Adenovirus Protein Involved in Virus Internalization Recruits Ubiquitin−Protein Ligases†

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ABSTRACT: Adenovirus penton base protein is involved in virus internalization. Searching for the cellular partners of this protein, we used dodecahedra, adenovirus subviral particles composed of 12 bases, for screening a human lung expression library. This screen yielded three ubiquitin−protein ligases, WWP1, WWP2, and AIP4, all of which belong to the HECT family and contain multiple WW domains. The xPPxY motif, known to interact with WW domains in partner proteins occurs twice in the N-terminal part of the base polypeptide chain. The recruitment of three ubiquitin-protein ligases was shown for two distinct virus serotypes, Ad2 and Ad3. The first N-terminal xPPxY motif in the base protein sequence is indispensable for the interaction. The association in vitro was shown by the protein overlay technique and in vivo by cotransfection followed by immunoprecipitation. The binding parameters studied by surface plasmon resonance confirmed the interaction of base protein with three ubiquitin−protein ligases. In case of WWP1 when the saturation of binding was achieved, the apparent dissociation constant of 65nM was calculated. This is the first demonstration of the interaction of nonenveloped viruses with ubiquitin-protein ligases of host cells.

Adenoviruses (Ad)[1] are a family of small nonenveloped icosahedral DNA viruses infecting humans as well as a variety of animals. On each of the 12 vertexes of the Ad virion is a penton, a complex of two oligomeric proteins: a penton base anchored in the capsid and an antenna-like fiber extending outward (*1*). The penton plays a dual role in the infection of host cells. The fiber attaches a viral particle to the cell surface through the interaction of its distal globular region (C-terminal head domain) with a primary receptor (*2*−*5*). The penton base protein is involved in virus internalization, as shown for serotypes 2, 3, 4, and 12, where an Arg−Gly−Asp (RGD) sequence in the penton base protein interacts with cellular integrins avβ3 and avβ5 (*6*, *7*). The Ad penton base protein is also implicated in the escape of the virus from endosomes; it was observed that upon Ad infection, foreign molecules co-endocytosed with virus were released from the endosomes (*8*−*10*). It has been suggested that the penton base undergoes an acid-induced conformational change in endosomes, resulting in the exposure of hydrophobic domains and leading to disruption of the endosomal membrane (*11*). Since the assembly of progeny virions takes place in the nucleus, viral proteins after being synthesized in the cytoplasm have to travel to the nucleus. It can be thus inferred that during their life cycle in the host cell penton proteins are involved in plethora of interactions that permit virus attachment, cell entry, release from endosomes as well as penton synthesis, folding, oligomerization, and nuclear transport.

Given the pivotal role of Ad base protein in virus internalization, it is essential to identify cellular partners of this protein. By expressing Ad3 base protein or base and fiber proteins, we have generated sub-viral particles, dodecahedra. The dodecahedra are composed either of 12 bases or of 12 pentons (complex of penton base and fiber) packed with icosahedral symmetry (*12*, *13*). Like the native virus, these particles enter cells efficiently via the endocytotic pathway and accummulate at the nuclear membrane (*12*), which suggests that during their passage toward nucleus they interact with adenovirus cellular partners. To search for cell proteins interacting with adenovirus penton base protein, we used dodecahedra for screening human lung expression library. This screen yielded three ubiquitin−protein ligases (Upls) belonging to the Nedd4-like group of E3 enzymes.

EXPERIMENTAL PROCEDURES

Adenovirus Structural Proteins and Mutants. Ad3 dodecahedron and Ad2 base protein were isolated after expression in the baculovirus system. Dodecahedra were purified as described by Fender et al. (*12*), and the base was purified with NaCl gradient on a Q-Sepharose column, followed by ammonium sulfate precipitation at 35% concentration. For the expression in human cells, Ad2 base gene (GenBank no. AJ293910), and its mutants were cloned into pcDNA 3.1 (Invitrogen). Two Ad2 base mutants, ΔPY1 (Ser_{17}−Phe_{571}) and Δ2PY (Arg_{47}−Phe_{571}), were obtained by PCR cloning of appropriately deleted base protein gene. The third mutant,

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[1] Abbreviations: aa, amino acid; Ad, adenovirus; Upl, ubiquitin-protein ligase; ON, overnight; RT, room temperature; SPR, surface plasmon resonance; RU, resonance units.

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PY2mut, had a second proline (Pro_{43}) of the second PPxY exchanged for alanine by PCR cloning with the upstream primer containing the appropriate mutation. All sequences were checked by DNA sequencing.

