Two Distinct Transport Motifs in the Adenovirus E3/10.4–14.5 Proteins Act in Concert to Down-modulate Apoptosis Receptors and the Epidermal Growth Factor Receptor*

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The adenovirus (Ad) early transcription unit E3 encodes immunosubversive functions. The E3 transmembrane proteins 10.4 and 14.5 form a complex that down-regulates the epidermal growth factor receptor and apoptosis receptors from the cell surface by diverting them to endosomes/lysosomes for degradation. The latter process protects infected cells from ligand-induced apoptosis. The mechanism by which 10.4–14.5 mediate re-routing remains elusive. We examined the role of putative XXYΦ and dileucine (LL) transport motifs within Ad2 10.4–14.5 for target protein modulation. By generating stable E3 transfectants expressing 10.4–14.5 proteins with alanine substitutions in these motifs, we show that 3 of the 5 motifs are essential for functional activity. Whereas tyrosine 74 in 14.5 appears to be important for efficient 10.4–14.5 interaction, the 122XXΦ motif in 14.5 and the dileucine motif Leu192-Leu193 in 10.4 constitute genuine transport motifs: disruption of either motif abolished binding to the cellular adaptor proteins AP-1 and AP-2, as shown by surface plasmon resonance spectroscopy, and caused missorting, dramatically altering cell surface appearance and the intracellular location of viral proteins. Fluorescence-activated cell sorter analysis and immunofluorescence data provide evidence that Tyr192 in 14.5 is essential for rapid endocytosis of the 10.4–14.5 complex, whereas the 10.4LL motif acts downstream and protects 10.4–14.5 from extensive degradation by rerouting it into a recycling pathway. Infection of primary cells with adenoviruses carrying the relevant point mutations confirmed the crucial role of these transport motifs for down-regulation of Fas, TRAIL-R1, TRAIL-R2, and epidermal growth factor receptor. Thus, two distinct transport motifs present in two proteins synergize for efficient target removal and immune evasion.

Proteins encoded within the early transcription unit E3 of human adenoviruses (Ads) play a key role in immune evasion (1–4). The great majority of E3 proteins are integral membrane proteins. For those characterized, a common activity has emerged, in that they subvert host defense mechanisms by rerouting of cellular target proteins (5): E3/19K interferes with antigen presentation and T cell recognition by mediating retention and retrieval of class I major histocompatibility complex antigens to the endoplasmic reticulum (ER) (6–10). The 10.4–14.5 proteins prevent Fas-mediated apoptosis by removal of Fas from the cell surface and by redirecting it to endosomal/lysosomal vesicles where it is degraded (11–13). 10.4–14.5 have also been reported to confer resistance to tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (14), by down-regulating TRAIL-receptor 1 (TRAIL-R1/DR4). Surprisingly, down-regulation of the related TRAIL-R2/DR5 required in addition the activity of a third E3 protein, 6.7K (15), that may also have an independent antiapoptotic activity (16). In infected human cells, 10.4–14.5 were also reported to contribute to inhibition of tumor necrosis factor-induced cytolsis. This is not achieved by tumor necrosis factor receptor modulation but rather by inhibition of tumor necrosis factor-induced translocation of cytosolic phospholipase A2 and possibly nuclear factor-κB (3, 11, 17, 18). 10.4–14.5 also removes the epidermal growth factor receptor (EGFR) from the cell surface by diverting constitutively internalized receptors into a degradation compartment (19–21). Initially, it was suggested that 10.4 alone is responsible for EGFR modulation (19). However, subsequent reports demonstrated that efficient down-regulation depends on both proteins (12, 13, 20). Taken together, the 10.4–14.5 complex, also named receptor internalization and degradation (RID), modulates a selective set of plasma membrane receptors involved in apoptosis and growth control (13, 22, 23). The molecular basis for the exquisite target specificity of 10.4–14.5 remains unknown.

E3/10.4 and 14.5 are encoded by human Ads from all subgenera and therefore are likely to be of general importance for Ad-host interaction (1, 2). 10.4 is expressed as two isoforms: in one the signal peptide is cleaved, whereas in the other it re-

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1 The abbreviations used are: Ads, adenoviruses; AP, adaptor proteins; EE, early endosomes; ER, endoplasmic reticulum; EGFR, epidermal growth factor receptor; FACS, fluorescence-activated cell sorting; Lamp, lysosome-associated membrane protein; LE, late endosomes; MVBs, multivesicular bodies; RID, receptor internalization and degradation; TGN, trans-Golgi network; TRAIL, tumor necrosis factor-related apoptosis inducing ligand; wt, wild-type; IP, immunoprecipitation; LL, dileucine; mAb, monoclonal antibody; SPR, surface plasmon resonance; Baf, bafilomycin A1; aa, amino acid(s).
mainly attached and serves as a second membrane anchor (Fig. 1A). The two 10.4 species form a disulfide-linked dimer via a conserved cysteine residue at position 31 (24) and associate non-covalently with the 14.5 protein in the ER (3). 14.5 is a type I transmembrane protein and the Ad5 product has been shown to be O-glycosylated and serine-phosphorylated (3, 25). Both proteins seem to be exposed on the plasma membrane (26), but a thorough quantitative assessment is lacking.

A wealth of information exists about trafficking and in particular ligand-induced down-regulation of the EGFR (27–29). EGFR binding to the receptor triggers its tyrosine kinase activity and enhances the rate of internalization into early endosomes (EE). From EE, the EGFR may rapidly return to the cell surface, whereas a significant pool of EGFR-EGF complexes is sorted to the inner vesicles of a late endosomal compartment, designated multivesicular bodies (MVBs). This pool is destined for degradation in lysosomes and is segregated from the pool of recycling receptors recruited to the limiting membrane of MVBs. This major sorting step is controlled by EGFR tyrosine kinase activity, ubiquitination, and a variety of cytosolic factors (30–33). Cell fractionation and immunocytochemical approaches (34) suggested that overexpressed 10.4 (by these authors called 13.7) may be located in EE and MVBs, and it was predicted that 10.4 may have signals for endosomal retention/retrieval and for directing EGFR to lysosomes. No attempt was made to experimentally identify these signals. Unlike EGFR, 10.4–14.5 do not appear to accelerate EGFR endocytosis, but rather modulate intracellular sorting of constitutively internalized EGFR (21). The underlying mechanism as to how this is achieved and which features of the viral proteins are responsible for the altered fate of EGF and death receptors remains elusive.

Interestingly, both viral proteins contain sequence elements in their cytoplasmic tails that conform to consensus transport motifs (Fig. 1): three putative YXXΦ motifs (where Φ is a tyrosine, Ψ any amino acid (aa), and Ψ (an aa with a bulky hydrophobic side chain) are present in 14.5 (Fig. 1B), whereas 10.4 proteins generally display two potential dileucine (LL) motifs (Fig. 1C).

Both tyrosine- and dileucine-based motifs have been shown to trigger rapid internalization from the cell surface, specify sorting to lysosomes or specialized endosomal/lysosomal compartments; they also mediate trafficking to the trans-Golgi network (TGN) as well as sorting to the basolateral plasma membrane and is involved in clathrin-dependent endocytosis, whereas AP-1 is found in coated buds of the TGN, but also on endosomes (40). AP-3 and AP-4 are also supposed to mediate trafficking from the TGN to endosomes. In addition to APs, several monomeric proteins have been described as adaptors and/or cooperating partners of APs for TGN-endosome and endosome-TGN cargo selection (36, 41, 42).

