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Retrovirus-Mediated Transfer of an Adenovirus Gene Encoding an Integral Membrane Protein Is Sufficient To Down Regulate the Receptor for Epidermal Growth Factor

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We have used retrovirus-mediated gene transfer to introduce sequences encoding a 10,400-molecular-weight (10.4K) adenovirus protein previously shown to down regulate the receptor for epidermal growth factor (EGF) into two murine cell lines that possess human EGF receptors (EGF-Rs). Assays for receptor expression showed that acute infection resulted in rapid, constitutive down regulation of the EGF-R via a pathway that appears to be endosome mediated. This represents the first demonstration that 10.4K expression in the absence of other virus-encoded proteins is sufficient to elicit this response. The usefulness of this approach for the study of 10.4K-mediated signal transduction in cells with a nontransformed phenotype is discussed.

The epidermal growth factor (EGF) receptor (EGF-R) is a ligand-activated protein tyrosine kinase involved in regulation of normal growth. We have recently shown that group C adenoviruses encode a protein of predicted molecular weight (MW) 10,400 (10.4K protein) that is not related to EGF but nevertheless causes EGF-R down regulation in the absence of ligand (5). The E5 transforming protein of bovine papillomavirus also appears to act on the EGF-R, but by inhibiting rather than promoting receptor down regulation (13); it will be interesting to compare the biological responses elicited by the E5 and 10.4K molecules, considering their antagonistic actions on the stability of EGF-R in the plasma membrane. The original observation that the 10.4K protein was required for EGF-R down regulation was based on analysis of an extensive series of virus mutants with deletions throughout the early region of the adenovirus genome, including each of the nine open reading frames within the E3 transcription unit that encodes 10.4K (5). It was impossible, however, to exclude the possibility that other viral proteins played an accessory role, particularly if there is some redundancy in the adenovirus genome. If the 10.4K protein is sufficient to elicit this response, then cells expressing this protein in the absence of other adenovirus proteins should undergo EGF-R down regulation. To test this hypothesis, we have isolated the 10.4K-encoding gene and introduced it into mammalian cells by using retrovirus-mediated gene transfer.

Psi-cre packaging cells with stably integrated DOL-10.4K DNA synthesize 10.4K-specific transcripts and proteins. Sequences encoding the 10.4K protein were excised from a plasmid containing the *Eco* D fragment of the adenovirus mutant pm760 (3) and ligated into pBR322. This manipulation eliminated coding sequences for two amino acids and the translational stop codon from the C terminus of the 10.4K protein. A *Bgl*II-*Eco*RI fragment containing nucleotides 566 to 2437 of the E3 transcription unit was excised and ligated into pGEM-4 (Promega Biotec), and synthetic *Bam*HI linkers (New England BioLabs, Inc.) were introduced at the unique *Stu*I site at nucleotide 2075. The wild-type 10.4K C terminus was reconstructed by ligating a 21-base-pair synthetic oligonucleotide (ATTCTTTAAGG ATCCGAATTC; Operon Technologies) to the *Eco*RI site at nucleotide 2437. The reconstruction also generated a new *Bam*HI site (underlined above), to facilitate ligation of the 10.4K-coding *Bam*HI fragment into the unique *Bam*HI site of the Moloney murine leukemia virus (MLV)-based DOL plasmid (12), with 10.4K transcription driven constitutively from the MLV long terminal repeat (LTR) (Fig. 1). DOL and DOL-10.4K plasmids were transfected into psi-cre retroviral packaging cells (8) by using lipofectin reagent (Bethesda Research Laboratories, Inc.) (9), and clones with stably integrated plasmid DNA were selected by using G418 (400 μg of active antibiotic per ml; Geneticin; GIBCO Laboratories). Cell lines were screened by slot blot analysis of RNA isolated from virions shed into culture supernatant, using probes specific for the neomycin resistance (Neo^r) or 10.4K gene sequence; RNA prepared from supernatants conditioned by DOL-containing cells hybridized to the Neo^r probe only, whereas RNA from supernatants conditioned by DOL-10.4K-containing cells hybridized to both probes (data not shown). Identical results were obtained when cytoplasmic RNA isolated from cells shedding retrovirus containing either DOL or DOL-10.4K was analyzed by using Neo^r and 10.4K-specific probes (data not shown).

