Involvement of cellular adhesion sequences in the attachment of adenovirus to the HeLa cell surface

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Immunofluorescence analysis of HeLa cells incubated with human adenovirus serotype 2 (Ad2) inoculum suggested that Ad2 receptors co-localized with the receptors of fibronectin (FN⁰⁰) and vitronectin (VN⁰⁰) at the cell surface. Ad2 adsorption also resulted in the occurrence of intracytoplasmic actin cables with submembranal anchorage. The cell binding of Ad2 virions, pentons and fibres was found to be efficiently inhibited by concanavalin A, laminin, anti-FN R and anti-VN R antibodies. Arginine--glycine-aspartyl tripeptide (RGD) and other related peptides reproducing cellular adhesion sequences of adhesion proteins also competed with Ad2 for cell adsorption, and drastically reduced the virus progeny yield at the end of the infectious cycle. Data from binding competition assays with Ad2 virions showed that the apparent affinity constants of RGD motif-containing peptides for Ad2 receptor ranged from $0.75 \times 10^8$ M$^{-1}$ to $2.2 \times 10^8$ M$^{-1}$, with a number of peptide recognizing sites varying from $1.5 \times 10^4$ to $9 \times 10^4$ per cell for the different peptides studied. Polypeptide analysis of labelled plasma membrane fractions isolated after cross-linking to unlabelled Ad2 virions showed three major protein species with apparent M r of 130K, 60K and 44K, respectively, reacting with anti-FN R and anti-VN R antibodies. These results suggested that Ad2 and extracellular matrix proteins recognize similar adhesion sequences at the surface of HeLa cells, or alternatively that integrins and Ad2 receptors have overlapping ligand specificity.

Introduction

The virions of adenovirus (Ad) reach their nuclear replication site within the susceptible cell via a complex, multistep route comprising (i) attachment of infectious virus particles to plasma membrane receptors (Defer et al., 1990; Svensson & Persson, 1984; Svensson et al., 1981), (ii) clustering of the virus receptors at the cell surface (Hennache et al., 1979, 1982; Persson et al., 1983; Silver & Anderson, 1988), (iii) internalization of receptor-bound virions by invagination into a clathrin-coated vesicle (FitzGerald et al., 1983; Seth et al., 1984), (iv) release of altered virions from the endocytotic vesicle and transit through the cytoplasm (Defer et al., 1990; Svensson, 1985), (v) binding to the nuclear pore complex (Morgan et al., 1969) and (vi) translocation into the nucleoplasm (Chardonnet & Dales, 1970; Morgan et al., 1969). Cellular permissiveness and tissue tropism of a virus in general are in part under the control of the first step, and hence depend upon the expression of virus receptors at the cell surface (reviewed in Crowell et al., 1988).

Results from earlier studies on the inhibition of Ad binding to erythrocytes by amino acid side-chain-blocking reagents have suggested that basic residues on Ad fibre interact with acidic groups of membrane glycoproteins (Neurath et al., 1970). The putative carboxyl groups involved in Ad binding did not seem to belong to sialic acids, as inferred from binding data using various neuraminidase-treated mammalian cells (Boulanger et al., 1972). The lectin concanavalin A (ConA), which has been identified as an integrin ligand (Albelda & Buck, 1990), has been reported to interfere negatively with the cellular attachment of Ad2 fibre (Hughes & Mautner, 1973). The number of receptor sites has been estimated to be $3 \times 10^3$ to $6 \times 10^3$ per HeLa cell for Ad2 (Persson et al., 1985; Phillipson et al., 1968), and $7 \times 10^8$ per KB or A549 cell for Ad3 (Defer et al., 1990). However, about $10^6$ receptor sites per cell have been calculated to bind to Ad2 soluble fibre (Phillipson et al., 1968). It has been hypothesized that this apparent discrepancy reflected a difference in the relative accessibility of receptor components to isolated fibres compared to capsid-inserted fibres (Boulanger & Phillipson, 1981). Six discrete protein species, migrating with apparent M r of 115K, 88K, 78K, 60K, 42K and 34K, have been identified in a cross-linked Ad2–KB cell receptor complex (Hennache & Boulanger, 1977). The
42K species could correspond to one of the two major glycoproteins (40K and 42K) purified from HeLa cell plasma membranes by affinity chromatography on a wheatgerm agglutinin column and immobilized Ad2 virions (Svensson et al., 1981).

