A Proteomic Approach To Identify Candidate Substrates of Human Adenovirus E4orf6-E1B55K and Other Viral Cullin-Based E3 Ubiquitin Ligases[▽]

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It has been known for some time that the human adenovirus serotype 5 (Ad5) E4orf6 and E1B55K proteins work in concert to degrade p53 and to regulate selective export of late viral mRNAs during productive infection. Both of these functions rely on the formation by the Ad5 E4orf6 protein of a cullin 5-based E3 ubiquitin ligase complex containing elongins B and C. E1B55K is believed to function as the substrate recognition module for the complex and, in addition to p53, Mre11 and DNA ligase IV have also been identified as substrates. To discover additional substrates we have taken a proteomic approach by using two-dimensional difference gel electrophoresis to detect cellular proteins that decrease significantly in amount in p53-null H1299 human lung carcinoma cells after expression of E1B55K and E4orf6 using adenovirus vectors. Several species were detected and identified by mass spectroscopy, and for one of these, integrin $\alpha 3$, we went on in a parallel study to confirm it as a *bone fide* substrate of the complex (F. Dallaire et al., J. Virol. 83:5329–5338, 2009). Although the system has some limitations, it may still be of some general use in identifying candidate substrates of any viral cullin-based E3 ubiquitin ligase complex, and we suggest a series of criteria for substrate validation.

During the past decade protein degradation has become increasingly recognized as a critical mechanism by which cells regulate a number of fundamental processes (reviewed in references 37, 57, and 59). Degradation frequently involves one of a variety of E3 ubiquitin ligase complexes in which a substrate recognition component introduces the target protein for ubiquitination and subsequent degradation by proteasomes (reviewed in reference 59). Several types of these complexes involve a member of the cullin family (reviewed in reference 59), and a considerable amount of information is known about those containing Cul2 or Cul5. In these cases the substrate recognition module is linked via elongins B and C to a sub-complex containing Cul2 or Cul5 and the RING protein Rbx1 (34, 58). This complex interacts with an E2 conjugating enzyme, often either Cdc34 or Ubc5, to conjugate ubiquitin chains to the substrate (44). With both Cul2- and Cul5-based complexes interaction with elongins B and C occurs via a single BC box sequence (42). The presence of either Cul2 or Cul5 is generally determined through the presence in the substrate recognition protein of specific Cul2- or Cul5-box sequences (35).

Many viruses have evolved to encode products that inhibit cellular E3 ligases to protect important viral or cellular species or, in some cases, that hijack these cellular complexes to target key substrates for degradation, including components of cellular host defenses, to facilitate the infectious cycle (reviewed in reference 4). These strategies are quite common among the small DNA tumor viruses (7), and one of the most

studied examples is the complex formed by the human adenovirus E4orf6 and E1B55K proteins. These proteins have been known for some time to interact (69) and to reduce the levels of the p53 tumor suppressor in infected cells (14, 47, 48, 62, 72, 73). In addition, they were shown to function in concert to block nuclear export of cellular mRNAs late in infection (2, 6, 29, 60) and to enhance the selective export of late viral mRNAs (2, 26, 29, 60, 78). Our group showed that the human adenovirus serotype 5 (Ad5) E4orf6 product interacts with several proteins (13), including components of what was at the time a unique Cul5-based E3 ubiquitin ligase containing elongins B and C and Rbx1 that degrades p53 (61). Curiously, Ad5 E4orf6 contains three BC boxes that we believe make it highly efficient in highjacking cellular elongin B/C complexes (8, 17, 41). The mechanism of selective recruitment of Cul5 by the Ad5 complex remains unknown as E4orf6 lacks a Cul5-box (17, 41). E1B55K seems to function as the substrate recognition module and, of considerable interest, both its association with E4orf6 and induction of selective late viral mRNA transport was found to depend on formation of the E3 ubiquitin ligase complex, suggesting that additional degradation substrates must exist (8, 9). This idea is not surprising since viruses, especially the small DNA tumor viruses, often evolve gene products that target multiple critical cellular pathways (32). In fact two additional E1B55K-binding substrates have now been identified, Mre11 from the MRN DNA repair complex (8, 75), and DNA ligase IV (3), the degradation of which prevent formation of viral genome concatemers, thus enhancing packaging of progeny DNA. Degradation of p53 has been suggested to promote enhanced progeny virus production by preventing the early apoptotic death of infected cells due to the stabilization of p53 by the viral E1A products (reviewed in reference 66). Nevertheless, degradation of these substrates seems unlikely to explain the observed effects on mRNA transport, suggesting that

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still more substrates remain to be identified. Although the studies described in the present report were in part launched to identify such substrates, as will become clear below, these targets remain to be identified.

In an attempt to identify new substrates of the Ad5 E4orf6/E1B55K E3 ubiquitin ligase complex, a proteomics-based approach was initiated involving two-dimensional difference gel electrophoresis (2D-DIGE) analysis and subsequent mass spectrometry. As is well known, this technique has the advantage of improved sensitivity and accuracy provided by its ability to separate samples under two different conditions on a single gel together with a reference sample, thus reducing significantly the analytical coefficient of variation. It allows the quantification of differentially abundant proteins in complex biological samples, providing a tool to detect decreases in the levels of proteins in the cell due to targeted proteolytic degradation. We report here our attempts to identify substrates of the Ad5 E4orf6/E1B55K complex by comparing the proteomes of human non-small cell lung carcinoma H1299 cells expressing, by means of adenovirus vectors, both E1B55K and E4orf6 proteins or E4orf6 protein alone. Ten candidate proteins were identified, most having functions seemingly unrelated to our current understanding of the roles of the E4orf6/E1B55K complex. At least three showed promising features characteristic of substrates, and one has now been confirmed in a parallel study to be a bona fide E4orf6/E1B55K substrate (20). We suggest that this approach could be utilized to identify candidate substrates, among relatively high abundance proteins, that are degraded by other viral cullin-based E3 ubiquitin ligase complexes.