We next examined psi-cre cells with stably integrated DOL or DOL-10.4K DNA for expression of 10.4K-related protein species. Previous studies have shown that the primary 10.4K translation product has an apparent MW slightly larger than that predicted from amino acid sequence; a second protein that migrates ahead of the primary translation product is also detected in adenovirus-infected cells (19). Extracts from metabolically labeled psi-cre cells were immunoprecipitated by using an antiserum directed against a

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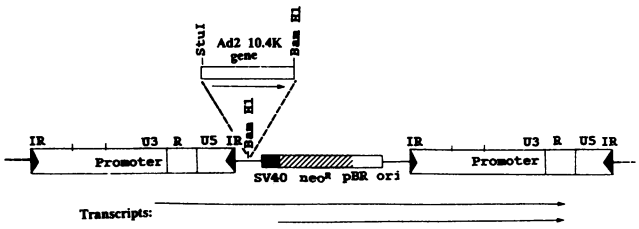


FIG. 1. Diagram of the DOL retroviral vector containing the 10.4K gene insert under regulation of the 5′ MLV LTR. This vector also has the Neo^r gene as a dominant selectable marker driven by the simian virus 40 early promoter, pBR322 *ori* for rescue of the vector as a bacterial plasmid, and the polyomavirus early region to facilitate transient rescue of virus (not shown). Arrows indicate the direction of transcription of the 10.4K gene from the MLV LTR and the Neo^r gene from the simian virus 40 promoter.

synthetic peptide representing the predicted C-terminal 15 amino acids of the 10.4K protein. Two bands of approximate MW 13,700 and 11,300 were detected in cells containing DOL-10.4K but not in psi-cre cells containing DOL only (Fig. 2A). The sizes of these proteins correspond to those of the 10.4K-related molecules detected in cells infected with adenovirus. Both 10.4K-related proteins also partitioned into Triton X-114 (Fig. 2B), a characteristic of integral membrane proteins (2).

10.4K transcripts and proteins are expressed in target cells after acute infection with retrovirus containing DOL-10.4K. Two murine cell lines that express human EGF-Rs were

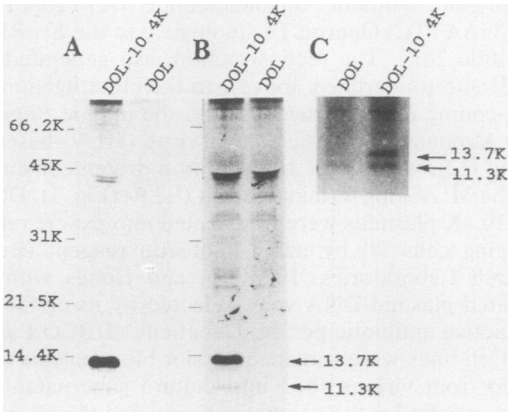
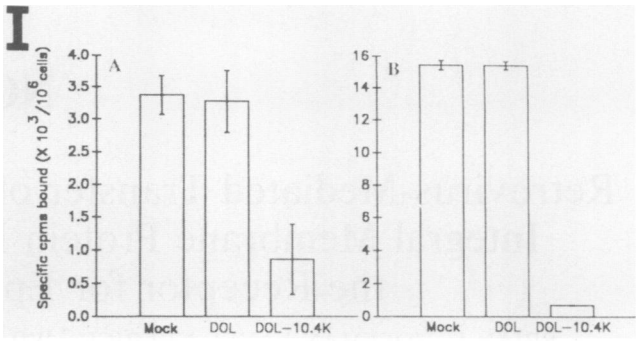