Using various adenovirus serotypes we have recently shown that the virus-induced alterations of the cytoskeletal network, the cytoplasmic pathways and the cell surface receptor components are different for serotypes of subgroups B (Ad3, Ad7) and C (Ad2, Ad5). For Ad2, results of homotypic competition binding assays between the virion and its soluble fibre suggested the existence of two classes of KB cell membrane proteins with respect to Ad2 ligand affinity on blot analysis. The first class, consisting of six discrete species, two major at 100K and 68K, and four minor at 120K, 88K, 80K and 60K, were found to bind to Ad2 with a high affinity. The second class, with a lower affinity for Ad2, was represented by a 130K band and multiple discrete molecular species migrating in the range of 56K to 15K (Defer et al., 1990).

Infectious virions or outer capsids of Ad have been successfully used as virion-packed (Rosenfeld et al., 1992) or unpacked (Cotten et al., 1992; Curiel et al., 1991; Wagner et al., 1992) gene-delivering systems. In spite of imminent applications to human gene therapy, the mechanisms involved in Ad attachment and subsequent cellular uptake remain poorly understood at the molecular level. The aim of the present study was to characterize further the polypeptide sequences involved in the molecular recognition between Ad2 virion elements and their protein partners in the HeLa cell plasma membrane, and hence identify some functional components of the Ad2 receptor. In blocking experiments using synthetic peptides, extracellular matrix proteins and antibodies against integrins, we found that antibodies to fibronectin and vitronectin receptors (FN and VN), arginine–glycine–aspartyl tripeptide (RGD), and RGD-related sequences competed with Ad2 for its binding to cell surface receptors. These peptide sequences are present in extracellular matrix proteins and are recognized by membrane integrins (Humphries, 1990; Ruoshlahti, 1988; Ruoshlahti & Pierschbacher, 1987; Tamkun et al., 1986; White & Littman, 1989; Woods et al., 1988; Yamada, 1989). Our results suggest that some adhesion receptor subunits are integral components of Ad2 receptors or that integrins and Ad2 receptors have overlapping ligand specificity.

**Methods**

**Cells and virus.** HeLa cells were cultured as monolayers in Eagle’s MEM supplemented with 5% fetal calf serum and 5% γ-irradiated newborn calf serum. Human adenovirus serotype 2 (Ad2) was produced in KB cells grown in suspension at a density of 2 x 10⁸ to 5 x 10⁸ cells per ml in Joklik’s-modified medium (F13, Difco) supplemented with 5% horse serum. Virus titre was determined by a fluorescent focus assay and expressed as fluorescent focus units (f.f.u.; D’Halluin et al., 1978). Cells were infected at an m.o.i. of 10 to 25 f.f.u./cell. Ad2 virions were extracted and purified as previously described (D’Halluin et al., 1978).

**Fluorescent and isotopic labelling.** Rhodamine labelling of adenoviruses was performed by the method described by Persson et al. (1983). Rhodamine B isothiocyanate (TRITC) was purchased from Sigma. Radioactive labelling of Ad2 virions and viral proteins was carried out with [14C]valine (18.5 kBq/ml) for 16 h at 12 h after infection. [14C]valine (≥ 9.25 GBq/mmol, CEA) was added to infected cell cultures maintained in a serum-free medium containing 10% concentration of valine in normal medium. For homologous Ad2 binding competition, the Ad2 ligand used was a suspension of virions labelled with [14C]formate. [14C]Formic acid, sodium salt (111 to 1.85 GBq/mmol, ICN), was added to Ad2-infected cell cultures in normal medium (74 kBq/ml), and labelling was performed for 16 h at 12 h after infection. The specific activity obtained with [14C]valine ranged between 0.7 x 10⁶ and 1.2 x 10⁷ c.p.m. per 10⁵ Ad2 virions, and between 3 x 10⁶ and 7 x 10⁷ c.p.m. per μg of isolated penton or fibre. With [14C]formate-labelled Ad2 virions, the specific activity was 500-fold higher, i.e. 10⁸ c.p.m. per 2 x 10⁶ particles.