In this study, we investigated the potential role of YXXΦ and dileucine motifs for the function of the Ad2 10.4–14.5 complex. We demonstrate that a conserved dileucine motif in 10.4 and a conserved YXXΦ-like motif in 14.5 bind AP-1 and AP-2 in vitro and are key determinants for receptor down-modulation in vivo. Evidence is provided that both function as transport motifs in a sequential manner. Whereas YXXΦ is important for endocytosis of the 10.4–14.5 complex, the LL motif contributes to the efficacy of receptor down-modulation by targeting 10.4–14.5 from a degradative into a recycling pathway. Our findings illustrate the highly sophisticated cooperation between two viral proteins, each providing a distinct transport signal required for efficient target removal and immune evasion.

**Experimental Procedures**

**Construction of Mutant Genes**—Plasmid pBSX-E3 (13) was used as template for PCR-mediated site-directed mutagenesis, generating alanine replacement mutants 14.5Y77A, 14.5Y79A, 14.5Y122A, and 10.4L1A. pBSX-E3 is a derivative of pBluescript II KS (Stratagene) containing the EcoRV fragment of Ad2, which encompasses the entire Ad2 region including the endogenous E3 promoter (43). pBSX-E3 was also utilized for introducing the FLAG tag DYKDDDDK downstream of the 14.5K signal sequence yielding pBSX-E3F14.5. This plasmid served as template for generating Ala replacement mutants of F14.5Y122, 10.4LL, and 10.4L in a two-step PCR using primers containing the relevant mutation together with flanking primers homologous to the 10.4 and 14.7 open reading frame (31). PCR fragments containing the mutation were cloned into the XhoI and HpaI sites of the pBSX-E3 and pBSX-E3F14.5 vectors, respectively. All primer sequences are available upon request.

For expression of 10.4 and 14.5 from the heterologous SV40 promoter/enhancer the vector pSG5 (Stratagene) was used. PCR fragments containing the Ad2 coding sequence of the 10.4, 14.5, or both genes were cloned between the EcoRI and BglII or the BamHI and BglII sites, respectively. For optimizing expression, the sequence 5′ of the ATG was modified to conform to the Kozak consensus for translation (44). All constructs were verified by dye terminator cycle sequencing.

**Cell Lines, Culture Conditions, Transfection**—HEK 293 (ATCC CRL 1575), A549 (ATCC CCL 185), a human lung carcinoma-derived epithelial cell line, and primary human foreskin fibroblasts (SeBu) (13) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin; Invitrogen). 293E5-45 cells (13) and SVOS, a human fibroblast cell line transformed with SV40 large T antigen and overexpressing the human Fas receptor (45) was propagated in the same medium supplemented with 200 μg/ml G418 (Invitrogen).

Cotransfection of human 293 cells with modified pBSX-E3 or pBSX-E3F14.5 plasmids and the pSV2-neo plasmid encoding the neomycin phosphotransferase gene as selectable marker, selection, and subsequent culture of stable transfecants was done as described (46). For transient transfections the calcium phosphate method was used, essentially as described in Ref. 47.

**Abs and Antiserum**—The following monoclonal antibodies (mAbs) were used: TW1.3 against E3/19K (48); W6/32 (ATCC HB 95), anti-HLA-ABC; B-G27 (Chemicon, Hofheim Germany) against human CD95; 528 (ATCC CRL 8509) directed to the extracellular portion of the M1 (Sigma) against the FLAG-octapeptide; 2D5 directed to Lamp2 (49), 6C4 against lysobisphosphatidic acid (50), HS101 against TRAIL-R1 (DR4), and HS201 against TRAIL-R2 (DR5) (Alexis Biochemicals, Groningen, Germany). Rabbit sera Burd and Bur3 raised against COOH-terminal 14.5 (CEISTYFNLTGDD) and 10.4 (CYRDRDIALRIL) peptides (Fig. 1, B and C, respectively, have been described (33), as was rabbit serum RaE19 against Ad2 E3/19K (46). To detect the 10.4LL mutant, peptide CYRDRDIALRIL was coupled to keyhole limpet hemocyanin to generate rabbit serum R71. Antiserum R59 was generated upon immunization with the peptide FDIVCVRICAYLRRHHPQYRDRDIALRIL (Sigma) recognizing the entire cytoplasmic tail of 10.4. The anti-human TG46 sheep serum was initially provided by S. Ponnambalam (University of Leeds, Leeds, UK) and was subsequently purchased from Serotec-Biozol, Munich, Germany. Fluorescein isothiocyanate- or rhodamine-conjugated donkey anti-rabbit, -mouse, or -sheep immunoglobulin G, respectively, for immunofluorescence and peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L) for Western blot detection were purchased from Dianova, Hamburg, Germany. For flow cytometry, goat anti-mouse and goat anti-rabbit fluorescein isothiocyanate IgG from Sigma were used.

**Viruses**—Ad2 mutant viruses were generated with a novel method combining ET cloning and transposition integration. This method allows the introduction of any mutation into Ad genomes independent of restriction sites. Infectious virus particles were reconstituted upon calcium phosphate transfection of linear wt or mutant Ad plasmid DNA into 293 cells. Viruses were propagated and titrated on A549 cells as described (51). In general, cells were infected with 100 plaque forming units/cell.

2 Z. Ruzsics, M. Wagner, U. Koszinowski, H.-G. Burgert, unpublished data.
Position and sequence of putative transport motifs are shown in circles. S-S indicates the proposed disulfide bond between the strictly conserved cysteine at position 31 of 10.4. Alignment of 14.5 cytoplasmic tail sequences from representative members of subgenera A–F (depicted on the right). C, alignment of 10.4 cytoplasmic tail sequences of representative members from each subgenus. The MegAlign software (DNASTAR Inc., Madison, WI) with the Clustal method and PAM250 residue weight table was applied. For a comparison of the full-length sequences and references, see Ref. 1. Above the alignment, the thick line denotes amino acids (aa) of the transmembrane segment (TM) and double arrows mark the various peptides used for immunization or SPR. The YXXΦ and dileucine motifs are indicated; strictly conserved ones are highlighted in boldface. Numbers in parentheses refer to the position of the Tyr or LL in the Ad2 sequence. Amino acids that conform to the consensus are shaded, a dash indicates the lack of the corresponding aa. Strictly conserved residues are given below the sequence comparison.

Flow Cytometry—Fluorescence activated cell sorting (FACS) was done essentially as described (13, 46) except that 3–5 × 10^5 cells/sample were used. In experiments using the anti-FLAG mAb M1 the FACS buffer was supplemented with 1 mM CaCl_2. Fluorescence profiles were obtained by analyzing 5000 viable cells in a FACSScanII flow cytometer using the CellQuest software (BD Biosciences).