MATERIALS AND METHODS

Cells, viruses, viral vectors, antibodies, and plasmids. Human non-small cell lung carcinoma H1299 cells (ATCC CRL-5803), which are p53-null, were grown in Dulbecco modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum (HyClone) at 37°C in 5% CO₂. The H1299 Cul5 knockdown cell line (H1299/Cul5 KD) and the H1299 control cell line (H1299 pCDNA3) were described previously (17) and grown as with the H1299 cells but with the addition of 1 mg of puromycin/liter.

The adenovirus vectors AdE1B55K and AdE4orf6 used to express the Ad5 E1B55K and E4orf6 proteins and the wild-type (wt) Ad5 strain H5pg4100 have been described previously (28, 43, 62).

The following antibodies were used: rabbit anti-E4orf6 (1807) (13), rabbit anti-Mre11 (catalog no. pNB 100-142; Novus Biologicals, Inc.), rabbit anti-Cul5 H-300 (catalog no. sc-13014; Santa Cruz) and rabbit anti-tryptophanyl tRNA synthetase (catalog no. ab31536; AbCam); mouse anti-E1B55K clone 2A6 (Western blots) (69) or rat monoclonal 7C11 (immunofluorescence) (31), mouse anti-integrin α 3A clone 29A3 (catalog no. MAB2290; Millipore), mouse anti-actin clone C4 (catalog no. 691001; MP Biomedicals), mouse anti-cortactin clone 4F11 (a generous gift from J. Lavoie, Université Laval), mouse anti-MEK2 clone 96 (catalog no. 610235; BD Biosciences), mouse anti-dynactin clone 1 (catalog no. 610473; BD Biosciences), mouse anti-lamin B2 clone LN43 (catalog no. MAB3536; Millipore), mouse anti-dynein clone 70.1 (catalog no. D5167; Sigma), mouse anti-hnRNP-K/J clone 3C2 (catalog no. R8903; Sigma), mouse anti-c-Myc clone 9E10 (catalog no. MMS-150R; Covance), and mouse anti-E2A DNA-binding protein (DBP) B6-8 (64). Secondary antibodies conjugated to horseradish peroxidase for detection in Western blotting analyses were goat anti-mouse and goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). Secondary antibodies for immunofluorescence-coupled Alexa fluorophores included anti-mouse Alexa 488 (A-11029) and anti-rat Alexa 594 (A-11007) (both from Invitrogen).

The pCDNA plasmid pCDNA3-E1B55K has been described previously (49). pCDNA3-GFP-FLAG-cortactin and pCH17-Myc-cortactin was a generous gift of J. Lavoie (Université Laval).

Cyanine fluorophores (Cy2, Cy3, and Cy5), immobilized pH gradient (IPG)

strips, IPG buffer, urea, thiourea, dithiothreitol (DTT), and iodoacetamide were obtained from GE Healthcare. CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} was obtained from BioShop. ReadyStrip IPG Strips and Bio-Lyte 3/10 Ampholyte were from Bio-Rad Laboratories. DAPI (4',6'-diamidino-2-phenylindole; Invitrogen), paraformaldehyde (Electron Microscopy Sciences), and Lipofectamine 2000 reagent (Invitrogen) were also used.

Infections and sample preparation. For 2D-DIGE analysis and time course validation, infections were performed as follows. H1299 cells were plated in six-well dishes and infected for 90 min with adenovirus vectors or wt virus diluted in infection medium (0.2 mM CaCl₂, 0.2 mM MgCl₂, and 2% serum in phosphate-buffered saline [PBS]) before the addition of normal growth medium. For the 2D Western blotting validation, a similar procedure was used with the wt virus except that cells were plated in 10-cm-diameter dishes. A multiplicity of infection (MOI) of 35 PFU per cell was used for viral vectors, whereas MOIs of 20 fluorescence-forming units/cell for wt virus were used. Cells were incubated for various periods and harvested according to the procedure described below.

Cells were washed with PBS and removed at different times postinfection (p.i.) by incubation for 5 min with gentle agitation with 0.53 mM EDTA. Cells were collected by centrifugation, and the pellets were incubated for 5 min on ice in CHAPS buffer (4% CHAPS, 30 mM Tris-HCl [pH 8.5], 50 mM NaCl, plus inhibitors [protease inhibitor cocktail; Sigma], 1 mM Na₃VO₄, 10 mM sodium PP_i, 10 mM NaF). Cells were then lysed by sonication, and protein concentrations of the lysates determined by using Bio-Rad DC protein assay reagents according to the manufacturer's protocol. Extracts were brought to 7 M urea–2 M thiourea (GE Healthcare Biosciences), clarified by centrifugation at 13,000 × g for 5 min, and protein concentrations were recalculated. Samples were maintained at –80°C until used.

2D-DIGE and image acquisition. 2D-DIGE analysis was performed as follows. Three independent sets of infections were carried out (biological replicates), with each set consisting of one control sample well of cells infected with the adenovirus vector AdE4orf6 alone and one sample well of cells infected with both AdE4orf6 and AdE1B55K. Cells were harvested at 24 or 48 h p.i., and cell extracts were prepared in CHAPS buffer. The following procedures were carried out by using the CIAN 2D-DIGE platform (McGill University). Cell extracts were labeled with Cy2, Cy3, and Cy5 fluorophores according to the manufacturer's procedure (GE Healthcare). Portions (50 µg) of protein (~10 mg/ml) were minimally labeled with 400 pmol of dye for 30 min in the dark at 4°C. Samples in each infection set were labeled with either Cy3 or Cy5, and dye swapping was also performed to minimize labeling-dependent bias. Internal control samples containing protein (50 µg) of each of the samples in the study were labeled with Cy2 to serve as a reference for spot normalization. The reaction was stopped by the addition of 10 mM lysine (1 µl/50 µg of protein) and incubation for 10 min at 4°C. Labeled samples from each set were combined (with 50 µg of Cy2-labeled control) in 2D-DIGE rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.002% bromophenol blue, 0.5% IPG buffer [pH 4 to 7], and 18 mM DTT).