**Cytochemistry, immunocytology and antibodies.** Cells were incubated with Ad2 at a ratio of 10⁴ virions per cell for 2 h at 4°C, then rinsed with PBS containing 1 mM-CaCl₂ and 1 mM-MgCl₂ (PBS-CaMg), fixed in 3.7% (v/v) paraformaldehyde in PBS-CaMg. When TRITC-labelled Ad2 was used, virions were localized at the surface of HeLa cells by direct examination of samples by fluorescence microscopy at the wavelengths and edge filters required for rhodamine (Defer et al., 1990). When unlabelled Ad2 was used, virions were detected by indirect immunofluorescence (IF) using anti-Ad2 rabbit polyclonal serum (home-prepared; Boudin & Boulanger, 1982) and TRITC-labelled anti-rabbit IgG goat antibodies (Jackson ImmunoResearch). FN and VN were visualized at the cell surface by using rabbit polyclonal antibodies against human FN (α5/β1), human VN (αV/β3), or anti-human β1-chain mouse monoclonal antibody (MAB) (clone 4B4, Coulter Immunology), and the corresponding fluorescein isothiocyanate (FITC)-labelled anti-IgG conjugate. All polyclonal sera were used at a dilution of 1:1000, whereas MAB was used at a dilution of 1:200. Anti-FN and anti-VN sera were both from Telios Pharmaceuticals, FITC- and peroxidase-conjugated anti-mouse IgG sheep antiserum was from BioSys. o-Dianisidine was the substrate for peroxidase-conjugated anti-mouse IgG antibody. Filaments of F-actin were observed after staining with FITC-conjugated phalloidin (Sigma). Cells were fixed as described above, permeabilized in PBS-CaMg containing 0.2% Triton X-100 for 1 min at 20°C, then treated with FITC-phalloidin (2 units/ml) in PBS at 20 min at 20°C. In binding competition reactions, the following control antibodies were used: anti-mouse IgG rabbit immunoglobulins (BioSys), anti-human CD4 mouse MAB (clone 13B8.2, Immunotech), and two different anti-HLA class I human sera reacting with all A, B and C haplotypes (from patients with graft rejection; a gift from F. Defossez, Centre de Transfusion Sanguine, Lille, France).

**Binding experiments.** Inhibition of the Ad2 virus particle or the Ad2 capsid protein attachment to HeLa cells by Ad2 virion itself, ConA, laminin, peptides and antibodies raised against receptors was performed as follows. Confluent HeLa cells grown as monolayers were rinsed twice with PBS. All subsequent operations were conducted at 4°C. Cell samples (5 x 10⁶ to 1 x 10⁶ cells) were pre-incubated with 100 μl of PBS (control) or 100 μl of unlabelled competitor solution in PBS for 30 min at 4°C, then further incubated with 100 μl samples of [14C]-labelled ligand for 2 h at 4°C. The ligands used were Ad2 virion...
suspension, Ad2 penton or fibre solution. The maximal amounts of Ad2 added corresponded to a ratio of 10^6 virions/cell, penton (3 mg/ml) to 6 x 10^6 molecules/cell and fibre (1.5 mg/ml) to 10^7 molecules/cell. The cells were then resuspended and resedimented at 10% TCA for 30 min at 4 °C. The TCA precipitate was collected and resuspended in a GF/C glass fibre filter and counted in a liquid scintillation spectrometer. This material was referred to as unadsorbed virus material. The cell pellet was resuspended and incubated in 0.5 ml of 50 mM-Tris–HCl pH 8.0, 1 mM-CaCl_2, containing 0.1 μg of nucleocapsid 5S (Boehringer), then dissolved by the addition of an equal volume of 50 mM-Tris–HCl pH 8.0, 10 mM-Na_2EDTA, 0.5% (w/v) Sarkosyl. After precipitation with 10% TCA, the acid-precipitable radioactivity was referred to as cell-adsorbed virus material. Correction for quenching was performed by counting known amounts of acid-precipitable radioactivity of [14C]-labelled virions mixed with cell samples treated under the same conditions (Defer et al., 1990).

Calculations. The calculations were made on a per-virion or per-receptor site basis (Persson et al., 1985). Considering one Ad2 virus particle or one receptor site as a molecular entity, 6 x 10^9 virions per l would represent an Ad2 concentration of 1 M, and 6 x 10^9 molecules/cell. An unbound virus radioactivity, the x-axis the cell-bound radioactivity, was referred to as cell-adsorbed virus material. For Scatchard, (1949) the y-axis represented the ratio of bound-to-free radioactivity and the x-axis the cell-bound radioactivity, expressed as the number of Ad2 virions per cell or as Ad2 molarity for the calculation of the apparent association constant (K_app). The affinity constant was derived from the slope of the Scatchard plot, its intercept with the y-axis giving the number of receptors per cell. As an example, the affinity constant of a SDGR sequence-containing viral protein for its specific receptor would be 1 x 10^9 M^-1, in terms of Ad2 molarity, and the number of the SDGR-binding elements would be 1.5 x 10^9 per HeLa cell (refer to Fig. 3b).