FIG. 2. Analysis of 10.4K protein expression in retroviral packaging cells (A and B) or HERc cells after infection with DOL or DOL-10.4K (C). Target HERc cells were plated in 100-mm dishes at a density of 5×10^4 cells 24 h before retroviral infections. Retrovirus was harvested from culture supernatants conditioned for 24 h by near-confluent packaging cells, filtered through 0.45-μm-pore-size Acrodisc filters (Gelman Sciences, Inc.), and supplemented with 8.0 μg of polybrene (Sigma Chemical Co.) per ml. Cells were metabolically labeled for 4 h with L-[^{35}S]cysteine and extracted with 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) (A and C) or 1% Triton X-114 by the method of Bordier (2) (B). Cell extracts were immunoprecipitated by using a 10.4K-specific synthetic peptide antibody, and immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The positions of 10.4K-related protein species are indicated by arrows to the right. Apparent MWs were derived from linear regression analysis based on the migration of MW standards: bovine serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400 (r = 0.99).



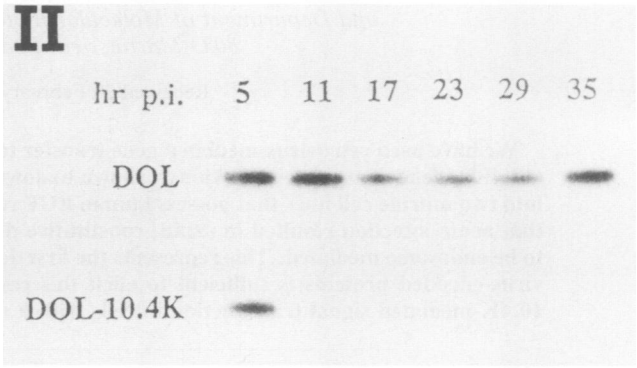


FIG. 3. Analysis of EGF-R abundance in cells expressing DOL or DOL-10.4K. (I) Psi-cre packaging cells transfected with DOL or DOL-10.4K (A) or cl 21 cells infected with DOL or DOL-10.4K (B) were analyzed for specific ^{125}I-EGF binding. ^{125}I-EGF binding assays were carried out for 30 min at 37°C, using receptor-grade mouse EGF (Sigma) radioiodinated by the chloramine-T method (11) (specific activity was approximately 2×10^8 cpm/μg of protein). Total binding was measured by using 5 ng of ^{125}I-EGF per ml of serum-free Dulbecco modified Eagle medium supplemented with 0.2% (wt/vol) bovine serum albumin (radioimmunoassay grade; Sigma); nonspecific binding was determined by using a 500-fold excess of unlabeled EGF and was less than 10%. Cells were solubilized with 1 M NaOH, and radioactivity was determined in a Beckman model 310 gamma counter. All determinations were done in duplicate. Error bars represent the sum of the standard deviations of total and nonspecific binding. (II) Time course of EGF-R down regulation in HERc cells after infection with DOL-10.4K retrovirus. Cells were infected and harvested for extraction with 1% Nonidet P-40 at the intervals indicated. Cell extracts were immunoprecipitated by using the EGF-R1 monoclonal antibody, and receptor autophosphorylation was catalyzed in the immune complex. Reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Control infections were carried out by using the DOL retrovirus.

used as targets for acute retroviral infections: HERc cells are NIH 3T3 cells devoid of endogenous EGF-R transfected with a plasmid containing the cDNA encoding the entire human, EGF-R, mouse dihydrofolate reductase, and Neo^r genes (18); Cl 21 cells are human-mouse hybrid cells that contain several copies of human chromosome 7 (7), which encodes EGF-R (14). Whereas cytoplasmic RNA prepared 24 h after acute infection of target cells with retrovirus containing DOL hybridized to probes derived from the parental plasmid (pBR322 *ori* for HERc cells and Neo^r for cl 21), RNA from cells infected with retrovirus containing DOL-10.4K hybridized to these probes as well as to a 10.4-specific probe (data not shown). We also found that both 10.4K-related protein species were expressed in target