Immunoprecipitation, SDS-PAGE, and Immunoblotting—Immunoprecipitation (IP) and SDS-PAGE were carried out essentially as described (6). Approximately equal numbers of cells (~4 × 10^6/sample) were lysed in 1% Triton X-100 (Roche Diagnostics) or digitonin (high purity; Calbiochem). The protein concentration was measured by the BCA method according to the manufacturer (Pierce). Equal amounts of protein were used for immunoprecipitation of 10.4 and 14.5, employing R59 and Bur4, respectively. Immunocomplexes were extensively washed and separated on 15% acrylamide SDS minigels (Bio-Rad). The proteins were blotted for 80 min at 0.8 mA per cm2 onto Hybond ECL transfer cell (Trans-Blot SD, Bio-Rad) and 48 mM Tris, 39 mM glycine, 0.37% SDS, 20% methanol as blotting buffer. Nonspecific binding sites were blocked overnight in phosphate-buffered saline containing 5% skim milk, 0.05% Tween 20, and 0.02% sodium azide followed by incubation of the membrane with primary antibody for 1 h at room temperature. For Western blotting of 10.4 and 14.5, Bur3 and Bur4 were used, respectively. As Bur3 exhibited only a marginal reactivity to the 10.4 mutants, R71 was applied for IP and Western blot detection of the LLAA mutant and R59 for the ILAA mutant. All antisera were diluted 1:1000. Proteins were visualized using peroxidase-conjugated goat anti-rabbit IgG (H+L) (1:10000). ECL detection reagent (Amerham Biosciences), and exposure to Bio-Max MR film (Kodak).

Confocal Immunofluorescence Microscopy—Cells were processed for immunofluorescence as described (52).

Surface Plasmon Resonance Spectroscopy (SPR)—The interaction of purified AP-1 and AP-2 adaptors with putative sorting motifs of 10.4 and 14.5 was recorded in real-time using a BIACore 3000 biosensor (BIACore AB, Sweden). Synthetic peptides, depicted in Fig. 1, were purified by reverse phase high performance liquid chromatography and immobilized to the surface of a CMS sensor chip via their NH2-terminal cysteine, using thiol coupling chemistry. Subsequently, AP-1 and AP-2 (at concentrations ranging from 100 to 500 nM) were passed over the sensor surface to monitor binding to the immobilized peptides and to determine the rate constants (k_a, k_d, and k_0 = k_d/k_a) of this interaction. The purification of AP-1 and AP-2 as well as the details and principles of SPR are described elsewhere (53–56).

RESULTS

Identification of Putative Transport Signals in the Ad2 10.4 and 14.5 Protein Sequences—To search for structural features with potential functional relevance, we have sequenced the 10.4 and 14.5 genes of subgenus D and E Ads (1, 2). Comparison of 10.4 and 14.5 protein sequences from Ads of all subgenus revealed only 9 aa in 14.5 and 15 aa in 10.4 that are strictly conserved (1). Some of these might be part of putative tyrosine- or dileucine-based transport motifs: in 14.5, two YXXΦ motifs, designated YXXΦ and YYXXΦ (according to the position of the Y in the Ad2 14.5 sequence), are strictly conserved, although the bulky hydrophobic aa in position +3 varies (Fig. 1B). The YYXXΦ motif is found in position −9 from the COOH terminus and Φ is Leu, Ile, or Phe, whereas YXXΦ might be located adjacent to or within the lipid bilayer and Φ is either Leu or Phe (Fig. 1, A and B). A third YXXΦ motif with Tyr at position 76, overlapping the YYXXΦ motif, is present only in 14.5 proteins of subgenus C Ads. Two sequence elements reminiscent of dileucine transport motifs can be recognized in the

![Diagram](image-url)
cytoplasmic tail of 10.4 (Fig. 1C). One LL motif (Leu<sup>87</sup>-Leu<sup>88</sup> in the Ad2 sequence) is conserved at position −4/-5 from the COOH terminus (in Ad12 at −5/-6) with the first Leu being replaced by Ile in subgenus D proteins. The last two aa, either IL in subgenus C or LI in subgenera B, D, and E may also constitute transport motifs.

**Three of the Five Motifs Are Crucial for 10.4-14.5 Function in 293 Cells**—To investigate whether these putative transport motifs are functionally relevant, the critical tyrosine and leucine residues were replaced by alamines. Mutations were introduced into plasmids encoding the entire E3 region of Ad2. Upon transfection into HEK 293 cells and selection of stable transfectants with G418, the effect of the mutation was quantitatively assessed by measuring Fas and EGFR cell surface expression using FACS analysis. This transfection system allows efficient expression of E3 proteins, because of the presence in 293 cells of E1A that transactivates the E3 promoter (6, 13, 43, 46). An additional advantage of the system is easy standardization of E3 protein expression levels by monitoring expression of E3/19K, an unrelated E3 protein not affected by the mutations introduced in 10.4 and 14.5 (13). Thus, transfectant clones were first screened for intracellular E3/19K expression. At least three independent clones from each transfection with expression levels comparable with transfectants expressing wild-type (wt) E3 proteins and with a similar HLA reduction on the cell surface were selected for further analysis. Compiling the data from five experiments, the mean value of fluorescence was calculated and related to that of untransfected 293 cells (Fig. 2A, HLA, open bars) or the control cell line 293E3-45 expressing wt E3 proteins (Fig. 2A, E3/19K, black bars). The average level of E3/19K expression was very similar among the selected clones and was comparable with that of E3-45 cells. This was confirmed by Western blotting and metabolic labeling/immunoprecipitation (data not shown). Accordingly, cell surface expression of HLA in these clones was reduced by at least 80% (open bars). Having established a comparable E3 expression level, the relative efficacy of each 10.4-14.5 mutant to modulate cell surface Fas and EGFR was determined by flow cytometry (Fig. 2B). Wt 10.4-14.5 proteins expressed in E3-45 cells reduce Fas surface expression by ∼80% relative to 293 cells. This modulating capacity is largely retained in cells expressing the alanine replacement mutant 14.5Y122A that exhibits about 10% higher Fas and EGFR levels as compared with E3-45 cells. By contrast, receptor down-regulation is essentially abolished when cells express 10.4LLAA, 14.5Y74A, or 14.5Y122A. In these transfectants receptor expression levels are similar to those seen in 293E3 transfectants that specifically lack expression of either 10.4 (10.4ko), 14.5 (14.5ko), or both (10.4-14.5ko). Small differences are detected regarding the effectiveness of the different mutations. For example, Fas levels were not fully reconstituted upon mutation of Tyr<sup>74</sup>. Likewise, in 10.4ko cells 14.5 alone may have some residual activity toward the EGFR. We conclude that the two strictly conserved tyrosines at position Tyr<sup>74</sup> and Tyr<sup>122</sup> in 14.5 and the dileucine at position 87/88 in 10.4 of Ad2 are of crucial functional importance in vivo, whereas Tyr<sup>86</sup> does not seem to be critical. For simplicity, we will refer to these mutants below as 10.4LL, 14.5Y74, 14.5Y76, and 14.5Y122.