**Co-localization of Ad2 and FN^R and VN^R at the cell surface**

HeLa cell monolayers were incubated with Ad2 virions, pentons or fibres, at a concentration of 10^6 virions/cell, and 5 x 10^9 to 10 x 10^9 molecules of penton or fibre per cell, for 2 h at 4 °C (Defer et al., 1990). Low temperature was used to inhibit the ligand penetration and recycling of receptors, and saturating amounts of virions were used to induce migration and clustering of receptors in the plane of the plasma membrane (Hennoch et al., 1979, 1982; Persson et al., 1983, 1985). After elimination of unadsorbed viral material, cells were further incubated for 10 min at 20 °C to allow re-arrangements of the membrane and cytoskeleton components to occur. Cells were then fixed, reacted with FITC-labelled phalloidin and examined using IF microscopy. A reorganization of the actin filament network was observed upon Ad2 adsorption, consisting of actin cables with submembranal insertions (Fig. 1b). Quan-
Fig. 1. Actin filaments in HeLa cells stained with FITC-conjugated phalloidin as revealed by immunofluorescence microscopy. (a) Control cells; (b) cells incubated with 10^4 Ad2 virions per cell; (c) cells incubated with 6 x 10^6 molecules of Ad2 penton per cell. Actin cabling was often localized at one pole of the cell, which explains why no cables are visible in the portion of the upper cell cut in (b).

Reversible versus irreversible binding of Ad2 to HeLa cells

Adenovirus attachment to its cellular receptor represents an equilibrium between free and membrane-bound virions. However, if some viral capsid proteins interact with membrane proteins (belonging or not to the viral receptor) with a high affinity such that the resulting virus-membrane complex cannot dissociate under physiological conditions, then irreversibly bound virions would not be delivered to the cytoplasm but stay sequestered in the plasma membrane component. In epithelial cells fully permissive to human adenoviruses, such as HeLa cells and Ad2, the possibility and extent of such a dead-end binding has never been evaluated, probably because this phenomenon has generally been considered as of negligible consequence. In order to estimate the degree of irreversible virus binding in the Ad2-HeLa cell system, binding of [14C]formate-labelled Ad2, used as the cell receptor ligand, was analysed in competition assays with increasing amounts of unlabelled Ad2 as the competitor. In the absence of
Fig. 2. Distribution of Ad2 virions, VN\textsuperscript{R} and FN\textsuperscript{R} at the surface of HeLa cells, analysed by single IF (a, b, c), or double IF microscopy (d, e, f, g). (a, b, c). Cell monolayers were incubated without (control, a) or with (b, c) Ad2 inoculum at 10\textsuperscript{4} virions/cell, then reacted with polyclonal antibodies against VN\textsuperscript{R} (b), or against FN\textsuperscript{R} (a, c), followed by fluorescent anti-rabbit IgG conjugate. The control pattern for VN\textsuperscript{R} antibody, similar to (a), is not shown. In (d) and (e), the cell culture was incubated with TRITC-labelled Ad2 virions, then reacted with anti-\textit{\textbeta}1-chain mouse MAb and FITC-labelled anti-mouse IgG conjugate. In (f) and (g), the cell sample was incubated with unlabelled Ad2 virions, then reacted simultaneously with anti-Ad2 rabbit antibodies and anti-\textit{\textbeta}1-chain mouse MAb, followed by TRITC-labelled anti-rabbit IgG conjugate and FITC-labelled anti-mouse IgG conjugate. The same cells are presented as photographed with the excitation wavelength and edge filters required for rhodamine (d, f) and fluorescein (e, g).
Table 1. Homologous competition between unlabelled and 14C-labelled Ad2 virions for binding to HeLa cells

