shown in Fig. 1, with a $^{32}\text{P}_i$ -labeled 80-kDa protein being immunoprecipitated from the lysates of all the human cells and the JA-1 cell line. The monkey COS-7 cells produced a slightly smaller specific protein of about 70 kDa which coelectrophoresed with the previously reported [^{35}S]methionine-labeled mPVR species (data not shown). To determine which membrane-bound isoform of PVR is phosphorylated, the samples were treated with *N*-Glycanase following immunoprecipitation of PVR. The results for Hep-2 (lane 1), COS-7 (lane 2), DiFi (lane 3), HeLa (lane 4), Henle (lane 5), JA-1 (lane 6), RD (lane 7), and SK-N-SH (lane 8) cells are shown in Fig. 2. In all cases the previously detected broad 80-kDa band shifted down to a lower molecular mass because of deglycosylation. For the human cell lines a single predominant band was found to occur at a molecular mass of about 46 kDa. This protein from the SK-N-SH cells is hardly visible unless longer autoradiographic exposures are performed, because of the relatively low levels of expression of hPVR in these cells, as previously reported (1, 3). In other experiments, this band comigrated with the previously

FIG. 2. N-deglycosylated immunoprecipitated PVR from the lysates of various cells labeled with $^{32}\text{P}_i$. The various cell types which are described in the text are indicated at the tops of the lanes. Cell labeling and immunoprecipitations were performed as described in the legend to Fig. 1. Immunoprecipitated PVR was boiled in TE buffer containing 0.1% SDS in order to release PVR from the protein A-monoclonal antibody P44 complex. Deglycosylation with *N*-Glycanase (Genzyme) was performed in the presence of SDS according to the recommendations of the manufacturer. Sample processing, SDS-PAGE, and autoradiography were performed as described previously (1) and in the legend to Fig. 1. Lane 9 contained ^{14}C -labeled protein molecular mass standards.

identified N-deglycosylated hPVR α isoform which was labeled with [^{35}S]methionine (data not shown).

For JA-1 cells the predominant 46-kDa band was always accompanied by a minor band of 43 kDa. The nature of this minor band is currently unknown. However, it appears that the occurrence of this protein is specific for this mouse-derived cell line (1), since even after prolonged overexposure, a band migrating at that position could not be detected in any human cell line examined (data not shown).

Lane 2 of Fig. 2 shows the phosphorylation of the deglycosylated isoforms of mPVR produced in monkey cells. One $^{32}\text{P}_i$ -labeled band occurs, with a molecular mass of 46 kDa, corresponding to the approximate molecular mass of the isoform AGM α 1, which is homologous to hPVR α . A second, stronger band occurs slightly below this band. This species would appear to represent AGM α 2, with the additional shift being caused by the 12-amino-acid deletion in the cytoplasmic portion of this protein (see Fig. 5). Therefore, the results shown here suggest that both isoform AGM α 1 and isoform AGM α 2 are phosphorylated and that the latter either is a preferred substrate for cellular kinase activity or is expressed at higher levels. Theoretical signal sequence analysis suggested that AGM α 2 has a higher efficiency of translocation than AGM α 1, supporting the idea that it is expressed at higher levels (2).

To determine which residue(s), serine, threonine, or tyrosine, of hPVR α is phosphorylated, $^{32}\text{P}_i$ -labeled PVR was immunoprecipitated from JA-1 cell lysate and, after thorough

FIG. 3. Two-dimensional thin-layer chromatography with electrophoresis in both directions of acid-hydrolyzed $^{32}\text{P}_i$ -labeled hPVR and comparison with *o*-phosphoamino acid standards. Two-dimensional analysis of phosphoamino acids was performed by using cellulose thin-layer plates (EM Separations) according to the technique described by Cooper et al. (6; see also reference 12). Immunoprecipitated $^{32}\text{P}_i$ -labeled hPVR was acid hydrolyzed by treatment with 6 N HCl for 2 h at 110°C. The origin is indicated by the small circle in the upper right-hand corner. Electrophoresis in the first dimension was from right to left in 7.8% acetic acid and 2.5% formic acid at pH 1.9. Electrophoresis in the second dimension was from top to bottom in 0.5% acetic acid and 0.5% pyridine at pH 3.5. The positions of the ninhydrin-stained marker phosphoamino acids are indicated by the circles and labels. Autoradiography was performed for 12 h.

hydrochloric acid hydrolysis, subjected to two-dimensional thin-layer chromatographic analysis with electrophoresis in both directions. The results shown in Fig. 3 indicate that the ^{32}P -labeled phosphoamino acids generated from hPVR migrated in a manner identical to that of the ninhydrin-stained *o*-phosphoserine standard marker. This result indicates that hPVR is phosphorylated only at a serine(s) and is therefore the substrate of a serine protein kinase. Whether one or multiple serines are phosphorylated per hPVR α molecule is presently unknown.

We found that the removal of growth factors contained in serum had no specific effect upon the phosphorylation of hPVR α and that labeling with $^{32}\text{P}_i$ significantly decreased only at time points after 12 h of starvation (data not shown). However, this decrease in PVR phosphorylation corresponded to a decrease in [^{35}S]methionine labeling, indicating that the reduction of label resulted from a general decrease in cell metabolism occurring in cells starved of serum for extended periods of time.