**Complex Formation of Mutant 10.4-14.5 Proteins**—Given that both 10.4 and 14.5 are required for down-regulation of Fas and the EGFR, and that they form a complex in infected cells (13, 20), it is conceivable that complex formation is a prerequisite for 10.4-14.5 function. Therefore, we examined whether loss of function of the 14.5Y74, 14.5Y122, and 10.4LL mutants was accompanied by a loss of 10.4 and 14.5 interaction. Complex formation was assayed by immunoprecipitation of 14.5 from detergent lysates and subsequent detection of associated 10.4 by Western blotting. One representative clone from each transfection was lysed in Triton X-100 and the less stringent detergent digitonin. Similar to wt, two bands representing the two forms of 10.4 (with uncleaved and cleaved signal sequence) are visualized in immunoprecipitates containing the Tyr<sup>122</sup> mutant (Fig. 3A, compare lanes 2 and 3). Thus, the interaction of the 14.5Y122 mutant with 10.4 was not significantly altered. The interaction of the 14.5Y76 mutant seemed to be reduced as 10.4 could only be detected after lysing cells in the mild digitonin buffer (Fig. 3A, lane 4). However, this reduced stability of the complex in Triton extracts appeared not to be critical for in vivo function (Fig. 2). Remarkably, we could not detect a significant interaction of 10.4 with 14.5Y74 in either detergent (Fig. 3A, lane 5). This was not caused by a low E3 expression in this particular clone, as E3/19K levels were comparable with wild-type and the other mutant cells (Fig. 3B). To examine whether the altered complex formation is caused by an altered expression of the individual subunits, we also determined the total amounts of 10.4 and 14.5 by immunoprecipitation/Western blotting. Whereas the total amount of 10.4 was very similar in all cells analyzed, steady-state levels of 14.5Y74 were greatly
caused by an impaired interaction of Tyr74 with 10.4, selectively reducing the stability of 14.5. The amount of 10.4 isolated in complex with 14.5Y122 was consistently higher than with wt 14.5 (Fig. 3A). This was accompanied by increased total amounts of immunoprecipitable 10.4 and 14.5Y122 proteins (Fig. 3C, 10.4 and 14.5), taking into account the lower E3/19K expression in these mutant cells (Fig. 3C, 19, compare lanes 2 and 3). The data therefore indicate an increased stability of 10.4 and 14.5 in the Tyr122 transfectant.

We next examined the role of the putative dileucine transport motifs in 10.4 for stability and 14.5 interaction. As the 10.4 antiserum proved to be ineffective for detection of mutant 10.4 proteins (data not shown), new antisera, R59 and R71, were generated directed to the entire 10.4 C-tail and the mutated 10.4AA peptide, respectively (Fig. 1C). The interaction of mutant 10.4 with FLAG-tagged wt 14.5 was assayed in digitonin lysates (Fig. 3D) and the total amounts of immunoprecipitable 10.4, 14.5, and 19K were determined in parallel (Fig. 3E). Alanine replacement of the COOH-terminal 2 aa (Leu90-Leu91) of 10.4 (mutant 10.4IL) had no significant influence on its interaction with 14.5 (Fig. 3D, compare lanes 3 and 7), or the overall stability of the individual subunits (Fig. 3E). This is reflected by a nearly unaltered functional activity of the 10.4IL-14.5 complex (see Fig. 4A). By contrast, the amount of detergent-resistant complexes between 10.4LL and wt 14.5 was negligible (Fig. 3D, lane 5). This might be due to a reduced stability of the individual subunits, whose levels were strongly reduced (Fig. 3E, lane 5). 19K levels were similar in all transfectants. The mutually dependent steady-state levels confirm the notion that 10.4 forms complexes with 14.5 and that both proteins have a common fate. We hypothesized that the dileucine motif in 10.4 may inhibit transport of the complex into a degradative compartment. In support of this hypothesis, steady-state levels of 10.4LL and 14.5 could be reconstituted to wt levels by treatment of 10.4LL transfectants with bafilomycin A1 (Baf), an inhibitor of the vesicular ATPase that is responsible for endosomal/lysosomal acidification (Fig. 3E, compare lanes 5 and 6 with 3 and 4). Baf caused an increase of the 10.4LL mutant protein and 14.5 by −10- and 8-fold, respectively. Interestingly, a 2–5-fold increase in signal intensity was also noted for the wt proteins (Fig. 3E, compare lanes 3 and 4). Remarkably, Baf restored levels of 10.4LL-14.5 complexes to those observed with wt 10.4 in untreated cells. The protective effect of Baf appears to be specific for proteins in post-ER compartments, because steady-state levels of the ER-located E3/19K protein are not significantly altered. Together, the data strongly suggest that mutation of the dileucine pair in 10.4 leads to enhanced lysosomal degradation of the 10.4–14.5 complex.

The Cellular Adaptor Proteins AP-1 and AP-2 Bind to 10.4 and 14.5 Cytoplasmic Tail Peptides in a Motif-dependent Fashion—As 14.5Y122 and 10.4LL are capable of forming complexes, yet were functionally defective, we hypothesized that Tyr122 and the dileucine might be critical residues of transport signals in the viral proteins. To test this hypothesis, we first investigated whether the cytoplasmic tails of 10.4 and 14.5 are able to bind to AP-1 and AP-2, cellular adaptor proteins that recognize dileucine- and YXXΦ-type motifs and are involved in clathrin-mediated transport (35, 36, 38). SPR using purified AP-1 and AP-2 and immobilized 10.4 and 14.5 cytoplasmic tail peptides (Fig. 1, B and C, SPR), encompassing the putative YXXΦ and dileucine transport motifs revealed a significant adaptor binding with affinities ranging from 300 to 400 nM for AP-2 to ~500 nM for AP-1 (Table I, wt). Binding required the integrity of the putative transport motifs, as alanine substitution of Tyr122 in 14.5 or the dileucine pair at position 87/88 in

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Fig. 3. Complex formation and stability of 10.4 and 14.5 mutant proteins. A, representative clones derived from transfection of 293 cells with the indicated PBSAX-E3 constructs were lysed in either Triton (Trit.) or digitonin (Dig.) buffer and subjected to immunoprecipitation (IP) with antiserum Bur4 against 14.5. Immunocomplexes were separated by SDS-PAGE and analyzed by Western blotting (WB) with Bur3 for the presence of 10.4. B, parallel Western blot analysis of E3/19K expression in 293 cells (lane 3) and the selected 293E3+ clones studied in A using Triton extracts and R59. C, total amounts of 10.4, 14.5, and E3/19K (19) in E3 transfectants. 10.4 steady-state levels were determined by IP of Triton extracts with Ab R59 and by Western blot with Bur3. For detection of 14.5, Bur4 was used in both IP and Western blot. The same lysates were subsequently reacted with R59 and blotted material was detected with the same Ab (19). Differences in the migration between mutants 14.5Y74 and 14.5Y76 versus wt and Tyr122 (14.5) are because of insertion of the FLAG tag in the latter two. D, association of 10.4IL and IL mutants with wt FLAG-14.5 without (−) or following Baf treatment (+) for 11 h (100 nM). 14.5-associated 10.4 in digitonin extracts of 293 cells (lanes 1 and 2) and 293E3F14.5–19 cells, containing the wt E3 transcription unit encoding FLAG-14.5 (wt, lanes 3 and 4), was detected after Western blotting with Bur3; the 10.4LL mutant from cell clone LL-11 (LL, lanes 5 and 6) was detected with antiserum R71, 10.4IL from clone IL-13 (lane 7 and 8) with R59. E, Western blot analysis of the total amounts of 10.4, 14.5, and E3/19K in the cell clones studied in D. 10.4, 14.5, and 19K were detected as in C. The same antibody combination, R59 for IP and Bur4 for Western blot, was used to detect 10.4 in 293, wt, and IL clones. For LL, R71 was used in IP and Western blots. Quantification was done with two exposures from two experiments using Labscan version 3.00 and Image master version 3.01 software (Amersham Biosciences).