<table>
<thead>
<tr>
<th>Ligand:competitor ratio</th>
<th>Total recovered</th>
<th>Free</th>
<th>Cell-adsorbed</th>
<th>Input (%)</th>
<th>Adsorbed (%)</th>
</tr>
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<tr>
<td>No competitor</td>
<td>50686</td>
<td>39738</td>
<td>10948</td>
<td>21.6</td>
<td>100.0</td>
</tr>
<tr>
<td>100:1</td>
<td>50020</td>
<td>41812</td>
<td>8208</td>
<td>16.4</td>
<td>75.9</td>
</tr>
<tr>
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<td>41424</td>
<td>38008</td>
<td>3416</td>
<td>8.2</td>
<td>37.9</td>
</tr>
<tr>
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<td>55770</td>
<td>52194</td>
<td>3576</td>
<td>6.4</td>
<td>29.0</td>
</tr>
<tr>
<td>1:10</td>
<td>55443</td>
<td>52091</td>
<td>3342</td>
<td>6.2</td>
<td>27.8</td>
</tr>
<tr>
<td>1:100</td>
<td>55544</td>
<td>52082</td>
<td>3462</td>
<td>6.2</td>
<td>28.7</td>
</tr>
</tbody>
</table>

* A constant input of [14C]formate-labelled Ad2 ligand (56000 c.p.m.; 1-1 x 10⁵ virions) was added per cell culture sample (1 x 10⁶ cells), after pre-incubation of the cells with increasing amounts of unlabelled Ad2 virions as the competitor. The conditions of binding and the radioactivity assays are described in Methods.

Inhibition of Ad2 attachment to HeLa cells by integrin-binding proteins

The adsorption of 14C-labelled Ad2 virion, penton and fibre to the HeLa cell surface was analysed in the presence of various competitors. Some molecules are known to bind to integrins: this is the case for ConA and adhesion protein laminin. Others have been designed for integrin recognition, such as antibodies against FNα and against VNβ. The results are summarized in Table 2. ConA decreased the amount of HeLa cell-bound Ad2 with a high efficiency: its inhibitory effect on viral attachment was found to be 85% of the control value at a concentration of 5 μg/ml, and higher than 97% at 10 μg/ml. The latter concentration corresponded to a 150-fold excess over Ad2 virions, in terms of protein ratio. Our results were thus consistent with previous data showing that inhibition of binding of radiiodinated Ad2 fibre by ConA reaches a maximum of 50 to 60% of control in KB cell samples where the lectin was in 2000-fold excess over the fibre (Hughes & Mautner, 1973). Laminin, used at 5 and 10 μg/ml, showed a less pronounced effect, although a significant inhibition was observed (60%). Anti-FNα, anti-VNβ and anti-β-chain antibodies inhibited adsorption of Ad2 virion, fibre and penton to a similar extent (50 to 66%). On the contrary, anti-HLA class I antibodies did not compete with Ad2 for its attachment to the HeLa cell surface.

Inhibition of Ad2 cellular binding by peptides mimicking cellular adhesion sequences

Peptides used in the following experiments represented consensus sequences identified as being involved in cell adhesion (Garcia-Pardo et al., 1990; Ruoslahti & Pierschbacher, 1987). RGD is present in laminin and is the core of the three following peptide sequences. RGDS is found in fibronectin and fibrinogen α-chain, GRGDS in fibronectin, and SDGR, which is antisense to RGDS peptide, in the α2 domain of MHC class I antigens. As a control, two peptides were used: RFDS and GRADSP. Both are known to be inactive for integrin binding (Ruoslahti & Pierschbacher, 1987). When HeLa cells were incubated with increasing molarities of peptides in PBS prior to addition of [14C]valine-labelled Ad2, the proportion of adsorbed Ad2 decreased for all four active peptides (Fig. 3a). The binding was 70 to 80% of the control level at 0.2 mM-peptide. At higher peptide concentrations, significant differences were observed in the Ad2-binding inhibitory effects: GRGDS reduced Ad2 binding to only 70 and 60% of the control at 2 and 20 mM, respectively, whereas 50 and 40% Ad2 binding were obtained with RGD and RGDS at the same
molarities. The highest inhibition level was observed with the antisense peptide SDGR, which reduced Ad2 adsorption to 44% at 2 mM, and to 34% at 20 mM. The latter value was close to the amount of irreversibly bound Ad2 observed in homologous competition assays (about 30%; Table 1). A similar factor of reduction of virus adsorption (2.5- to three-fold) have been obtained with competing peptides in other viral systems (Fox et al., 1989).