To get an idea of which serine protein kinase phosphorylates hPVR α and which cellular pathway might be involved, we used pharmacological agents which inhibit, either specifically or in a broader range, the activity of a variety of cellular protein kinases including cyclic AMP-dependent protein kinase, protein kinase C, cyclic GMP-dependent protein kinase, myosin light-chain kinase, calcium/calmodulin-dependent protein kinase II (CaMK II), casein kinase I, and casein kinase II. The inhibitors chosen were A-3, chelerythrine, HA-1077, and staurosporine (Biomol). JA-1 cells were used for these experiments

FIG. 4. The effects of various protein kinase inhibitors on the phosphorylation of hPVR. JA-1 cell monolayers were treated for 2 h with the various kinase inhibitors described in the text at the concentrations shown at the top for 2 h prior to being labeled with $^{32}\text{P}_i$, as described in the legend to Fig. 1. Immunoprecipitation, N deglycosylation, sample processing, SDS-PAGE, and autoradiography were performed as described in the legend to Fig. 2. The positions of molecular mass markers are indicated at the right.

because the comparatively high levels of hPVR expression resulted in the requirement of a smaller amount of radioactive material for labeling and detection. First, cells were treated overnight with different concentrations of the drugs to determine the maximum concentrations at which no deleterious effects upon the cells could be observed by light microscopy. On the basis of these results, cells were then treated for 2 h with A-3 at concentrations of 20 and 200 μM , chelerythrine at a concentration of 5 μM , HA-1077 at concentrations of 10 and 100 μM , and staurosporine at concentrations of 10 and 50 nM. The effects of the various drugs upon the phosphorylation state of hPVR were then analyzed by labeling of cells with $^{32}\text{P}_i$, followed by immunoprecipitation and N deglycosylation. The results are shown in Fig. 4. None of the inhibitors had a noticeable effect on the phosphorylation state of hPVR α except staurosporine at a concentration of 50 nM. No observable differences were detected by SDS-PAGE analysis of total $^{32}\text{P}_i$ -labeled protein from cell lysates which were treated with 50 nM staurosporine when this protein was compared with protein from control untreated cells (data not shown).

Staurosporine is a potent inhibitor of cyclic AMP-dependent protein kinase (K_i of 7 nM), protein kinase C (K_i of 0.7 nM), cyclic GMP-dependent protein kinase (K_i of 8.5 nM), and myosin light-chain kinase (K_i of 13 nM). It is a noncompetitive direct inhibitor of CaMK II only at concentrations of above 20 nM (11, 14, 22, 26, 28). As shown in Fig. 4, no inhibitory effect could be observed at staurosporine concentrations of 10 nM. These considerations together with the lack of effect of the other inhibitors and the known specificities of these inhibitors for the various protein kinases suggest that PVR could be phosphorylated by CaMK II. Other preliminary evidence to support this theory includes the inhibition of phosphorylation of hPVR by treatment of cells with medium which doesn't contain calcium for 4 h prior to labeling (data not shown).

The phosphorylation of hPVR α as well as the mPVR

FIG. 5. Alignment of the cytoplasmic portions of the monkey and human PVR isoforms. The deduced amino acid sequences for the predicted cytoplasmic portions of hPVR isoforms α and δ (16, 19) and the mPVR isoforms AGM α 1, AGM δ 1, and AGM α 2 (17) are shown in the alignment. Gaps, indicated by periods, were inserted to maintain alignment of the conserved residues. The name of the particular isoform is indicated at the left with a schematic to indicate the position of the transmembrane domain. The lengths of the respective cytoplasmic domains of the isoforms are indicated by the column of numbers on the right. The serines present in the 25 most C-terminal amino acids of hPVR α , AGM α 1, and AGM α 2, which are the most likely possible substrates for phosphorylation, are in bold and underlined, and the conserved consensus sequence (RXXS) for CaMK II is enclosed by the box.

isoforms AGM α 1 and AGM α 2 is most likely to occur at a kinase substrate site in the cytoplasmic portion of the molecule. An alignment of the cytoplasmic domains of two hPVR isoforms and three mPVR isoforms is shown in Fig. 5. As shown, only the hPVR α isoform is serine phosphorylated. Moreover, the data suggest that both the AGM α 1 isoform and the AGM α 2 isoform are phosphorylated. In contrast, phosphorylation of the δ isoforms could not be detected. There are seven serine residues contained within the 25 C-terminal cytoplasmic amino acid residues of hPVR α which are unique and do not occur in hPVR δ . Remarkably, all of these serine residues are conserved in the monkey AGM α 1 isoform and five of them are conserved in the AGM α 2 isoform. One of these conserved serines occurs as part of the known consensus substrate phosphorylation site, RXXS, for CaMK II (9, 13, 21). Interestingly, a corresponding consensus site is also present in the cytoplasmic domain of the mouse homolog of PVR (MPH), a cell surface polypeptide that does not function as a poliovirus receptor (20). However, we could not investigate a possible phosphorylation of MPH, since the monoclonal antibody used in our immunoprecipitations is specific for monkey and human PVR and an MPH-specific antibody was unavailable.

Multifunctional CaMK II is an important neural-cell protein kinase thought to modulate synaptic vesicle trafficking and the level of neurotransmitter responsiveness (8). It is particularly concentrated in areas of the spinal cord, hippocampus, and motor cortex. It localizes subcellularly to membrane fractions of synaptosomes (9). It is interesting that the histopathology of poliomyelitis correlates well with this pattern (4). Furthermore, viral binding to neural tissue homogenates was reported to be highest in synaptosomes (5), suggesting that hPVR expression and CaMK II activity colocalize with respect to their distribution in the central nervous system. Although we could not detect any inhibition of viral propagation upon treatment of JA-1 cells with the above-mentioned protein kinase inhibitors (data not shown), these results obtained with mouse tissue culture cells do not exclude an important role of hPVR phosphorylation in the poliovirus infectious cycle taking place in the neural tissue context. That hPVR α may be phosphorylated by CaMK II is an intriguing observation that may relate to its cellular function.

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