First-dimensional isoelectric focusing (IEF) of labeled proteins was performed on an Ettan IPGPhor II electrophoresis system (GE Healthcare) using 24-cm IPG strips, pH 3–11NL or pH4–7 (GE Healthcare). All steps were performed in the dark to avoid bleaching of the dyes. Strips were rehydrated with Cy-labeled samples at 30 V 50 µA/strip at 20°C for 15 h, followed by the addition of a small piece of hydrated IPG electrode strip paper between the strip and the electrode to limit electroendosmosis. Focusing was continued for 0.5 kV · h and stepped up to 500 V, followed by a 0.8-kV · h gradient stepped up to 1,000 V, a 13.5-kV · h gradient stepped up to 8,000 V, and 25-kV · h (pH 3 to 11NL) or 37.5-kV · h (pH 4 to 7) stepped up to 8,000 V. Proteins in the IPG strips were reduced and alkylated with 1% DTT and 4% iodoacetamide, respectively, in equilibration buffer (50.25 mM Tris-HCl [pH 8.8], 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate [SDS], and 0.00125% bromophenol blue) for 15 min. The second-dimension separation was performed by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% polyacrylamide gels on an Ettan DALT VI electrophoresis system (GE Healthcare) cooled at 15°C with a slow entry phase at 30 V for 1 h, followed by 1 W/gel until the bromophenol blue dye reached the bottom of the gel. The 2D gels were scanned on a Typhoon 9400 imager (GE Healthcare) at a 100-µm resolution with $\lambda_{\text{ex}}/\lambda_{\text{em}}$ values of 488/520, 532/580, and 633/670 nm for Cy2, Cy3, and Cy5, respectively. The ImageQuant software version was used to preview gel images. Photomultiplier tube voltages were set to ensure that intensities were within the linear range of the instrument and to correct minor errors in protein loadings.

Gel imaging and analysis. Each gel image was cropped to remove undesired regions and gel artifacts by using the ImageQuant software. DeCyder Software (v6.5; GE Healthcare) was used to perform intra-/intergel matching, statistical analysis (Student *t* test [*P* < 0.05]), and spot filtering. The differential in-gel analysis module was used to perform spot detection, spot volume quantitation,

and volume ratio normalization of different samples in the same gel. Exclusion filters and manual detection of spots were applied to each gel in order to obtain the most representative gel image. Gels were exported to the biological variation analysis (BVA) module. Fifteen spots were manually landmarked to allow the software to perform intergel matching. Manual editing was done to ensure correct matching of spots, to get rid of streaks and speckles, and to reduce the spot-matching quality value as much as possible. Two groups were defined for the analysis: the control group consisting of samples from cells expressing E4orf6 alone and those from cells expressing both E4orf6 and E1B55K. Protein spots were assigned a "protein-of-interest" (POI) status based on the following criteria: a volume ratio change of ≤ -1.0 (decreased) or ≥ 1.0 (increased) in at least two of the three biological replicates and a statistically significant quantitative change and Student *t* test at 95% statistical confidence ($P < 0.05$). The "PICK" status was assigned to the POI spots with a volume ratio change of ≤ -1.2 or ≥ 1.2 , visual confirmation of the change, and clear separation from other spots for accurate spot excision.

Protein identification. Preparative 2D gels for protein identification were used to analyze 500 to 1,000 μ g of unlabeled protein samples as described above. Samples were rehydrated individually on 13-cm IPG strips (pH 4 to 7; GE Healthcare) for the first-dimension IEF according to the following procedure: rehydration at 30 V 50 μ A/strip 20°C for 15 h, addition of hydrated IPG electrode strip paper between the strip and the electrode, 0.5 kV $\cdot$ h stepped up to 500 V, a 0.8-kVh gradient stepped up to 1,000 V, a 11.3-kV $\cdot$ h gradient stepped up to 8,000 V, and 5.4 kV $\cdot$ h stepped up to 8,000 V. The second-dimension SDS-PAGE was performed by using a Hoefer Ruby SE600 electrophoresis system (GE Healthcare) after reduction and alkylation of disulfide bonds using a slow entry phase at 30 V for 1 h at room temperature, followed by a constant 50 to 75 V at 4°C until the dye front exited the gel. The 2D gels were silver stained by a procedure adapted from Blum et al. (10).

Protein spots were manually excised from the gel. All of the following procedures were performed by the McGill University and Genome Quebec Innovation Center. In-gel trypsin digestion was performed on a MassPrep robotic workstation (Perkin-Elmer), and digested peptides were processed by using tandem mass spectrometry on an LC-QToF Micro apparatus (Waters). Subsequent identification of peptides and proteins from complex mixtures was done by using Mascot v2.1 (Matrix Science) and the following parameters: tandem mass spectrometry ion search, trypsin digestion, carbamidomethyl fixed modification, oxidation variable modification, monoisotopic mass values, unrestricted protein mass, a peptide mass tolerance of ± 0.5 to 1.5 Da, a fragment mass tolerance of ± 0.5 to 0.8 Da, one missed cleavage, and the NCBI nonredundant database as reference.

Immunoblotting of 1D and 2D gels. Equal amounts of unlabeled protein samples prepared as described above were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore) that had been blocked using 5% skim milk. Primary antibodies were added on membranes for 2 to 3 h at room temperature or overnight at 4°C. Membranes were washed with PBS containing 0.1% Tween 20, and the secondary antibody was added for 1 h at room temperature. Detection was performed using the Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer).

Gels for 2D Western blotting analysis were prepared as described above with 750 μ g of protein from unlabeled samples rehydrated individually on Bio-Rad ReadyStrip strips (pH 4 to 7), 7 cm, in 2D Bio-Rad rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.002% bromophenol blue, 1 $\times$ Bio-Lyte 3/10 Ampholyte, 18 mM DTT). The first-dimension IEF was performed by using a Protean IEF Cell (Bio-Rad Laboratories). Active rehydration was done at 50 V 50 μ A/strip at 20°C for 15 h, followed by the addition of hydrated IPG electrode strip paper between the strip and the electrode. Focusing was continued for 30 min with a slow increase to 500 V and then a 9-kV $\cdot$ h rapid increase to 4,000 V. After reduction and alkylation of disulfide bonds the second-dimension SDS-PAGE was performed in a Mini-Protean 3 electrophoresis system (Bio-Rad Laboratories) using a slow entry phase of 30 V for 1 h at room temperature and then 90 V until the dye front exited the gel. The 2D gels were transferred to polyvinylidene difluoride membranes (Millipore) as described above and blotted accordingly.