Screening of the Expression Library. A human lung cDNA expression library in lambda gt11 (Clontech) was used throughout this work. Phages were allowed to attach to *E. coli* cells and were spread according to the manufacturer's instructions. The phages were lifted onto a nitrocellulose membrane soaked in 10 mM IPTG. All subsequent solutions contained 10 mM $MgCl_2$. The membranes were rinsed with the Tris-borate buffer containing 0.05% Tween 20 (TBST), saturated for 30 min at 42 °C with TBST containing 5% de-fatted milk (TBSTM), and allowed to interact ON at 4 °C each with 3 mL of TBSTM containing 1 μg of fiber dodecahedra or of penton dodecahedra. The membranes were washed with TBS buffer three times for 10 min, and the revelation with the polyclonal antibody against Ad3 penton diluted at 1:4 000 was followed by ECL. Positive clones were plaque-purified and DNA was isolated from phage lysates with Qiagen lambda midi kit and sequenced.

Upls and Their Fragments. WWP1 (AIP5.1: Thr_{258}−Arg_{528}, see GenBank no. NP_008944), WWP2 (AIP2.2: Gly_{111}−Iso_{776}, see GenBank no. NP_008945) and AIP4 (AIP4.1: Gly_{124}−Iso_{697}, see GenBank no. NP_113671) (see ref *14*) as well as four WWP domains of WWP1 (see ref *15*) cloned in pGEX-4T-2 (Amersham Pharmacia Biotech) were expressed as GST-fusion proteins in *E. coli* as follows. The DH5-α bacteria bearing the appropriate plasmid were grown until $OD_{600\ nm}$ equal 0.4 at 37 °C for pGEX-AIP5.1, pGEX-AIP4.1, and pGEX-WW_{1-4} of WWP1 or at 30 °C for pGEX-AIP2.2, in the presence of 100 μg/mL of ampicillin and induced with 40 μM IPTG for 3 h. Cells from 1 L cultures were harvested by centrifugation and resuspended in 40 mL of 50 mM Tris-HCl, pH 8, supplemented with 1× complete protease inhibitor cocktail (Roche Molecular). The bacteria were lysed by sonication (10 min of 0.2 s pulses at 70% power) on ice. The lysates were then centrifuged at 38 000 g for 30 min at 4 °C and the supernatants were filtered through 0.2 μm filters before protein purification on Glutathione Sepharose-4B column. GST-fusion proteins were eluted with 10mM glutathione. WWP proteins were then obtained by cleavage of the GST-WWP proteins with human plasma thrombin (Sigma), followed by Glutathione Sepharose-4B purification.

For the expression in human HeLa cells, an incomplete WWP1 gene obtained during our screen was cloned into pcDNA 3.1 (Invitrogen), resulting in a truncated protein (aa 345−922; GenBank no. NP_008944), devoid of C2 domain. Another variant of WWP1 (aa 345−531) deleted additionally of HECT domain and thus only constituted of the four WW domains, was similarly constructed by PCR cloning in the same vector. All sequences were checked by DNA sequencing.

Blot Overlay Assay. Similar amounts (1 μg) of GST-fusion proteins or transfected cell lysates (0.1×10^7 cells) were applied to the denaturing polyacrylamide gels, run and electroblotted onto PVDF membrane. The membrane was saturated with TBSTM buffer and was allowed to interact ON at 4 °C with the purified ligand or with the extract of insect cells expressing an appropriate ligand. After washing with TBS, the membrane was allowed to interact with the appropriate primary antibody for 90 min at RT. After interaction with the HRP-conjugated secondary antibody, the reaction was developed with 4-chloro-1-naphthol or by ECL.