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3 J. Lindberg, unpublished data.
10.4 protein. Upon transfection of pBS stable E3 A intracellular sorting of 10.4 peptides interestingly, both peptides exhibited a higher affinity for AP-2 10.4 dramatically reduced binding by more than 100-fold. In- observed loss of AP binding to the mutant tail peptides in vitro —expression of the Complex in Vivo Motif in 14.5 Results in Opposing Effects on Cell Surface Ex- is reflected by an altered trafficking of 10.4 and 14.5 related to that of 293E3F14.5 FLAG mAb M1. Mean value of fluorescence for each cell line were analyzed in wt (E3F14.5), and untransfected 293 control cells. 14.5 surface expression was monitored with the anti- 14.5 function. The FLAG tag allowed us for the first time to quantitatively assess 10.4–14.5 expression on the cell surface. Flow cytometry with the FLAG-specific mAb M1 revealed that cells expressing the 10.4LL mutant exhibited a significant reduction of 14.5 cell surface expression (Fig. 4A). The comparable down-modulation of Fas and the EGFR demonstrated that FLAG-tagged 14.5 exhibits a functional activity equivalent to unmodified 14.5 (Fig. 4A, compare E3F14.5–19 with E3-45, black and white bars). This was fur- ther substantiated by the efficient association of F14.5 with 10.4 in digitonin extracts (Fig. 3D). We then introduced FLAG into the functionally defective, putative transport mutants 14.5Y122 and 10.4LL. As expected from the phenotype of cells expressing untagged versions, stable 293 transfectants expressing the corresponding FLAG-tagged alanine replacement mutants showed no loss in Fas and EGFR surface expression (Fig. 4A, black and white bars). By contrast, the capacity of 10.4–14.5 to down-modulate Fas and EGFR was largely retained upon mutation of the last two amino acids (IL) of 10.4. This confirms that the IL sequence element is not essential for 10.4–14.5 function.

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**Missorting of Mutant 14.5Y122 to the Cell Surface and 10.4LL to Intracellular Vesicles**—To analyze the intracellular distribution of the viral proteins in greater detail, we sought to express 10.4 and 14.5 in cells more suitable for immunofluo- rescence than 293 cells. This required a 10.4–14.5 expression system independent of AdE1A products. To this end, Ad2 10.4 and F14.5 were separately cloned into pSG5 expression vectors to drive 10.4 and 14.5 synthesis by the SV40 promoter/en- hancer. Equimolar amounts of 10.4 and FLAG-14.5 expression plasmids were transiently transfected into SV80Fas cells, a human fibroblast cell line expressing SV40 T antigen and Fas, and the intracellular localization of proteins was determined. Wt 10.4 and 14.5 proteins colocalized in a perinuclear, Golgi- like structure. In cells expressing high amounts of 10.4 and 14.5, 14.5 could also be detected at the cell surface and in clusters underneath the plasma membrane (Fig. 5A). Coexpression of wt 10.4 with the F14.5Y122 mutant resulted in a marked increase in the number of cells exhibiting 14.5 plasma membrane staining, confirming the FACS data obtained with

| Peptide     | AP-1  | AP-2  |
|-------------|-------|-------|
| 10.4 wt     | 0.52  |        |
| 10.4LL      | 80.0  | ×154  |
| 14.5 wt     | 0.48  | 0.3   |
| 14.5Y122    | 52.0  | 50.0  |

**Affinity reduction**

*Kd* equilibrium dissociation constants in micromolar.

**Fold reduction.**

293 cells, stable clones (e.g. E3F14.5–19) were selected that expressed similar levels of E3/19K and HLA molecules as the E3* reference cell line (E3-45) expressing untagged 14.5 (Fig. 4A). The comparable down-modulation of Fas and the EGFR demonstrated that FLAG-tagged 14.5 exhibits a functional activity equivalent to unmodified 14.5 (Fig. 4A, compare E3F14.5–19 with E3-45, black and white bars). This was fur- ther substantiated by the efficient association of F14.5 with 10.4 in digitonin extracts (Fig. 3D). We then introduced FLAG into the functionally defective, putative transport mutants 14.5Y122 and 10.4LL. As expected from the phenotype of cells expressing untagged versions, stable 293 transfectants expressing the corresponding FLAG-tagged alanine replacement mutants showed no loss in Fas and EGFR surface expression (Fig. 4A, black and white bars). By contrast, the capacity of 10.4–14.5 to down-modulate Fas and EGFR was largely retained upon mutation of the last two amino acids (IL) of 10.4. This confirms that the IL sequence element is not essential for 10.4–14.5 function.

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The prominent vesicular staining, together with the loss of cell surface expression upon mutation of the LL motif, could be explained by missorting of the 10.4LL-14.5 proteins either at the TGN or following endocytosis. To distinguish between these possibilities, the 10.4LL and 14.5Y122 mutant proteins were coexpressed. If the LL mutation caused missorting at the TGN prior to the proposed activity of the 122YXXΦ motif in internalization at the plasma membrane, the double mutant would be expected to be localized in intracellular vesicles and not at the cell surface. If it acted subsequently to endocytosis, a cell surface phenotype similar to that of the 14.5Y122 mutant would be expected. Fig. 5D shows that the latter is indeed the case. The majority of the cells exhibited a strong cell surface staining of both proteins. This indicates that the two mutant proteins are capable of interacting with each other and are transported together to the cell surface. Moreover, the failure to detect a prominent vesicular staining in this combination suggests that 10.4LL-14.5 complexes are not sorted into vesicles at the TGN, but rather following Y122Y-mediated endocytosis. We conclude that an intact 122YXXΦ motif in 14.5 is required for generation of the vesicular phenotype induced by the 10.4LL mutant. Hence, the 122YXXΦ motif acts upstream of the LL motif.

Enhanced Transport of 10.4–14.5 to Late Endosomes/Lysosomes in the Absence of the 10.4 Dileucine Motif—To identify the compartments in which wt 10.4–14.5 and their derivatives are localized, the two proteins were coexpressed from a single vector by transfection of pSG5/10.4-F14.5 (Fig. 6, A–C and G–I) or pSG5/10.4LL-F14.5 (Fig. 6, D–F and J–L), followed by double immunofluorescence with cellular markers. This vector system was used to ascertain that both viral proteins were coexpressed in the same cell. With this expression system 10.4 was exhibited, apart from the perinuclear staining seen before (Fig. 5A), a prominent ER staining (Fig. 6A). This is presumably due to lower amounts of 14.5 synthesized (relative to 10.4), as evidenced by the reduced 14.5 cell surface expression as compared with cotransfection of single expression vectors (data not shown). The perinuclear compartment was identified as the Golgi/TGN, because it stained with mAbs against TGN46 (Fig. 6C) and galactosyltransferase (data not shown). Partial colocalization was also observed with the transferrin receptor, a marker for the recycling endosome that is visualized in close proximity to the TGN in a perinuclear location. No costaining was seen with EEA1, a marker for early endosomes (data not shown). The few 10.4 and 14.5 positive vesicles did not colocalize appreciably with vesicles expressing late endosomal/lysosomal markers Lamp2 (Fig. 6A), lyso(bisphosphatidic acid (Fig. 6B), or TGN46 (Fig. 6C). The 10.4LL mutant was no longer detectable in the ER, but primarily in the Golgi/TGN where it colocalized with 14.5 (Fig. 6, D–F). This phenotype suggested enhanced export of the dileucine mutant from the ER or a reduced steady-state expression level, possibly caused by increased degradation. The latter suggestion is supported by the restoration of 10.4LL to wild-type levels upon Baf treatment of 293 transfectants (Fig. 3, D and E). Therefore, we tested whether Baf influenced steady-state localization of 10.4–14.5. Baf treatment of cells transfected with wt 10.4–14.5 induced the appearance of a small but significant number of 14.5 positive vesicles that colocalized with Lamp2 and partly with lyso(bisphosphatidic acid, indicating that wt 14.5 enters Baf-sensitive late endosomal/lysosomal compartments. No changes in ER/Golgi localization of 10.4 (Fig. 6, G–I) were observed. In cells expressing 10.4LL, Baf triggered the appearance of a large number of 10.4—14.5 vesicles that were identified as late endosomal/lysosomal compartments, as they colocalized with Lamp2 and partially also with lyso(bisphosphatidic acid (Fig. 6,}