DNA transfections. Cells were transfected with pCDNA3-E1B55K and either pCDNA3-GFP-FLAG-cortactin or pCH17-Myc-cortactin plasmid DNAs using the Lipofectamine 2000 reagent according to the manufacturer's recommendations. The final amount of DNA per well was equalized by using the plasmid vector pCDNA3.

Immunofluorescence microscopy. For colocalization experiments, H1299 cells were transfected with pCDNA3-E1B55K and either pCDNA3-GFP-FLAG-cortactin or pCH17-Myc-cortactin plasmid DNAs. Cells grown on coverslips and transfected as described above were washed once in PBS and fixed with 4% paraformaldehyde diluted in PBS for 15 min at room temperature. Cells were

washed twice with PBS and permeabilized by 0.5% Triton X-100 in PBS for 15 min at room temperature. Triton X-100 was then replaced by TBS-BG blocking buffer (20 mM Tris-HCl [pH 7.4], 137 mM NaCl, 3 mM KCl, 1.5 mM MgCl₂, 0.05% Tween 20, 66 mM glycine, 5 g of bovine serum albumin/liter) for 20 min at room temperature. Cells were incubated for 2 h at room temperature with primary antibody diluted in PBS and then washed two times for 5 min each time with PBS, followed by incubation for 1 h at room temperature in the dark with secondary antibody diluted in PBS. Coverslips were washed again and incubated for 10 min at room temperature in the dark with DAPI diluted in PBS. Cells were washed twice with PBS and once with water before being mounted on slides in mounting media (Immu-Mount; Thermo Scientific). Images were acquired at the McGill Life Sciences Complex Imaging Facility using an Axiovert 200M fully motorized confocal microscope and the Zeiss LSM 5-Pa software.

RESULTS

Establishment of standard protocol to identify E4orf6/E1B55K substrates. In previous work we studied the activity of the Ad5 E4orf6/E1B55K E3 ubiquitin ligase complex using p53-null H1299 cells transfected with plasmid DNAs expressing these Ad5 proteins (8, 9, 61, 63). These studies have involved measuring the degradation of both exogenously added substrate (p53) and endogenous substrates (Mre11). In the present study we wanted to identify new substrates of this ligase complex within the endogenous proteome of H1299 cells by using 2D-DIGE, which allows separation and visualization of abundant cellular proteins to permit evaluation of variations in protein levels on gels. The power of the 2D-DIGE technology relies on the reproducibility of the results combined with strong statistical analysis to provide clear, reliable, and easy-to-use data. Figure 1A illustrates the strategy used. Identification of substrates in wt virus-infected cells is complicated by the fact that, during the course of infection, host protein levels may change for a variety of reasons, including those that are unrelated or only indirectly linked to the specific actions of E4orf6 and E1B55K. Thus, it was critical to examine changes in protein levels in the presence of E4orf6 and E1B55K alone, in the absence of other viral gene products. To optimize the chances of detecting putative substrates, we wanted to express Ad5 E4orf6 and E1B55K proteins at high levels in every cell in the culture, conditions that were not possible by DNA transfection but that could be achieved after infection with the adenovirus vectors AdE4orf6 and AdE1B55K that in large measure express only these viral gene products (27). We performed an initial characterization by examining the degradation of the known substrate Mre11 and eventually found that, at an MOI of 35 PFU/cell for each of the vectors AdE4orf6 and AdE1B55K, virtually all of the Mre11 was degraded by 24 h, whereas little degradation was observed with AdE4orf6 alone (data not shown).

We initially evaluated the protein profile at 24 h p.i. using gels for the first dimension in the pH 3 to 11 range; however, because most of the resolved species were concentrated within pH 5 to 8, we focused on this region by using a pH 4 to 7 range IPG strip. Although our objective was to look at proteins that decreased in the presence of E4orf6 and E1B55K, we also noted a number of species that clearly increased in intensity. The latter could represent proteins that were either produced at higher levels or stabilized by E4orf6 and/or E1B55K proteins directly or as a consequence of the degradation by the E4orf6/E1B55K ligase of substrate species that reduced their synthesis and/or stability. Such species may be of interest (see

FIG. 1. Experimental design and analysis. (A) Flow chart showing the details on the 2D-DIGE strategy for the identification and validation of the candidate substrates. The labeling and mixing strategies are shown in the top right panel. (B) Abundance of protein spots at each step of the selection criteria based on DeCyder analysis. The POI status has been assigned to spots with a volume ratio change of ≤ -1.0 (decreased) or ≥ 1.0 (increased) in at least two of the three biological replicates and a statistically significant quantitative change and Student *t* test at 95% statistical confidence ($P < 0.05$). Spots with the “PICK” status are POI spots having a volume ratio change of ≤ -1.2 or ≥ 1.2 , visual confirmation of the change, and clear separation from other spots for accurate spot excision. The average (AVG) ratio criteria corresponds to further manual filtering based on the AVG ratio and the number of PICK spots identified. Mass spectrometry candidates are species that could be submitted for mass spectrometry identification. D, decreased; I, increased; NL, nonlinear.

below). As shown in Fig. 1B, a low number of species (spots) detected at 24 h p.i could be assigned the so-called PICK status: i.e., having a volume ratio change of ≤ -1.2 or ≥ 1.2 in at least two of the three 24-h biological replicate; a Student *t* test at 95% statistical confidence ($P < 0.05$), visual confirmation of the change, and clear separation from other spots for

accurate spot excision. In addition, the maximum average volume ratio was only -1.6 for PICK spots showing decreases and 1.6 for the PICK spots showing increases. We thus considered for further identification only the spots reproducibly assigned as PICK in at least two of the three studies summarized in Fig. 1B done on extracts prepared at 24 h p.i. Because the degradation of some substrates may take longer than 24 h, we performed another 2D-DIGE study using extracts from cells harvested at 48 h p.i. Figure 1B shows that a very large number of spots could be assigned as PICK, and thus we only considered for further study those with the highest average ratio, i.e., below -1.5 for spots showing decreases and above 2.9 for spots showing increases. Only spots showing these characteristics and identifiable on preparative silver-stained 2D gels from the 24- and 48-h studies were extracted for identification by mass spectrometry, as summarized in the last column of Fig. 1B.