Transfection and Immunoprecipitation. HeLa cells grown in EMEM/10% of fetal calf serum under 5% CO_2 were plated in 6-well plates and transfected at 4×10^5 cells/well for 36 h with 1 μg of plasmid DNA in the presence of 3 μL of cationic lipid Fugene 6 (Roche Molecular). In case of cotransfection with two plasmids, 0.5 μg of each plasmid were used. Twenty-four hours after transfection, cells were fed with 40 μCi/ml of Redivue Pro-mix (Amersham, sp. act. 1000Ci/mmol) containing radioactive methionine and cysteine. After appropriate growth period, cells washed with PBS were lysed with 20 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 10 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitors. After 30 min incubation on ice, supernatant was recovered by 15 min centrifugation at 13 000 rpm. Preclearing was done for 2 h at 4 °C with 20 μL of Protein G-Sepharose beads (Amersham Pharmacia). Resulting supernatant was allowed to interact for 2 h at 4 °C with 20 μL of Protein G Sepharose carrying anti-Ad2 base antibody or anti-Nedd4 antibody, which was obtained by ON interaction of 0.1 μg of the appropriate antibody with 20 μL of beads. Beads were collected by centrifugation and washed 4 times with the lysis buffer. Attached proteins were extracted with 3× Laemmli buffer (Laemmli, 1970), boiled for 5 min, and run on SDS/PAGE. Gels were dried and autoradiographied for 1 week. Anti-Nedd4 antibody used for WWP1 immunoprecipitation is a polyclonal one raised against the three WW domains of mouse Nedd4 protein (*15*), which share a high degree of homology with WW domains of WWP1, WWP2 and AIP4 (*14*, *16*). This antibody has been successfully tested for its cross reactivity against the three studied Upls, as well as against each of the four WW domains of WWP1 protein (data not shown).

Assessment of Interaction Strength by Surface Plasmon Resonance (SPR). SPR measurements were carried out using a Biacore apparatus (Biacore, St. Quentin-en-Yveline, France). Flow cells of a Biacore F1 sensor chip were activated with a cross-linking mixture of EDC/NHS (50 μL 0.2 M *N*-ethyl-*N*′-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride and 0.05 M N-hydrosuccinimide), after which Ad2 penton base protein at 5 μg/mL or GST-WWP1 (aa 258−528) at 10 μg/mL, both in 10 mM sodium acetate, pH 4.2, were injected. Remaining activated groups were blocked with 50 μL of 1 M ethanolamine, pH 8.5. These procedures allowed to couple 1300 resonance units (RU) of Ad2 penton base protein, or 450 RU of GST-WWP1. Negative controls consisted of EDC/NHS activated then blocked surface, or surface coupled to GST (200 RU). Binding assays were carried out at 25 °C, at a constant flow rate of 10 μL/min. The proteins, GST-WWP1 (aa 258−528), GST- WWP2 (aa 111−776), GST-AIP4 (aa 124−697), or Ad2 penton base, were diluted in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM sodium chloride, 3 mM EDTA, 0.005% P20), and a range of protein concentrations were injected over the surface for 5 min each, after which the surface was washed with HBS-EP for 15 min. Protein complexes containing the penton base and WWP proteins were then dissociated with sodium hydroxide (10−12 mM for 1 min), to regenerate the surface. Binding curves were analyzed with the Biacore BIAevaluation 2.1 software,