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**Fig. 5. Intracellular localization of 10.4 and 14.5 analyzed by laser scanning confocal microscopy.** SV80Fas cells were transfected with different combinations of 10.4 and 14.5 wt and mutant expression plasmids: A, pSG5–10.4 and pSG5–14.5; B, pSG5–10.4 and pSG5–14.5Y122A; C, pSG5–10.4LLAA and pSG5–14.5; or D, pSG5–10.4LLAA with pSG5–14.5Y122A. Cells were stained for 10.4 and 10.4LLAA with Baf and R71/fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (left column) and for 14.5 with FLAG-specific mAb M1/rhodamine-labeled donkey anti-mouse IgG (right column). Bars = 10 μm.

stable 293 transfectants. In this combination, the 10.4 protein could also be detected at the plasma membrane and colocalized with 14.5 (Fig. 5B). We conclude that 10.4 and 14.5 are expressed at the cell surface, presumably as a complex. The increased cell surface staining of the 14.5Y122 mutant indicated that the 122YXXΦ motif in the cytoplasmic tail of 14.5 might be required for endocytosis of the 10.4–14.5 complex and that in its absence the dileucine pair in 10.4 alone is unable to mediate efficient endocytosis. When the 10.4LL mutant is coexpressed with wt F14.5, an increased staining of intracellular vesicles, but no surface staining of 10.4 or 14.5 was observed (Fig. 5C). Interestingly, 14.5 colocalized extensively with 10.4LL vesicles, indicating that the dileucine mutation in 10.4 also affected trafficking of 14.5. This mutual dependence of 10.4 and 14.5 localization on the integrity of the studied trafficking motifs confirms previous data that the proteins act as a complex and strongly suggests that trafficking of the complex depends on signals in both proteins.
Disruption of the dileucine motif in 10.4 influences the steady-state localization of 10.4 and 14.5. SV80Fas cells were transfected with pSG5/10.4-F14.5 (A–C and G–L) and pSG5/10.4LL-F14.5 (D–F and J–L), respectively, and processed for confocal microscopy 40 h post-transfection. Localization of 10.4 or 14.5K (indicated on top, green) was compared with that of marker proteins (depicted on the left, red) for different cellular compartments: Lamp2 (LE/lysosomes), lysobisphosphatidic acid (LBPA), TGN46 (TGN), with (G–L) or without (A–F) prior Baf treatment (11 h, 100 nM). Bars = 10 μm. The antibodies used are given in Fig. 5 and under “Experimental Procedures.”
J and K), but not with TGN46 (Fig. 6L). These findings corroborated our previous conclusion that mutation of the dileucine motif of 10.4 causes enhanced degradation of both 10.4 and 14.5 and suggest that the dileucine motif actively prevents efficient transport to lysosomes, thereby protecting 10.4–14.5 from degradation. We hypothesize that the LL motif achieves this by directing the viral proteins into a recycling compartment.

Recombinant Ads Expressing 10.4–14.5 Mutants Are Defective in Receptor Down-modulation—To examine the phenotype of mutant 10.4–14.5 proteins in their natural viral context and to extend the analysis to primary cells and cells expressing other 10.4–14.5 target molecules, we incorporated the mutations that caused a loss of 10.4–14.5 function in stable transfectants into the Ad genome. With a novel method that is independent of any restriction sites we created recombinant adenoviruses expressing wt (Ad2/F14.5) and mutant versions of FLAG-tagged 14.5. Similar to wt Ad2 (13), Ad2/F14.5 infection of A549 lung epithelial cells essentially abrogated cell surface expression of Fas, EGFR, DR4, and DR5 (>90% reduction), as measured by FACS analysis at 21 h post-infection (Fig. 7, A–D). By contrast, recombinant Ads not expressing 10.4 (Ad2/10.4ko-F14.5), 14.5 (Ad2/14.5ko), or both (Ad2/10.4–14.5ko) were unable to down-modulate apoptosis receptors, but rather induced their cell surface expression. The latter can be explained by Ad-induced activation of NF-κB, which stimulates Fas and TRAIL-R promoters (57–59) or by transient up-regulation of Fas on the cell surface by Ad-induced p53 activation (60). Independent of which 10.4–14.5 knock-out Ad was utilized, EGFR levels consistently declined to about 80% of that in uninfected cells, indicating an additional 10.4–14.5-independent modulation, presumably caused by E1A-induced transcriptional repression (61). Of note, the Ad2/14.5ko virus reduced EGFR surface expression levels by an additional 20%, indicating some residual activity of 10.4 alone (19). Confirming the data obtained in transfectants, Ad2/F14.5Y122 was unable to down-regulate any target receptor. DR4 was even substantially up-regulated. Thus, the Y122A mutation completely abolished the function of the 10.4–14.5 complex. Furthermore, in line with the accumulation seen in 293 transfectants, the Y122A-substituted F14.5 protein accumulated to 2.7-fold higher levels at the plasma membrane compared with wt F14.5 (Fig. 7E). In contrast, F14.5 surface expression levels reached only 13% of wt after infection with Ad2/10.4LL-F14.5, a level similar to that seen in 10.4LL transfectants or upon infection with the double knock-out virus. However, whereas down-regulation of the EGFR and DR5 were as effectively compromised as upon infection with 14.5ko virus, Fas and DR4 levels were reduced by 56 and 32%, respectively, compared with infection with the double knock-out virus. Thus, Ad2/10.4LL-F14.5 retained some functional activity toward Fas and DR4. We conclude that during infection the 122YXXΦ motif is required for down-regulation of all target proteins, whereas the LL motif of 10.4 is essential for the modulation of the EGFR and DR5 and is less critical for modulation of DR4 and Fas. This differential effect indicates mechanistic differences in targeting of the two sets of receptors. A differential effect was also observed for the Ad2/14.5Y74 mutant that exhibited normal F14.5 surface expression and retained the capacity to down-regulate Fas and DR4, but induced only a partial down-modulation of the EGFR and DR5 (activities: 62 and 73%, respectively; Fig. 7). This pheno-

![Graph](image-url)

**Fig. 7. Functional activity of 10.4 and FLAG-14.5 mutants upon incorporation of mutations into the Ad2 genome.** A549 cells were infected with equal plaque forming units of wt Ad2/F14.5 or mutant Ads, Ad2/10.4–14.5ko, Ad2/10.4ko-F14.5, Ad2/14.5ko, Ad2/10.4LL-F14.5, Ad2/F14.5Y122, and Ad2/F14.5Y122 as indicated below the figure. At 21 h post-infection cell surface expression of Fas (A), DR4 (B), EGFR (C), DR5 (D), and FLAG-14.5 (E) was measured by FACS analysis and related to that of uninfected A549 cells (A–D) or Ad2/F14.5 wt-infected cells (E), analyzed in parallel and set to 100%. Data are compiled from at least three independent experiments. Error bars represent mean ± S.E.
Fig. 8. 10.4–14.5K complex formation upon infection of A549 cells with recombinant Ads. A, digitonin extracts of infected (21 h post-infection) and mock-infected A549 cells were subjected to immunoprecipitation with the 14.5 specific antiserum Bur4 and the presence of 10.4 was visualized as described in the legend to Fig. 3. Ad2 mutants are depicted on the top. B, total amounts of 10.4, 14.5, and 19K in Triton extracts were determined as described in the legend to Fig. 3.