Reduction of Ad2 progeny yielded by cells treated with ConA, RGD-related peptides and anti-fibronectin receptor antibodies

The effect of ConA, anti-FN R antibodies and adhesion peptides on the cellular adsorption of Ad2 virions was further analysed by assaying the virus progeny yielded at the end of the infectious cycle. As shown in Table 3, incubation of cell samples with ConA resulted in a 100-fold reduction of Ad2 progeny at 40 h after infection.
Peptides RGD, RGDS, GRGDS and SDGR also drastically reduced the virus yield (92 to 97% reduction). The strongest effect was obtained with RGD and GRGDS. No effect on the virus progeny yield was observed with control GRADSP, or with any competing peptide when added after Ad2 infection. RGD peptide solution (10 mM) proved to be almost as effective for inhibition of Ad2 production as a solution of ConA at 10 μg/ml. When used at decreasing dilutions, a rabbit anti-FN serum showed a progressive reduction of the virus yield at 40 h after infection (from 95 to 99%; Table 3). The fact that no reduction of Ad2 progeny was observed when cells were pre-incubated with ConA at 1 h before the addition of Ad2 inoculum or when ConA, peptide or receptor antibody was added at 3, 8 or 16 h after Ad2 infection, suggested that the inhibitory effect was not due to some ConA-, antibody- or peptide-induced cytotoxic effect, but rather to direct competition between Ad2 and ConA, antibody or peptide for the same receptor site. Since the difference in the inhibitory efficiency of the various peptides tested could be due to some variation in the stability of the receptor–peptide complex, the parameters of the binding competition reaction between Ad2 virions and peptides were analysed.

**Parameters of the binding competition reaction between Ad2 and RGD peptides**

Considering an Ad2 virion and a cellular adhesion peptide as two ligands competing for a common cellular receptor, the binding data of competition reactions performed at different peptide molarities as shown in Fig. 3(a) were graphically represented (Scatchard, 1949), and analysed by regression (Fig. 3b). The Scatchard plot for GRGDS and antisense sequence SDGR showed a straight line (y = -0.034 + 0.016x, with R = 0.99 for GRGDS; y = -0.020 + 0.015x, with R = 0.100 for SDGR) with a similar apparent affinity constant for Ad2 receptor, K_{app} = 1 × 10^8 M^{-1}. The intercept of the curves with the x-axis gave the number of 20000 to 15000 recognition sites per cell for GRGDS and antisense SDGR respectively. The data obtained with RGDS were also compatible with one line (y = -0.048 + 0.036x) and one slope (K_{app} = 2.25 × 10^8 M^{-1}), with a significant value for the R coefficient (R = 0.96; P ≤ 0.05). The intercept of the RGDS curve with the x-axis gave the number of 15000 for complementary sites per cell. The Scatchard plot for RGD–Ad2 binding competition assays was not a straight line, and the calculations gave a low probability of linearity (R = 0.91; P = 0.10). The curve better fitted a two-slope line (y = -0.0496 + 0.012x, R = 0.96, and y = -0.3031 + 0.0344x, R = 0.97, respectively), with P ≤ 0.05, suggesting the existence of an heterogeneous population of RGD-binding sites on HeLa cell membrane proteins. One class, present at a copy number of 9 × 10^4 per cell, would have an affinity for the RGD peptide of K_{app} = 2.20 × 10^8 M^{-1}; the other class, less abundant (5 × 10^4 per cell), would bind to RGD with a threefold lower affinity (K_{app} = 0.75 × 10^8 M^{-1}). These results would imply that the outer components of the Ad2 virion do not recognize a unique complementary sequence on a single receptor protein at the surface of HeLa cells, but rather a variety of sequences present in multiple copies in one or several receptor proteins. One common feature of all the Ad2-competing sequences tested was the presence of an RGD peptide core.