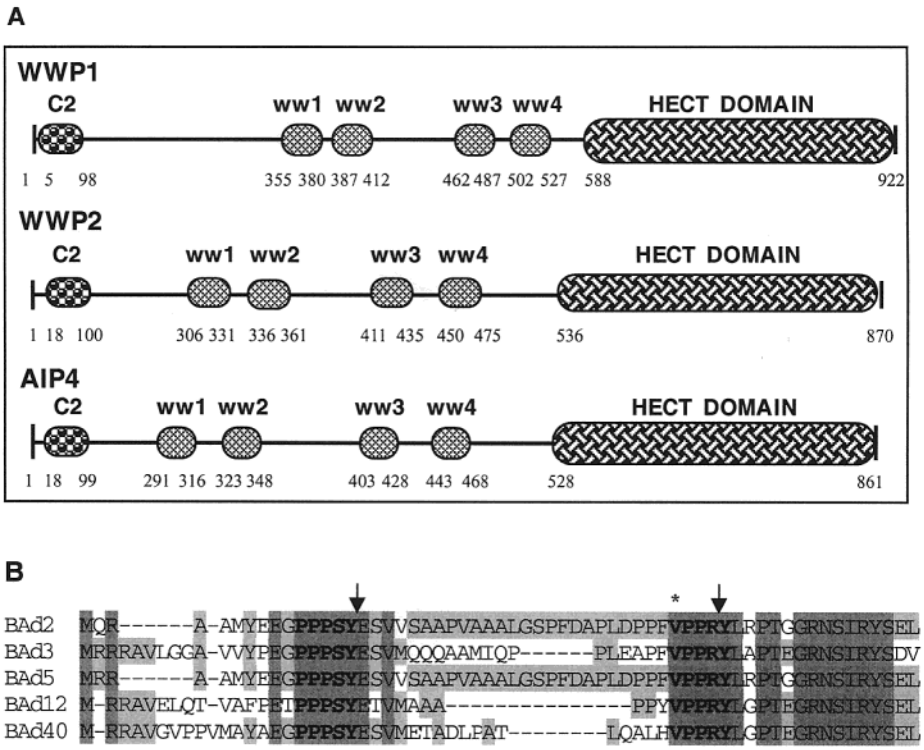

FIGURE 1: (A) Organization of ubiquitin-protein ligases obtained by library screen with Ad3 base dodecahedron. C2 is the domain responsible for the association with membranes, ww is the WW domain, HECT is the catalytic domain of Upl. (B) Primary sequence of Ad bases of different serotypes. xPPxY motif is in bold, and conserved amino acids are in gray. Arrows indicate the first amino acid in Ad2 base deletion mutants ΔPY1 and Δ2PY, and the asterisk indicates Pro_{43} changed to Ala in PY2mut.

and when possible, equilibrium data were extracted from the sensorgram at the end of each injection and used to calculate the equilibrium dissociation constant.

RESULTS

Screening of Human Expression Library. By expression in the baculovirus system of Ad3 either the penton base protein alone or the base and fiber proteins, we obtained symmetrical icosahedral particles composed either of 12 bases or 12 pentons (complexes of base and fiber) (*12*, *13*). Like the native virus, these particles enter cells efficiently via the endocytotic pathway and accumulate at the nuclear membrane showing that they are functional and presumably able to interact with the same partners as the virus. We used these particles in order to screen the human respiratory tract expression library for proteins interacting with pentons. Screening of some 600 000 clones yielded 20 or so positive clones interacting with both base and penton dodecahedrons, showing that the interaction depends on the presence of the base protein in the ligand. Among these clones, several coded for three recently described Upls of the HECT domain family, WWP1, WWP2, and AIP4 (*14*, *17*) (Figure 1A).

Interaction of Base Protein with WW Domains in Vitro. HECT domain Upls contain usually several WW domains. These domains are known to recognize in partner proteins four kinds of proline-rich or proline-containing core motifs, including one with the xPPxY consensus (*18*, *19*). It is relevant in this context that the adenovirus base protein contains in the N-terminal part of the polypeptide chain two xPPxY motifs, which are conserved in all sequenced serotypes (Figure 1B). To assess the involvement of four WW domains of WWP1 in the interaction, these domains were expressed in form of GST-tagged proteins in *E. coli*, purified, and used separately in the blot overlay assay with different adenovirus structural proteins (Figure 2). WW domains of WWP1 interact with Ad2 base protein, with dodecahedron made of 12 Ad3 bases but not with the adenovirus fiber protein. All four WW domains are apt to interact with base proteins, albeit with the fourth one interacting slightly weaker.

First xPPxY Motif in the Base Protein Is Responsible for the Interaction. To find out if both xPPxY motifs in the base protein are required for the interaction with WW domains, we constructed three mutants of the Ad2 base protein (Figure 3F). ΔPY1 and Δ2PY were respectively deleted of either the first or both xPPxY motifs, and the PY2mut had a second proline of the second PPXY exchanged for the alanine. The rationale for this last mutation is based on studies identifying this residue in the xP**P**xY motif as indispensable for interaction with WW domains (*17*, *20*).

All three Upls and also the family prototype Nedd4 were able to interact in vitro with the adenovirus base protein (lanes Ad2 base in Figure 3A−D). However, the removal of the first xPPxY was sufficient for this interaction to be abolished (lanes ΔPY1 in Figure 3A−D). On the contrary, the mutation of the essential proline in the second xPPxY motif did not alter the interaction (lanes PY2mut in Figure 3A−D) suggesting that the second xPPxY is not essential for the interaction.

Association in Vivo. Co-immunoprecipitation experiments were performed to determine in vivo association of Upl with adenovirus base protein. To this end HeLa cells were first transfected separately with plasmids encoding these proteins, and the optimum time of expression was established as being