Analysis and identification of putative E4orf6/E1B55K substrates. Figure 2A shows the master 2D gel generated from the study involving extracts of cells harvested at 24 and 48 h p.i., indicating species identified by the Decider software. The 13 PICK spots of the 48-h-p.i. study showing decreases have been annotated in yellow, and the 3 PICK spots of the 24-h-p.i. studies are indicated in blue. These species all were present in a region containing proteins in the pH 4 to 7 range and had molecular masses from approximately 45 to 120 kDa. Examples of images representing 3D images of spot intensity for some of increased or decreased spots are shown in Fig. 2B. Material containing 16 of these species was excised from preparative gels and analyzed by mass spectroscopy, and Table 1 shows the 12 of the 16 species showing decreases that were identified. Of the known substrates of the E4orf6/E1B55K complex, p53 of course was not identified since it is not expressed in H1299 cells; however, Mre11 should have been, and reasons why it was not detected, and thus some of the limitations of this approach, are presented in the Discussion. Of the 29 species showing increases in the 48-h-p.i. study, only the 5 with the highest average ratio were analyzed by mass spectrometry, together with 7 species from the 24-h-p.i. studies. Eleven of these proteins were successfully identified and are shown in Table 2. The reasons for the failure to identify the other species may include problems in peptide elution, low amounts of protein, or scoring below the threshold in the Mascot search engine. Tables 1 and 2 indicate that species from both classes all are relatively high abundance proteins (see Discussion) and are implicated in a wide range of cellular pathways. Spots 1575, 1580, and 1587 were all identified as being heterogeneous nuclear ribonucleoprotein (hnRNP) K isoform B, perhaps illustrating the resolution capacity of the approach. Also of potential interest was the observation that the five proteins showing the largest increases in level were either heat shock proteins or pyruvate kinase, suggesting perhaps that degradation by the ligase or expression of E1B55K may induce a stress response and/or production of ATP. Clearly, more studies will be required to determine whether these processes are indeed affected.

Preliminary validation of putative E4orf6/E1B55K substrates. It was possible that reductions in species could be explained by changes in posttranslational modifications rather than from degradation since such changes often affect gel migration. To evaluate this possibility and to determine whether

FIG. 2. Analysis by 2D-DIGE. (A) Representative 2D-DIGE control Cy2 gel image from the 48 h p.i. study run on 24-cm pH 4 to 7 IPG strips (first dimension) and SDS-10% PAGE (second dimension). The positions of the 16 decreased candidates for identification by mass spectroscopy have been annotated. Numbers correspond to the master gel number of each spot in the respective studies (yellow, 48-h-p.i. study; blue, 24-h-p.i. studies). (B) Examples of DeCyder software generated 3D images of some species that either decreased or increased in amount in H1299 cells expressing E4orf6 and E1B55K versus E4orf6 protein alone. 3D peaks representing spots of interest (master numbers on the left) in each panel set is circled in red. The AVG ratio for each spot is indicated on the right.

these candidate species actually decrease in level during productive viral infection, two different initial validation assays were conducted on eight of the identified proteins. First, the levels of integrin $\alpha 3$, lamin B2, cortactin, dynactin, dynein, MEK2, hnRNP K, and TrpRS were examined by Western blotting analysis of extracts from wt Ad5-infected H1299 cells

harvested at various times up to 72 h p.i., using appropriate antibodies, with Mre11 and the early viral protein DBP serving as controls. Figure 3 shows that the infectious cycle proceeded well, as indicated by the appearance by 16 h p.i. of DBP, and that Mre11 indeed decreased dramatically in amount, as shown previously (9). Of the eight proteins tested, integrin $\alpha 3$ subunit

TABLE 1. Summary of the proteins exhibiting decreases in response E4orf6 and E1B55K

Master no.	Protein AC	Name	Alternate name	Theoretical		<i>p</i> ^b	Mascof score	Coverage (%) / no. of peptides	Avg ratio	Function
				Size (Da)	pI					
386 ^a	AAH71583	DCTN1 protein	Dynactin 1	127,370	5.31	0.049	335	8/7	-1.34	Cofactor for the dynein microtubule-based motor protein
1097	AAB26248	EMS1 gene product	Cortactin isoform a	61,770	5.24	0.004	307	14/7	-3.06	Implicated in actin cytoskeletal organization
1238	AAK67638	Cytoplasmic dynein intermediate chain		68,655	5.16	0.0002	124	5/3	-2.89	Microtubule-based motor protein
1587	NP_112552	hnRNP K isoform B		51,230	5.39	0.0002	795	35/14	-2.34	Control of mRNA translation and mRNA stability; chromatin remodeling; transcription, splicing, and translation processes
1890	AAA35763	Cytokeratin 8		53,529	5.52	6.21E-05	1170	44/20	-1.89	Member of the intermediate filament gene family
1575	NP_112552	hnRNP K isoform B		51,230	5.39	0.0015	730	29/12	-1.77	Cell surface transmembrane glycoproteins; adhesion; receptors in cell-ECM interactions;
612	BAA00845	VL-A-3 alpha subunit	Integrin $\alpha 3$ subunit	114,517	6.13	0.0269	109	2/2	-1.69	link matrix proteins to the cytoskeleton
1496	NP_116126	Lamin B2		67,762	5.29	0.0020	1199	33/21	-1.62	Intermediate filament; mechanical stability to the nucleus; chromatin organization; might be involved in DNA replication
2141	NP_109587	Mitogen-activated protein kinase kinase 2	MAPKK2 or MEK2	44,681	6.12	0.0004	169	16/6	-1.61	Regulation of mammalian cell cycles through mitogen-activated protein kinase pathway
1580	CAA51267	Transformation upregulated nuclear protein	hnRNP K isoform B	51,325	5.13	0.0058	498	26/10	-1.58	
1845	1ULH_B	Chain B, a short peptide insertion crucial for angiostatic activity of human tryptophanyl-tRNA synthetase	Tryptophanyl-tRNA synthetase isoform a	44,991	6.41	0.0002	708	33/11	-1.55	Links protein synthesis to signal transduction pathways; protein biosynthesis and angiogenesis
544	P12109	Collagen alpha-1(VI) chain precursor		109,621	5.29	0.0113	117	2/2	-1.55	Constituents of the subendothelium that determine the thrombogenicity of the vessel wall