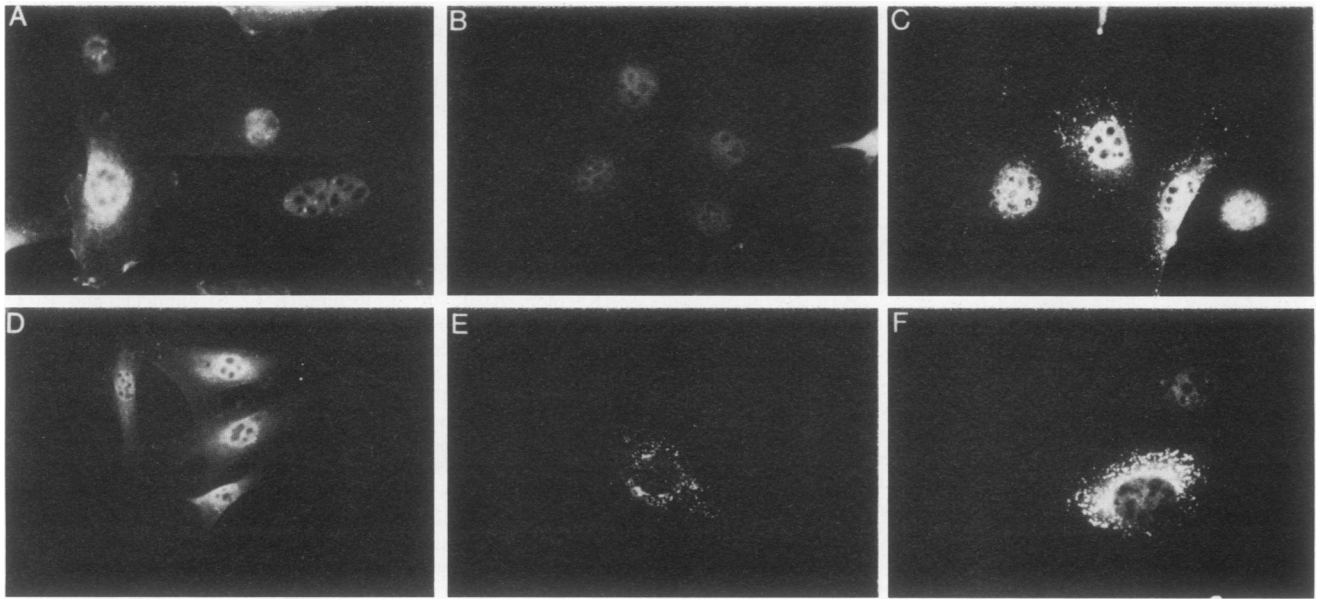


FIG. 4. Indirect immunofluorescence analysis of intracellular EGF-R distribution in HERc cells. Cells were fixed with 3.7% paraformaldehyde and permeabilized with 0.2% Triton X-100. Staining was with the EGF-R1 monoclonal antibody (1:100) for 1 h, followed by affinity-purified fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (1:200; Organon Teknika) for 30 min; control staining was carried out by using second reagent only. Cells received the following treatments: (A) None; (B) stimulation with EGF (100 ng/ml) for 30 min, staining with secondary reagent only; (C) stimulation with EGF for 30 min; (D) infection with DOL retrovirus; (E and F) infection with DOL-10.4K retrovirus. Cells in panels D to F were fixed at 11 to 12 h postinfection. Magnification, ×210.

cells after acute infection with retrovirus containing DOL-10.4K (Fig. 2C).