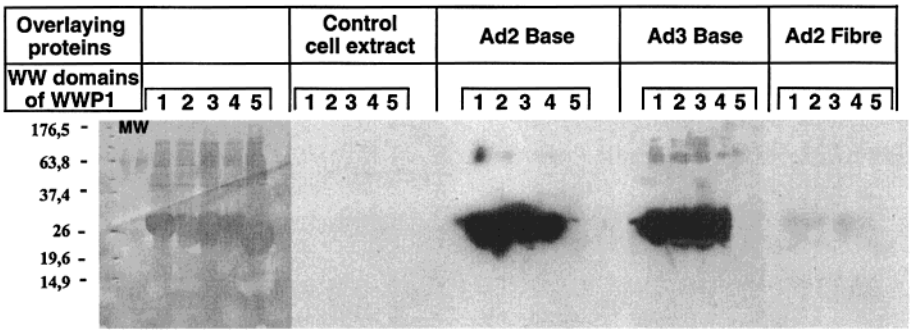

FIGURE 2: Interaction of Ad proteins with four WW domains of WWP1. Equal amounts (10 μg) of each of the four WW domains of WWP1, expressed as fusions to GST (lanes 1−4), as well as GST alone (lane 5), were separated by denaturing PAGE and transferred to PVDF membrane. Membranes were incubated with the indicated proteins. (A) Protein staining with Coomassie Brilliant Blue. (B) Blot overlay performed with the indicated proteins and developed with appropriate antibodies.

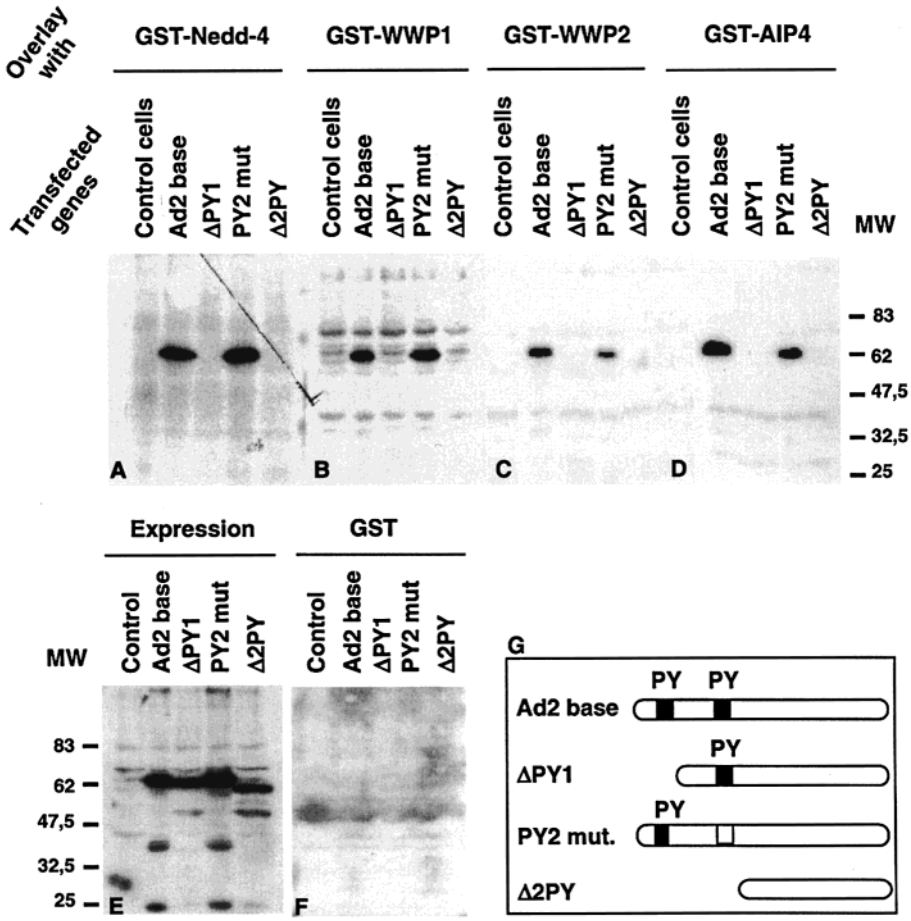

FIGURE 3: First xPPxY motif in the Ad2 penton base protein is indispensable for the interaction with WW domains-containing ubiquitin-protein ligases. (A−F) Identical samples of HeLa cells transfected with plasmids coding for Ad2 penton base protein (lanes 2), ΔPY1 (lanes 3), PY2 mut. (lanes 4), and Δ2PY (lanes 4) as well as untransfected control cells (lanes 1) were lysed, subjected to 10% PAGE/SDS, and transferred onto PVDF membranes. Membranes were allowed to interact ON at 4 °C with *E. coli* extracts expressing appropriate GST fusions of Ulps (A−D), as well as GST alone (F), and revealed with anti-GST monoclonal antibody followed by ECL. (E) Expression of base proteins in HeLa cells, shown by western blot with anti-Ad base antibody. (G) Ad2 base protein and its mutant. ΔP1 has the first xPPxY (PY) motif deleted, PY2mut has the crucial proline mutated in the second xPPxY motif, and ΔP2 is devoid of both xPPxY motifs (see Figure 1B).