^a Spot coming from the 24-h studies.
^b *As* determined by the Student *t* test.

TABLE 2. Summary of proteins exhibiting increases in response to E4orf6 and E1B55K

Master no. ^a	Protein AC	Name	Alternate name	Theoretical		<i>P</i> ^b	Mascot score	Coverage (%) / no. of peptides	Avg ratio
				Size (Da)	pI				
595/521*	AAI06072	Transforming, acidic coiled-coil containing protein 3		91,159	4.97	0.00177	473	15/8	1.53
2302/2292/1512*	NP_115754	Eukaryotic translation elongation factor 1 delta isoform 1	EEF1D protein	71,834	4.95	0.00646	545	19/10	1.32
	CAA25833	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	36,202			491	30/7	1.32
2334/2321*	AAH00678	EEF1D protein		61,244	4.95	0.01441	314	12/5	1.29
2220/2198/1465*	CAA34809	B23 nucleophosmin (280 aa)		31,090	4.64	0.00214	840	50/11	1.26
2260/1497*	NP_003012	Small glutamine-rich tetratricopeptide (SGT)	Annexin I	34,270	4.81	0.00561	385	30/8	1.26
	AAV38720	Annexin A1	Annexin I	38,918	6.57		239	26/7	1.26
2429/2373/1608*	AAH11792	Peroxisomal enoyl-coenzyme A hydratase-like protein		36,647	8.16	0.00684	505	26/8	1.27
1635	CAA39849	Pyruvate kinase		58,411	7.58	3.1E-04	326	18/8	7.6
842	AAC18044	Antigen NY-CO-25	Heat shock 105-kDa protein	99,347	5.29	4.1E-04	139	3/2	9.2
1321	AAH00478	Heat shock 70-kDa protein 9B (mortalin-2)		73,967	6.03	1.27E-06	997	30/15	11.6
1450	AAA67526	MTHSP75	Heat shock 70-kDa protein 9B (mortalin-2)	74,019	5.97	3.78E-05	78	1/1	30.4
1655	CAA39849	Pyruvate kinase		58,411	7.58	2E-05	190	9/4	73.5

^a *, Spots with several master numbers come from the 24-h studies.^b As determined by the Student *t* test.

and lamin B2 showed significant decreases by 16 to 24 h p.i. At 48 h p.i. in two separate studies the cortactin species were seen to change significantly in two ways. First, a decrease in overall amounts was evident. Second, a change in the ratios of faster- and slower-migrating forms was apparent. Little change was apparent in the levels of the other species until 72 h p.i., when a more global reduction in protein levels is often seen, the result of virus-induced shutoff of host cell protein synthesis.

To study these species further, several were analyzed using extracts from wt Ad5-infected H1299 cells collected at 0 and 48 h p.i. on 2D gels, followed by Western blotting with appropriate antibodies. Figure 4 shows that Mre11 migrated as more than a half dozen species across the pH gradient, the levels of which all dramatically decreased by 48 h p.i., thus confirming the results in Fig. 3 and previous studies that indicated that global Mre11 levels decrease in response to E4orf6/E1B55K (8, 9, 75). Mre11 is known to be modified by phosphorylation (19, 22, 24, 40, 74, 80, 84) and methylation (11, 12, 21), and these results indicated that all forms of the protein appear to be degraded by the E4orf6/E1B55K complex. A similar global decrease (although only ca. 50%) was observed in all species of lamin B2. For hnRNP K and TrpRS, only the minor, slower-migrating species (circled in Fig. 4) appeared to decrease at 48 h p.i., whereas the main collection of species across the pH gradient remained relatively unaffected. These proteins are both known to be modified by phosphorylation (50, 52–56, 71) and in the case of hnRNP K also by methylation (15, 16, 51) and sumoylation (39). It is possible that only some of these modified forms are targeted by the E4orf6/E1B55K complex, and further study will be required to examine this possibility. Both dynactin and dynein are modified by phosphorylation (67, 76, 77; reviewed in reference 70), generating the multiple spe-

cies observed on 2D gels. Some decrease was observed in the major species. However, another population appeared that migrated at a higher pH, therefore perhaps making the global amounts shown in Fig. 3 to appear unchanged. Thus, it was unclear whether any of these species are degraded by the E4orf6/E1B55K complex, making further studies necessary. MEK2 is modified by phosphorylation (18, 38, 76) and acetylation (46); however, none of the multiple species appeared significantly affected, leaving the decrease observed in the 2D-DIGE analyses unexplained. Figure 4 also shows the cortactin profile obtained in two studies. Both indicated loss of the species migrating in the more basic region of the gels, and one showed a more global loss of all species, suggesting that cortactin should be studied further as a potential substrate. In summary, all proteins identified by 2D-DIGE as being decreased, apart from MEK2, appeared to be affected by infection with wt Ad5, although integrin α 3, lamin B2, and cortactin appeared to be the best candidates as substrates of the E4orf6/E1B55K E3 ubiquitin ligase complex. As discussed below, we have now confirmed in further separate studies that integrin α 3 is a bone fide substrate of this Ad5 ligase (20).