**Immunological analysis of DTBP cross-linked Ad2–receptor complex**

To further characterize the polypeptide composition of the adenovirus receptors, [14C]valine-labelled HeLa cell membrane proteins were reversibly cross-linked to unlabelled Ad2 virions. Ad2-containing complexes were isolated by ultracentrifugation in a combined CsCl–glycerol gradient, taking advantage of the sedimentation coefficient of the Ad virion in velocity gradient (750S), and of its apparent density in CsCl gradients, (ρ = 1.345 g/ml). As controls, labelled HeLa cell membrane proteins were cross-linked with DTBP in the absence of Ad2 virions, and unlabelled membrane proteins were cross-linked to [14C]valine-labelled Ad2 virions. Samples from the gradient fractions corresponding to Ad2 density were processed in parallel, and immunoselected on S. aureus Protein A, using anti-Ad2 virion, anti-VN R, anti-FN R or anti-β1-chain antibodies. The precipitates were analysed by SDS-PAGE and autoradiography. In control samples of labelled virus cross-linked with unlabelled cells, radioactive Ad2 material was found in precipitates with anti-FN R, anti-VN R and anti-β1 (not shown), implying that some integrins were present in the complex and had mediated the immunoprecipitation of Ad2 particles. Conversely, anti-Ad2 virion antibodies precipitated labelled membrane proteins cross-linked with unlabelled Ad2 virions. Analysis of these immunoprecipitates showed three major labelled bands at 130K, 60K and 44K (Fig. 4a, lane 1). The same pattern of major polypeptide species was found when the gradient fractions were immunoprecipitated with anti-FN R or anti-VN R antibodies (Fig. 4a, lanes 2, 3). Other minor discrete bands of lower Mr were also visible on prolonged exposure of the gel autoradiogram (Fig. 4b). In immunoblotting analysis, the 130K species was found to react with anti-β1-chain antibody (Fig. 4c). When control labelled HeLa cell plasma membranes were treated with DTBP in the absence of Ad2 virions, no detectable signal was found in the gradients at the density of the virus, and...
with the results of a previous report showing that cytochalasin B, an inhibitor of microfilament functions, blocked Ad5 internalization (Patterson & Russell, 1983). (iii) Attachment of Ad2 virions, pentons and fibres onto HeLa cells was efficiently inhibited by molecules which are naturally or synthetically designed for binding to integrins, e.g. ConA, laminin, antibodies against FN<sup>R</sup> or VN<sup>R</sup>, and peptides reproducing the cell attachment sequences of adhesion proteins (Table 2). (iv) Analysis of radioactively labelled plasma membrane proteins isolated in a CsCl gradient via Ad2 virion-cross-linking showed that complexes sedimenting at the virus density contained three major labelled polypeptide species of respective Mr, 130K, 60K and 44K immunoprecipitated with antibodies against FN<sup>R</sup>, VN<sup>R</sup> or Ad2 virions (Fig. 4). The apparent Mr of 130K is consistent with that of integrin β subunit, whereas some of the low Mr species could represent reduced polypeptide subunits or proteolytically cleaved fragments from a larger α-type subunit (Ruoshlahti, 1988; Springer, 1990a; Staquet et al., 1990). Alternatively, they could be integral components of Ad2 receptor sites, not necessarily related to the integrin family but co-precipitated with integrin β subunits present in cross-linked viral complexes.

Several viral receptors have been functionally or biochemically characterized. Data from competition binding experiments have shown that adsorption of coxackievirus B3 to HeLa cells is inhibited by both Ad2 virion and Ad2 fibre (Lonberg-Holm et al., 1976). Human rhinovirus 2 (HRV-2) attachment to HeLa cells (minor group receptor; Uncapher et al., 1991) is only partially inhibited by the Ad2 virion, but not by soluble Ad2 fibre, and more interestingly, HRV-14 (major group rhinovirus) and poliovirus 2 are both competed by Ad2 virions to some extent (approx. 10%; Lonberg-Holm et al., 1976). A receptor protein of about 50K, isolated from the HeLa cell, has been shown to combine specifically with group B coxsackieviruses (Mapoles et al., 1985), and a HeLa cell membrane component of 120K has shown specific binding activity to HRV-2 on blot analysis (Mischak et al., 1988). More recently, a 44K glycoprotein has been found to be involved in the attachment of echovirus 11 (EV-11) to KB cells (Mbida et al., 1992).

RGD and related peptide sequences have been found to mediate the binding of fibronectin, vitronectin, fibrinogen, type I collagen and several other extracellular matrix proteins to adhesion receptors localized at the surface of various epithelial, endothelial and fibroblastic cell lines and belonging to the integrin family (reviewed in Humphries, 1990; Hynes, 1987; Ruoshlahti, 1988; Ruoshlahti & Pierschbacher, 1987; Springer. 1990a; b; Yamada, 1989). VLA-2 integrin has been shown to be involved in the attachment of EV-1 (Bergelson et al.,...
Table 3. Influence of ConA, adhesion peptides and anti-FN R antibodies on Ad2 progeny yield