24−48 h for base proteins and about 36 h for WWP proteins. Cells were then cotransfected with appropriate plasmids, 24 h later fed with radioactive amino acids and immunoprecipitated 12 h later.

In the initial immunoprecipitation experiments the longer WWP1 clone was used (aa 345−922); however, this protein with a theoretical molecular mass of 68 kDa surprisingly ran with the same mobility as the main proteolytic fragment of Ad2 base, around 50kDa when compared to molecular weight marker. Therefore, WWP1 variant containing WW domains only (aa 345−531), and resulting in a protein of about 22 kDa was used for the experiment shown in Figure 4. Immunoprecipitation of cells cotransfected with WWP1 and either Ad2 base or PY2mut yielded both proteins in the complex (Figure 4, lanes 2 and 4). Additionally the major proteolytic fragment of the base protein could be seen, identified separately by western blot (data not shown) as well as nonspecific bands, seen also in the control immuno-

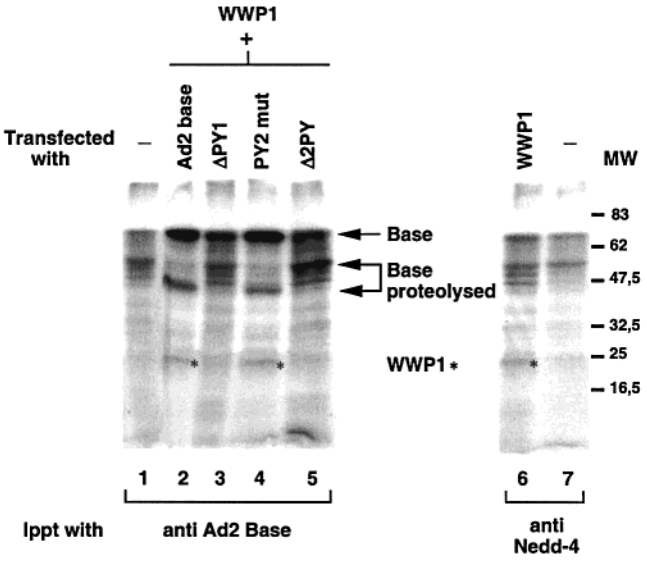

FIGURE 4: In vivo interaction of Ad2 base protein and WWP1 (aa 69−331). HeLa's were transfected or cotransfected with indicated plasmids. After 24 h cells were fed for 12 h with a mixture of [S35]-labeled methionine and cysteine. Cells were then lysed and immunoprecipitated with protein G-Sepharose beads presaturated either with an antibody against Ad2 base protein (lanes 1−5) or with an antibody against Nedd4 protein (lanes 6 and 7). Proteins were extracted, analyzed on a 10% SDS-PAGE, and autoradiographied as described in Materials and Methods.

precipitates with anti-base and anti-Nedd4 antibodies. Contrary to that, immunoprecipitation of cells cotransfected with WWP1 and the base deletion mutant ΔPY1 did not show complex formation (Figure 4, lane 3). These results corroborate those of gel overlay (Figure 3) and confirm that the removal of the first PPxY motif in the base protein is sufficient to invalidate the interaction with the WWP1 partner. Furthermore, use of WWP1 containing WW domains only further confirms that the interaction with base protein is mediated by WW domains of Upls.

Characterization of Interaction. To investigate the interaction of the Ad2 penton base with WWP1 (aa 258−528, see Figure 1), SPR with the Biacore apparatus as a detection system was used. Ad2 penton base was injected over a surface on which GST-WWP1 fusion protein (aa 258−528) has been immobilized. Figure 5a shows an overlay of sensorgrams obtained when increasing concentrations of Ad2 penton base were injected over a surface containing 450 RU of GST-WWP1. Visual inspection of the sensorgrams showed that Ad2 penton base bound rapidly to the immobilized WWP1 protein and formed stable complexes (low dissociation). Injection of Ad2 penton base, in the same concentration of range, over a surface functionalized with GST alone did not produce any signal (data not shown), demonstrating the specificity of the binding. When this set of data was analyzed using the A+B ↔ AB model, the data were found to deviate from this simple model. This may be caused by factors that derive from the technology itself (such as rate-limiting mass transport effect or rebinding of the ligand during the dissociation phase), or, more likely, because the A+B ↔ AB binding model does not apply well to penton base-WWP1 interaction. It is relevant in this context that the penton base is pentameric, and thus could potentially bind up to five molecules of each ligase. Moreover, ligase contains four potentially interactive WW binding motifs (see