Analysis of degradation of candidate substrates by E4orf6 and E1B55K alone and with increased MOI of wt Ad5. Further characterization of cortactin and lamin B2 was conducted by Western blotting of extracts from H1299 cells expressing E4orf6 and E1B55K from adenovirus vectors in the absence of other viral proteins. Cortactin is highly modified by phosphorylation and acetylation (82, 83; reviewed in reference 1), thus explaining the complex pattern on 2D gels as in Fig. 4. Figure 5A shows that cortactin migrates on 1D SDS-PAGE as two closely migrating species of about 80 to 85 kDa and that the expression of both E4orf6 and E1B55K

FIG. 3. Analysis of candidate substrates by Western blotting. H1299 cells were infected with wt Ad5 at an MOI of 20 fluorescence-forming units/cell, and cells were harvested at several times. Cell extracts were analyzed by SDS-PAGE, followed by Western blotting with appropriate antibodies indicated on the right.

using vectors led to a clear loss of the faster-migrating species, a result consistent with the findings shown in Fig. 4. Figure 5B shows that in cells infected at MOIs ranging from 1 to 50 the faster-migrating cortactin species diminished in a somewhat dose-dependent manner, suggesting that some forms of cortactin may represent bone fide substrates of the E4orf6/E1B55K ligase.

Similar experiments were done on lamin B2. Figure 5A and B shows that very significant reductions in lamin B2 were evident when both E4orf6 and E1B55K were expressed; however, in cells infected with wt Ad5 at increasing MOIs the reduction appeared more modest. Since lamin B2 is modified by phosphorylation (5, 25), farnesylation, and methylation (36, 68), further studies will be required to determine whether lamin B2, or at least certain forms of this protein, is degraded by the E4orf6/E1B55K complex.

Figure 5B also demonstrates dramatic reductions in integrin $\alpha 3$ levels with increasing MOIs of wt Ad5. The full validation of this protein as a substrate of the E4orf6/E1B55K ligase is discussed below.

It was shown previously that degradation of Mre11 and integrin $\alpha 3$ is partially relieved when assays were conducted in H1299/Cul5KD cells in which Cul5 levels had been knocked down by constitutive expression of an appropriate RNA interference (17, 20). Figure 5C shows results comparing the degradation of substrates in H1299/Cul5 KD cells and control H1299 cells. Degradation of both Mre11 and integrin $\alpha 3$ was clearly reduced in H1299/Cul5KD cells, as found previously (20). At best only a modest effect was evident with lamin B2, suggesting that further studies will be required to establish the validity of this protein as a bone fide substrate. Degradation of cortactin by the E4orf6/E1B55K complex was reduced in H1299/Cul5KD cells, although repeat studies of this kind sometimes showed lower levels of reduction (data not shown). Nevertheless, cortactin exhibited properties typical of a bone fide substrate.

Colocalization of cortactin with E1B55K. E1B55K is believed to act as the substrate recognition module of the complex since its interaction with p53 is essential for the efficient p53 degradation (63, 65). Thus, we determined whether or not cortactin colocalizes with E1B55K when expressed alone in the absence of E4orf6. H1299 cells were transfected with plasmid DNAs expressing E1B55K and green fluorescent protein (GFP)-tagged cortactin (Fig. 6A) or Myc-tagged cortactin (Fig. 6B), and the colocalization of these proteins was determined by confocal immunofluorescence microscopy using appropriate antibodies against E1B55K (Fig. 6A) or E1B55K and Myc (Fig. 6B). E1B55K was seen clearly to colocalize with the GFP-tagged cortactin (Fig. 6A) in typical perinuclear aggresomelike structures (9) in virtually all of the cells examined. This colocalization was also observed with Myc-tagged cortactin (Fig. 6B), although a somewhat weaker signal made it difficult to detect in all cells. Nevertheless, this colocalization supported the idea that cortactin represents a bone fide substrate of the E4orf6/E1B55K ligase.

DISCUSSION

The 2D-DIGE technology is now recognized as a very powerful tool to address changes related to a variety of effects on

FIG. 4. Analysis of candidate substrates by 2D gels and Western blotting. An experiment similar to that shown in Fig. 3 was performed except that cells were harvest at either 0 or 48 h p.i., and extracts were analyzed on 2D gels, followed by Western blotting with appropriate antibodies, including two different experiments for cortactin. Dotted circles indicate spot population varying with virus infection.

the cellular proteome. It allows detection of most posttranslational modifications, and coupled to mass spectroscopy it is possible to identify proteins that change under specific conditions in structure or concentration. This technology seemed applicable to the identification of putative new substrates for the adenovirus E4orf6/E1B55K Cul5-based E3 ubiquitin ligase since degradation of all known substrates of this complex has been shown to occur in cells expressing just these two proteins in the absence of other viral products (3, 8, 63, 65). The E4orf6 protein highjacks elongins B and C to form a functional Cul5-based E3 ligase to which substrates appear to be introduced by E1B55K (8, 30, 61). This targeted degradation should therefore cause a limited number of decreases in the global cellular proteome of cells expressing both E4orf6 and E1B55K, relative

to those expressing E4orf6 alone that could be detected and analyzed by using the 2D-DIGE system.