The recombinant adenovirus mutants allowed us to express and localize mutant 10.4–14.5 proteins also in primary cells (Fig. 9). In comparison to the ER/Golgi/PM staining seen for wt proteins (Fig. 9A), infection with the Ad2/F14.5Y122 mutant resulted in a prominent labeling of cellular extrusions, indicating a markedly increased surface expression of 10.4–14.5 (Fig. 9B). This accumulation is in accord with the FACs data and the proposed role of the YXXΦ motif in mediating internalization of the 10.4–14.5 complex. In contrast, primary fibroblasts infected with Ad2/10.4LL exhibited a highly vesicular staining of 10.4LL-14.5 (Fig. 9C), even without Baf treatment. After Baf treatment, many of these 10.4LL+ vesicles either coalesced with or were closely attached to vesicles expressing the late endosomal/lysosomal marker Lamp2 (Fig. 9D). The profound redistribution of 10.4LL-14.5 into these vesicles accompanied by the reduced ER staining indicated an increased ER export rate and a more efficient delivery of the complex to lysosomes. We hypothesize that the LL motif prevents efficient sorting of the 10.4–14.5 complex to lysosomes by facilitating its retrieval from the endosomal/lysosomal compartment into a recycling pathway to the plasma membrane. In its absence, the vast majority of 10.4LL complexes are destined for degradation in lysosomes.

The proposed role of LL for recycling implies that a considerable fraction of 10.4–14.5 dissociates from and only transiently interacts with target molecules. Therefore, we investigated whether or not 10.4–14.5 reached the same endosomal/lysosomal vesicles in which Fas accumulates upon bafilomycin treatment (13). A comparison of the steady-state localization of 14.5 and Fas in infected Baf-treated SV80Fas cells (Fig. 9E) revealed an enhanced transport of 14.5 into vesicles, but strikingly only a few of these colocalized with Fas+ late endosomes/lysosomes.
lysosomes. This distinct vesicular distribution of Fas and 10.4–14.5 at steady-state suggests that the association of Fas with the viral proteins is indeed short-lived.

DISCUSSION

We have identified two trafficking motifs that are crucial for 10.4–14.5 function. To be recognized transport motifs have to retain a minimal distance of 5–7 aa from the lipid bilayer (36). Of the three XXXΦ motifs present in Ad2 14.5, two with Tyr at positions 74 and 76 might be located adjacent to or even within the lipid bilayer. Therefore, it was not surprising that mutation of the non-conserved Tyr76 had no profound effect on the capacity of 10.4–14.5 to down-regulate Fas and the EGFR (Fig. 2). However, mutation of the strictly conserved tyrosine Tyr74 nearly abrogated functional activity in transfected 293 cells (Fig. 2B). This deficiency correlated with a marked reduction of 10.4–14.5Y74 complexes (Fig. 3A). Upon infection a similar reduction of Triton-stable complexes was noted although in digitonin extracts complex formation was hardly affected. Accordingly, surface expression was unaltered. The increased amount of 14.5 degradation product (Fig. 8, lane 3) hints to a higher degradation rate. At present, it is unclear whether the decreased amount of complexes is a direct consequence of the lower affinity of Ad2 14.5Y74 to 10.4 or of the decreased levels of mutant 14.5 in the ER, caused by inefficient complex formation and subsequent degradation. We suggest that, rather than being part of a transport motif, Tyr74 is required for efficient interaction with 10.4 in the ER or in an endocytic compartment. In Ad2/F14.5Y74-infected cells, target receptors were differentially affected. Whereas EGFR and DR5 modulation was inefficient, Fas and DR4 removal from the cell surface was similar to that in Ad2/F14.5-infected cells (Fig. 7). This indicates that (i) increased expression of 14.5Y74 (and 10.4) during infection can overcome inefficient 10.4–14.5 association, and (ii) differential sensitivity of target molecules to the mutation may be due to their differential affinity for RID (Fas/DR4 > EGFR/DR5); the higher the affinity of target receptors the more easily the negative effect of the mutation on modulation is compensated by higher expression of mutant 10.4–14.5. Alternatively, the mechanism of target receptor modulation may not be identical.

The XXXΦ motif in 14.5 with Tyr in position 122 is absolutely crucial both for function and trafficking. In 293 transfectants and in all cells infected with Ads expressing the 14.5Y122 mutant, down-regulation of apoptosis receptors and the EGFR was abolished (Figs. 2B and 7). The importance of the equivalent residue (Tyr122) in Ad5 14.5 was noted in a study using a heterologous overexpression system in COS7 cells aimed at identifying residues involved in signal transduction (25). The authors speculated that Tyr122 may have a function in endocytosis although trafficking of 14.5 was not investigated. We show here by directly monitoring 14.5 trafficking that mutation of Tyr122 caused a dramatic increase in F14.5Y122 cell surface expression as detected by FACS (270–400%; Figs. 4 and 7) and immunofluorescence, which also reveals a corresponding increase of 10.4 cell surface expression. This is accompanied by significantly increased steady-state levels of 10.4 and 14.5 and higher amounts of detectable complexes (Figs. 3 and 8). Together with the crucial importance of Tyr122 for efficient binding of the 14.5 tail peptide to AP-1 and AP-2 in vitro and the higher affinity of the 14.5 tail for AP-2, the data suggest that Tyr122 is the key residue of a strong tyrosine-based transport motif that is recognized by AP-2 and thereby triggers endocytosis of the 10.4–14.5 complex. Our data do not exclude the possibility that Tyr122 has an additional function in intracellular sorting.

Interestingly, the 10.4 tail also contains a sequence element important for function and transport. Mutation of the dileucine motif at position 87/88 in 10.4 abolished the function of RID in 293 transfectants (Fig. 2). In contrast to 14.5Y122, lack of receptor modulation was associated with a drastic reduction of 10.4LL-14.5 cell surface expression both in transfected and infected cells (Figs. 4 and 7) and a dramatically decreased stability of 14.5 and 10.4–14.5 complexes (Fig. 3). Baf treatment rescued 10.4LL-14.5 from degradation and induced the accumulation of the proteins in intracellular vesicles that colocalized mostly with late endosomal and lysosomal markers (Figs. 6 and 9). Baf also considerably increased the stability of wt RID, suggesting that normally a proportion of RID is transported to and degraded in LE or lysosomes. Although interpretation of the infection experiments was complicated by viral-induced regulatory phenomena (58, 61, 62), the important role of the LL motif for trafficking and target protein modulation was confirmed. Interestingly, target molecules are differentially affected by substitution of 10.4LL. Modulation of DR5 and the EGFR is essentially abrogated, whereas DR4 and Fas surface levels are significantly reduced. Strikingly, cell surface expression of 14.5 reaches only ~15% of that seen upon infection with wt Ad2 (Fig. 7). As the LL mutation has no detrimental effect on 10.4LL-14.5 association in infected cells (Fig. 8), and 10.4LL is efficiently transported to the cell surface on coexpression with 14.5Y122 (Fig. 5D), the dramatically reduced cell surface expression with wt 14.5 is likely to be caused by subsequent events requiring endocytosis. This is supported by the dependence of the vesicular LL phenotype on an intact 122XXΦ internalization motif. The increased amounts of 14.5 degradation products (Fig. 8, lane 5) together with the vesicular localization (Fig. 9) and Baf sensitivity suggests that replacement of the LL motif strongly enhances RID degradation in an late endocytic compartment. Together with the SPR data it is suggested that LL functions as a transport motif to prevent extensive degradation of the 10.4–14.5 complex in LE/lysosomes.