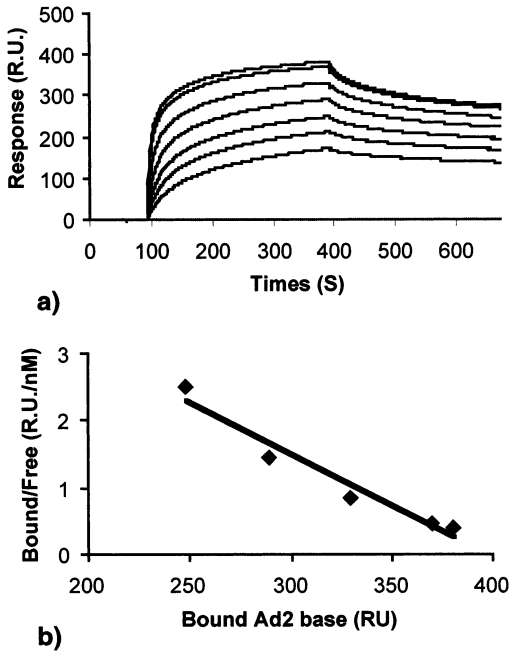

FIGURE 5: Binding of Ad2 penton base to immobilized GST-WWP1 fusion protein. (a) Overlay of sensorgrams obtained with the injection of Ad2 penton base at (top to bottom) 1000, 800, 400, 200, 100, 50, 25, and 0 nM injected at a flow rate of 10 μL/min during 5 min over a surface containing 450 RU of GST-WWP1. Similar injection over a surface containing GST only did not produced any binding response (not shown). (b) Scatchard analysis. The levels of bound Ad2 penton base obtained at the end of the injections of five highest base concentrations have been extracted from the sensorgrams divided by the concentration of unbound analyte (bound/free in RU/nM) and plotted against bound penton base (in RU).

Figure 1), all together accounting for a particularly complex binding mechanism. To get an affinity value independent of the kinetic analysis, equilibrium data were extracted from the sensorgrams and plotted according to the Scatchard representation. The resulting straight line ($R^2 = 0{,}95$) enables us to estimate an affinity of $K_d = 65$ nM (Figure 5b) characterizing the Ad2 penton base binding to WWP1.

In the second series of experiments, the Ad2 penton base was immobilized and allowed to bind either of GST-protein ligases injected over the surface. All proteins bound well to the immobilized penton base; however, the sensorgrams did not reach equilibrium (data not shown). Since in these cases the binding curves could not be adequately described by any of the models available in the evaluation software, no affinity value could be calculated. The comparison of binding curves obtained for all three Ulps shows nevertheless that WWP1 interacts more strongly with Ad2 base protein than the two other ligases.

DISCUSSION

Several recently solved structures of the WW domains (*21*−*25*) show a compact, slightly bent three-stranded antiparallel β-sheet. The aromatic groove of WW domain composed of conserved Tyr and Trp forms the recognition site against which packs polyproline helix of the ligand. The mechanism of ligand recognition by WW domain seems to focus on proline N-substitution, which excludes other amino acids, since proline is the only natural N-substituted amino acid. As a consequence, it allows a highly selective recogni-

tion of rather low affinity (*26*). Some functional roles of the WW domain have been described even that it is clear that it mainly serves as an adapter in forming functional complexes containing at least three proteins. The domain is present in a variety of cytoskeletal and signal-transducing proteins, which might be associated with plasma and organellar membranes. Hypotheses concerning functions of these domains include substrate recognition, control of subcellular localization, involvement in nuclear transport, control of epithelial sodium channels, transcriptional regulation in erythroid and possibly other cells, retrovirus budding, cell adhesion, and signal transduction in brain *(27*−*35*). Recently, the transcription-promoting activity has been demonstrated for the isolated WW domains of Yap, Pin1, and Npw38 (*33*), and the co-activating role has been shown for the Yap WW domain interacting with the polyoma enhancer binding protein 2 (PEBP2) transcription factor in the two-hybrid system (*36*).

In this study we found that penton base protein of human adenoviruses is able to interact through its first PPxY motif with three human Upls containing partner WW domains and belonging to the E3 group of Nedd4-like enzymes, characterized by the presence of the catalytic HECT domain. This interaction was discovered by screening the cDNA expression library with the base protein and confirmed by the gel overlay technique and immunoprecipitation. The recruitment of three Upls, WWP1, WWP2, and AIP4, has been shown for two physiologically different virus serotypes, Ad2 and Ad3. Moreover, the interaction of the three Upls with penton base is specific, as these enzymes do not bind the adenovirus fiber protein. Using mutants of the Ad2 base protein, we observed that the first N-terminal xPPxY motif in the base protein sequence is indispensable for the interaction.