Although this platform allows detection of very small changes in spot intensity due to protein degradation, it has some limitations that therefore require careful follow-up validation experiments. First, there could be some or considerable overlapping of protein spots due to similarities in molecular mass and pI, thus masking differences between treated samples and controls. Second, the 2D-DIGE approach allows detection of only a portion of the proteome, namely, proteins that are expressed at relatively high levels. In our study all of the candidates identified were proteins of fairly high abundance. Hence, potentially important substrate proteins that are expressed at levels below the detection limit of 2D-DIGE may be

FIG. 5. Analysis of candidate substrates by Western blotting of extracts from cells expressing E4orf6 and E1B55K or infected by wt Ad5. (A) H1299 cells were infected with viral vectors expressing E4orf6, E1B55K, or both and harvested at 48 h p.i. Cell extracts were analyzed by Western blotting with appropriate antibodies. (B) H1299 cells were infected with wt Ad5 at increasing MOI and harvested at 24 h p.i. Cell extracts were analyzed by Western blotting with appropriate antibodies. (C) H1299 cells with reduced levels of Cul5 protein (H1299/Cul5 KD) or control H1299 (pCDNA3) were infected with viral vectors expressing E4orf6, E1B55K, or both and harvested at 48 h p.i. Cell extracts were analyzed by Western blotting with appropriate antibodies. The anti-Cul5 antibody H-300 was used for the detection of Cul5 protein. Arrows indicate Cul5 species.

missed. In the present studies these problems may explain why we failed to identify the known E4orf6/E1B55K substrates Mre11 and DNA ligase IV in our screens. Third, some decreases could result as an indirect consequence, for example, by the degradation of a factor that regulates transcription or splicing, or the translation and/or stability of other cellular proteins, all of which could also lead to reductions in amount in 2D-DIGE analysis. Another example of such an indirect

FIG. 6. Cortactin colocalizes with E1B55K in H1299 cells. (A) H1299 cells were transfected with pCDNA3-E1B55K and pCDNA3-GFP-FLAG-cortactin DNAs and at 24 h cells were fixed stained for E1B55K (in red). Two equally representative localization are shown. (B) H1299 cells were transfected with pCDNA3-E1B55K and pCH17-Myc-cortactin DNAs, and at 24 h the cells were fixed and stained for E1B55K (in red) and c-Myc (green).

effect would be if the E4orf6/E1B55K complex acts on a post-translational modifying enzyme (such as a protein kinase, acetyltransferase, etc.), thus inducing perhaps widespread changes in protein migration on 2D gels that would appear as reductions of these species. Fourth, E1B55K might only interact with, or the complex degrade, specific isomers or modified forms of substrate proteins, as may have been the case with several of the candidate proteins identified in the present study.

Regardless of these potential problems or shortcomings, we believe that the 2D-DIGE approach provides a useful avenue to identify new ubiquitin ligase substrates, provided that sufficient follow-up validation studies are performed. In addition, identification of heat shock proteins and pyruvate kinase as species that increase significantly in amount in the presence of the E4orf6/E1B55K complex (or perhaps E1B55K alone) may offer additional insights.

We believe that the following seven criteria need to be fulfilled to validate a candidate protein revealed by this approach as a bone fide viral E3 ubiquitin ligase substrate, in this case the E4orf6/E1B55K ligase complex. (i) Significant decreases in protein levels should be detected in a majority of 2D-DIGE screening studies involving expression of the viral protein(s) present in the ligase complex (in this case Ad5 E4orf6 and E1B55K) in the absence of other viral (Ad5) proteins, relative to the pattern obtained in cells that do not express the substrate recognition module (expression of E4orf6 alone). In cases where only a single viral protein is associated with the ligase, the comparison could be made with cells infected with a control viral vector. (ii) Decreases in candidate substrates should be confirmed in similar studies by Western blotting with antibodies specific for the candidate protein. (iii) Decreases should be apparent following wt (in this case Ad5) virus infection. (iv) Decreases in protein in relevant subcellular compartments, or when appropriate on the cell surface, should be detectable using suitable approaches such as flow cytometry or cell fractionation in vector experiments, wt infection, or both. (v) Degradation of the candidate protein should be inhibited in cells in which levels of the appropriate cullin (in this case Cul5) have been knocked down using small interfering RNAs (siRNAs) (8, 17) or after expression of a dominant-negative form of the cullin (79). Alternatively, a proteasome inhibitor (MG132, lactacystin) should be shown to reduce degradation of the candidate protein. (vi) The candidate should ideally be shown to bind the substrate recognition factor (in this case E1B55K) in coimmunoprecipitation studies. However, this association may not always be possible, even in the presence of proteasome inhibitors or using appropriate mutants, if binding is either transient or at low affinity. In these cases especially, the candidate should be shown to colocalize in cells with the substrate recognition factor (in this case E1B55K). (vii) Finally, some biological effect on virus replication should ideally be observed after knock-down by siRNA or overexpression of the candidate substrate. Alternatively, siRNA treatment may mimic a cellular phenotype observed following the formation of the viral ligase.

Based on the preliminary data presented here, we carried out such a series of studies to confirm integrin $\alpha 3$ as a bone fide substrate and have published them separately (20). That study showed, by Western blot analysis, (i) that the total cell levels of

integrin $\alpha 3$ decreased dramatically upon expression of E4orf6 and E1B55 alone or infection of cells by wt Ad5; (ii) that some reductions occurred in the cell surface pool of integrin $\alpha 3$; (iii) that infection by adenovirus particles played an as-yet-undefined role in the process; (iv) that degradation was inhibited in H1299Cul5 knockdown cells; (v) that integrin $\alpha 3$ colocalized with E1B55K; and (v) that integrin $\alpha 3$ degradation by the E4orf6/E1B55K ligase appeared to affect cell adhesion. We believe that this confirmation of integrin $\alpha 3$ as a substrate validates the proteomic approach we have been describing.

Clearly, this approach, and the validation protocols suggested above, could be used for other cullin-based viral E3 ubiquitin ligases and perhaps other classes of such complexes. Study of viral complexes is aided by the ability to set up appropriate controls and to use infection by wt virus as a validation tool. The E4orf6/E1B55K complex is unique in that separate protein species are responsible for forming the complex and providing the substrate recognition module, as most often a single protein contributes both of these functions. Examples include the Vif protein of human immunodeficiency virus that forms a Cul5-based complex (45, 81), and the Cul2-based complexes formed by high-risk human papillomavirus E7 protein (33) and the NS1 product of respiratory syncytial virus (23). Coupled with other approaches, such as tandem affinity purification using the viral substrate recognition module, application of 2D-DIGE should help in the identification of new and potentially important substrates for these and other E3 ubiquitin ligases.

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