The EGF-R is down regulated in target cells after acute infection with retrovirus containing DOL-10.4K. We next performed ^{125}I-EGF binding assays, using the psi-cre packaging cells described above and target cells after retrovirus-mediated gene transfer. Binding by psi-cre cells expressing DOL-10.4K was reduced by approximately 75% compared with binding of untransfected cells or cells containing DOL DNA only (Fig. 3IA). When acutely infected cl 21 cells were analyzed 30 h after addition of fresh supernatant containing DOL-10.4K retrovirus, there was essentially no EGF-binding capacity (Fig. 3IB). This result was in sharp contrast to that for cells which had been exposed to DOL retrovirus, in which binding was equivalent to that seen in mock-treated cells. Similar results were obtained when HERc cells were used as targets of acute infection (not shown).

The reduction in EGF-binding capacity was shown to be due to loss of functional receptor protein in an immune complex assay for receptor autophosphorylating activity. Target HERc cells were exposed to retrovirus containing either DOL or DOL-10.4K, and cell extracts prepared at several intervals after infection were immunoprecipitated by using a monoclonal antibody specific for the human EGF-R (EGF-R1; 20). When immune complexes were labeled with [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont NEN Research Products) (4), EGF-R autophosphorylating activity was virtually absent by 11 h after infection with DOL-10.4K and did not reappear during the period tested in this experiment (Fig. 3II). In contrast, autophosphorylating activity was unchanged in cells exposed to DOL retrovirus.

Finally, morphological evidence strongly suggests that removal of EGF-R from the surface of acutely infected cells occurs via an endosome-mediated mechanism. HERc cells exposed to retrovirus containing either DOL or DOL-10.4K were fixed, permeabilized, and stained for immunofluorescence by using EGF-R1. Staining of cells that had been infected with DOL-10.4K retrovirus revealed a punctate pattern identical to that seen shortly after stimulation with EGF (Fig. 4); these brightly staining vesicles are thought to represent endosomes containing internalized ligand-receptor complexes (1). In some acutely infected cells, receptor molecules appeared to have accumulated in larger vesicles, suggesting that endosomes had fused with lysosomes (Fig. 4F). Ligand-receptor complexes are also delivered to lysosomes in cells stimulated with EGF (6). Putative endocytotic vesicles began to appear approximately 10 h after addition of DOL-10.4K retrovirus and could be seen in some cells as late as 12 to 13 h postinfection. Hence, there is good agreement between results presented here and those in Fig. 3II, which showed that loss of functional receptor molecules occurs during the same interval. The intracellular distribution of EGF-R molecules in cells infected with DOL retrovirus was unchanged (Fig. 4D).

To summarize, we have used retrovirus-mediated gene transfer to demonstrate that constitutive expression of a 10.4K protein encoded by human group C adenoviruses is sufficient to induce endosome-mediated EGF-R down regulation. EGF-R-binding capacity is also reduced in packaging cells transfected with the DOL-10.4K plasmid, although not to the same extent as in target cells assayed after acute infection with retrovirus; it is interesting to consider whether the packaging cells have activated a regulatory mechanism(s) that allows for steady-state expression of 10.4K and EGF-R. The finding that the 10.4K protein is membrane associated (19) has led us to postulate that there is a direct or indirect interaction between EGF-R and 10.4K within the microenvironment of the plasma membrane. Studies with adenovirus, however, did not rigorously exclude the possibility that 10.4K induces expression of an EGF-like molecule which then acts as an autocrine growth factor. Evidence presented here, however, suggests that this is probably not

the case, since receptor internalization would presumably occur within minutes after the addition of media conditioned by packaging cells expressing 10.4K if a secreted growth factor were present. Future studies to evaluate the ability of 10.4K to induce late events in the EGF-R signaling pathway can be addressed in a straightforward manner by using human diploid fibroblasts grown in serum-free defined media (e.g., reference 17). Although these cells do not stably integrate plasmid DNA (10), foreign genes are expressed at high frequency after retrovirus-mediated gene transfer (15, 16). Results presented here show the feasibility of exploiting this approach to study the consequences of 10.4K expression in nontransformed cells.

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