For the more detailed characterization of this interaction, we used SPR techniques. However, the interaction between base protein and ubiquitin−protein ligases cannot be described in simple kinetic terms. Assuming that only first xPPxY in base protein is involved in this interaction, five possible sites of interaction will be available since the base protein is pentameric. In addition, each of three interacting ligases contains multiple WW domains, which complicates even more the interaction. Furthermore, the domain content of used proteins is different. WWP1 (aa 258−528) consist of four WW domains only: WWP2 (aa 11−776) is the most complete protein with C2, WW and nearly total HECT domains, whereas AIP4 (aa 124−697) in addition to four WW domains encompasses nearly half of HECT domain (see Figure 1A). The presence of C2 or HECT domain could influence the folding of WW protein and so possibly also the interaction. The complexity of the binding mechanisms that characterized these interactions prevented extraction of quantitative values, in particular from a kinetic point of view. Nevertheless, in the case of WWP1/Ad2 penton base complex, the binding curve reached equilibrium. From these data an apparent equilibrium dissociation constant of 65 nM was estimated (Figure 6B). As explained above, this value is necessarily approximate, but it gives an idea of the concentration range in which the interaction takes place.

The human genome encodes over 20 and the genome of *Caenorhabditis elegans* encodes at least seven different HECT domain proteins (*23, 37*). They seem to be quite ubiquitous proteins which, however, are not necessarily redundant in their function, since knock-out in protein similar to human WWP1 results in embryonic lethality of *C. elegans* (*23*) which shows that other HECT domains-containing protein cannot substitute for it. Similarly, the HECT domain of E6-AP cannot be replaced by other HECT domains in the ubiquitination of p53 (*37*). By library screening we identified WWP1, WWP2, and AIP4 but not other protein−ubiquitin ligases. However, the interaction specificity with base protein does not to seem to be very narrow since Nedd4, another member of this family of Upls, is able to interact with the base protein in vitro (Figure 3). Interestingly, the same three ligases are the partners of atrophin-1, a protein containing 5 xPPxY motifs (*14*, *38*), which appears to be causal in the hereditary neuro-degenerative disorder termed dentatorubral-pallidoluysian atrophy (DRPLA) linked to an abnormal glutamine expansion *(39*, *40*).

Specific functions have not been attributed yet to these three Upls. WWP1 seems to be involved in the interactions with xPPxY domains-containing transcription factors such as hematopoietic transcription factor NF-E2 and lung Kruppel-like transcription factor, modulating their activity (*30*, *41*). For WWP2, it has been shown that overexpression of its dominant negative mutants increased surface expression and currents of ClC chloride channel (*42*). AIP4 might have some role in inflammation and the regulation of epithelial and haematopoietic cell growth, as has been shown for the murine AIP4 homologue gene, itch, that it is disrupted in mice with the genetic disease characterized by multiple inflammatory disorders (*43*). However, none of these studies showed the enzymatic activity of these Upls resulting in the ubiquitination of their partners.

Some enveloped viruses have been shown to interact with Upls. LMP2A, a latent membrane protein of Epstein−Barr, is able to interact with WWP2, AIP4, and Nedd4 (*44*). This raft-associated viral protein contains in its cytoplasmic amino terminus two PPxY motifs, both of which seem to be necessary for the interaction. LMP2A serves as a scaffold able to recruit Upls and B-cell signal transducing molecule lyn, which results in the degradation of both LMP2A and lyn, which in turn modulates B-cell signal transduction (*44*, *45*). The polyubiquitination of specific viral protein resulting from the interaction of this protein with HECT-type Upls Nedd4 and yeast Rsp5 has been shown for Ebola and Marburg viruses (Filoviridae) as well as for vesicular stomatitis and rabies viruses (Rhabdoviridae) (*46, 47*). Ubiquitination is dependent on xPPxY motif in the viral protein and its disruption abolishes the interaction and results in decreased virus budding.

In contrast, nothing is known about the interaction of the nonenveloped viruses with E3 ubiquitin-protein ligases. Our communication is the first demonstration of the interaction of the adenovirus protein responsible for the virus internalization, penton base, with this class of enzymes. Further studies will show if base protein undergoes ubiquitination by the action of E3 enzymes, resulting in modulation of virus internalization or if the base protein serves as a scaffold permitting ubiquitination of the third, viral or cellular, partner.

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