Exactly how LL mediates protection remains to be defined. The phenotype of cells coexpressing 10.4LL and 14.5Y122 mutants (accumulation of RID on the cell surface, lack of RID+ vesicles) shows that the LL motif functions downstream of the 122XXΦ motif. We propose that LL rescues RID from degradation by directing a substantial pool of RID into a recycling pathway to the plasma membrane, possibly via the Golgi or the recycling endosome. The latter is considered as some colocalization with the transferrin receptor was observed (data not shown). The proposed defect in recycling is consistent with the dramatic reduction of 14.5 cell surface display in cells expressing the 10.4LL mutant (Figs. 4 and 7). The compartment in which LL may act and mediate recycling (EE, the limiting membrane of MVBs, and recycling endosome) remains to be identified. In any case, the SPR data suggest that LL is recognized by AP-1 or AP-2. This is consistent with the function of AP-1A in retrograde endosome-TGN transport (40). We cannot rule out that AP-3 and AP-4 (39) contribute to sorting of 10.4–14.5. No evidence was found that LL mediates endocytosis or synergizes with LL signals of the EGFR by “signal multimerization” to direct EGFR to the internal membranes of MVBs (34, 63). Instead, LL might be the postulated endosomal retrieval/retention signal in 10.4 (34). The distribution of 10.4LL-14.5 differs from that of the LL mutant of the cationic-dependent mannose 6-phosphate receptor that accumulates in EE, but also at the plasma membrane because of impaired sorting within endosomes and a failure to return to the TGN (64). Recycling of 10.4–14.5 complexes might be beneficial for the virus for two reasons: first, recycling might enhance down-modulation efficiency by increasing the available concentration.
of RID and by allowing its repeated usage. Second, recycling may also enable the specific targeting of receptor populations in recycling compartments.

In summary, we suggest that full activity of 10.4–14.5/RID requires the combined action of two sorting motifs, Leu$_{67}^{10.4}$–Leu$_{68}^{14.5}$ in 10.4 and Leu$_{122}^{14.5}$ in 14.5. The data also imply that entry of RID into the endosomal system occurs via the plasma membrane and not from the TGN. The following mechanism for 10.4–14.5-mediated down-modulation of target receptors is suggested (Fig. 10). 10.4–14.5 assemble in the ER, which is required for efficient transport of both proteins to the cell surface. The AP-2 adaptor may recognize the 14.5Leu$_{122}$ sorting signal at the cell surface, thereby recruiting the complex into coated pits and inducing its rapid internalization into early endosomes. It is still unclear whether RID associates with its target proteins at the plasma membrane (1) or only after internalization in EE (2). If RID interacts with target molecules on the cell surface, then changes in 14.5 cell surface expression induced by mutations should be followed by a corresponding modulation of its target receptors. This is not the case. Instead, we observe disparate cell surface phenotypes for the F14.5Y122 and 10.4LL mutants and their target molecules. This is particularly obvious in cells expressing 10.4LL. Here high expression of target receptors contrasts with a minimal 14.5 surface expression (Figs. 4 and 7). The 4-fold up-regulation of 14.5Y122 is also not followed by a comparable increase in Fas, EGFR, and DR5. The 1.7-fold higher cell surface expression of DR4 in cells infected with the 14.5Y122 mutant virus may indicate though that some association with DR4 can occur at the plasma membrane. Therefore, these data provide strong evidence that interaction between RID and most target receptors takes place in EE, rather than on the plasma membrane. Our findings are consistent with our earlier data (13) and with an immunochemical study by Carlin and co-workers (34) who could detect 10.4 complexed with constitutively internalized EGFR in EE and MVBs after overexpression of both molecules. No other experimental evidence has been found as yet for a direct physical interaction between RID and its targets. One explanation might be that the interaction is either mediated by other cellular proteins (Figs. 10, × ) or is short-lived. Support for the latter comes from the limited colocalization of 14.5 and Fas in endosomal/lysosomal compartments (Fig. 9). We propose that RID-associated receptors dissociate as they encounter the progressively lower pH in endosomes or following their modification. After segregation from RID (possibly in MVBs) target proteins proceed to lysosomes where they are degraded.

It thus appears that 10.4–14.5 redirect the EGFR and apoptosis receptors to the same degradative compartment as their ligands. The molecular mechanism as to how the 10.4–14.5 membrane proteins may mediate this sorting step remains to be clarified. Segregation of cargo destined for degradation in lysosomes from material to be recycled occurs in MVBs (27, 28, 32, 33, 65). One sorting tag directing proteins, such as the EGFR, into the luminal membranes of MVBs has been identified as ubiquitin (66). It will be interesting to test whether 10.4–14.5 also stimulate ubiquitination of the EGFR and the other RID target molecules.

Alternatively, RID target molecules might be sorted to internal vesicles of MVBs and lysosomes independent of ubiquitin. One mechanism proposed is based on segregation of lipids. Integral membrane proteins with special properties of the transmembrane domain, e.g. containing polar residues, may be partitioned together with certain lipids to the internal vesicles of MVBs (67, 68). Interestingly, the transmembrane domains of both 10.4 and 14.5 proteins contain an unusually high number (6–10) of polar residues, like serine, threonine, cysteine, or tyrosine. This feature is conserved in RID proteins of Ads from all subgenera (1). Therefore, it is attractive to speculate that 10.4–14.5 may reroute target proteins to the luminal membranes of MVBs by binding to and providing a polar shield to the intrinsically hydrophobic TMDs of Fas, DR4/5 (3 polar aa), and the EGFR (none). Such a scenario is consistent with the observed Baf sensitivity of wt RID levels and implies that a fraction of wild-type 10.4 and 14.5 is sorted to lysosomes.

It is puzzling how RID can target and reroute these structurally very diverse molecules; EGFR with tyrosine kinase activity required for sorting, and apoptosis receptors that lack such an activity, but have instead a death domain. Little is known about trafficking of Fas and TRAIL receptors (69), although they are generally regarded as plasma membrane receptors. Therefore, it was surprising that in untransformed vascular smooth muscle cells Fas was detected predominantly in the Golgi/TGN and to a lesser degree on the cell surface (60). More recently, it was shown that Fas stimulation results in microaggregation and clustering, followed by internalization and transport into transferrin receptor-positive endosomes (70). Thus, Fas can enter the endocytic pathway in the presence of ligand. Inspection of the Fas, TRAIL-R1, and TRAIL-R2 sequences reveals a YXXΦ motif and multiple potential LL transport motifs. Although it is unclear whether these motifs are involved in death receptor trafficking, a common property of RID target proteins emerges, in that all target molecules seem to share the intrinsic capacity to enter the endosomal/lysosomal system upon ligand binding. Therefore, it will be interesting to test whether these putative transport motifs contribute to target modulation by 10.4–14.5.

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