<table>
<thead>
<tr>
<th>Ad2 binding competitor</th>
<th>Ad2 yield*</th>
<th>Progeny reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F.f.u. ((\times 10^{-4}))± s.D.</td>
<td>%</td>
</tr>
<tr>
<td>ConA (10 µg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No competitor added</td>
<td>46.6±1.1</td>
<td>100.0</td>
</tr>
<tr>
<td>ConA control, -1 h</td>
<td>46.2±2.6</td>
<td>99.1</td>
</tr>
<tr>
<td>ConA control, +3 h</td>
<td>44.9±3.6</td>
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<tr>
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<td>103.2</td>
</tr>
<tr>
<td>ConA</td>
<td>0.5±0.1</td>
<td>1.07</td>
</tr>
<tr>
<td>Peptide (10 mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No competitor added</td>
<td>39.6±0.9</td>
<td>100.0</td>
</tr>
<tr>
<td>GRADSP</td>
<td>40.0±1.0</td>
<td>101.0</td>
</tr>
<tr>
<td>RGD, control +3 h</td>
<td>38.9±2.1</td>
<td>98.2</td>
</tr>
<tr>
<td>RGD, control +3 h</td>
<td>39.3±0.3</td>
<td>99.2</td>
</tr>
<tr>
<td>RGD, control +8 h</td>
<td>40.1±0.8</td>
<td>101.2</td>
</tr>
<tr>
<td>RGD, control +8 h</td>
<td>39.4±2.3</td>
<td>99.7</td>
</tr>
<tr>
<td>SDGR, control +8 h</td>
<td>39.6±4.5</td>
<td>100.0</td>
</tr>
<tr>
<td>GRGDS, control +8 h</td>
<td>40.6±3.7</td>
<td>102.3</td>
</tr>
<tr>
<td>RGD</td>
<td>1.06±0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>RGDS</td>
<td>2.90±2.7</td>
<td>7.3</td>
</tr>
<tr>
<td>SDGR</td>
<td>3.20±2.0</td>
<td>8.1</td>
</tr>
<tr>
<td>GRGDS</td>
<td>2.10±0.3</td>
<td>5.3</td>
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<tr>
<td>Anti-FN R rabbit serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control antiserum (1:25)</td>
<td>224.0±7.2</td>
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<tr>
<td>Anti-FN R (1:100)</td>
<td>13.3±2.4</td>
<td>5.9</td>
</tr>
<tr>
<td>Anti-FN R (1:50)</td>
<td>6.4±3.6</td>
<td>2.8</td>
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<tr>
<td>Anti-FN R (1:25)</td>
<td>1.1±0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Anti-FN R (1:25; control +8 h)</td>
<td>238.0±3.6</td>
<td>106.0</td>
</tr>
</tbody>
</table>

* HeLa cell monolayers \((3 \times 10^8 \text{ cells})\) were incubated with ConA, antibody or competing peptide solution in serum-free medium for 30 min at 4 °C. Ad2 inoculum was then added at an m.o.i. of 20 f.f.u./cell, and the cells were further incubated for 30 min at 37 °C. The medium was rinsed off, replaced by prewarmed culture medium, and the virus cycle allowed to proceed for 40 h at 37 °C. Ad2 production was assayed by counting cells presenting late fluorescent adenovirus antigens, and expressed as f.f.u. \pm s.D.; \(n = 6\).

† Zero inhibition was given by untreated cell cultures, with no competitor added. Possible ConA-, antibody- or peptide-induced cell-detaching or cytopathic effects were monitored by incubating Ad2-infected cell samples with ConA (10 µg/ml), antiserum (1:25) or peptide (10 mM) for 30 min at 4 °C followed by 30 min at 37 °C, at 3 h (control +3 h), 8 h (control +8 h) or 16 h (control +16 h) after Ad2 infection. ConA control −1 h consisted of pre-incubating the cells with ConA for 30 min at 4 °C for 30 min at 37 °C, and transferring them to prewarmed, normal medium 1 h prior to Ad2 inoculum.

‡ Antiserum against human FN R was used at the indicated dilutions in PBS. Control antiserum was an anti-mouse IgG rabbit antiserum.

1992) to susceptible cells, and RGD-binding molecules participate in the cellular binding of foot-and-mouth disease virus (FMDV; Fox et al., 1989). Similarly, members of the immunoglobulin superfamily have been identified as cellular receptors for other viruses (reviewed in White & Littman, 1989), e.g. poliovirus (Mendelsohn et al., 1989), murine ecotropic retrovirus (Albritton et al., 1989), HRV-14 (Greve et al., 1989; Register et al., 1991; Staunton et al., 1989) and human immunodeficiency virus 1 (Dalglish et al., 1984). In addition, primary blood T lymphocytes, which lack detectable Ad receptors as resting cells, become permissive to Ad upon activation (Silver & Anderson, 1988), and show discrete qualitative changes in the pre-existing VLA integrin receptors expressed at their surface (Shimuzu & Shaw, 1991). Another human bacterial pathogen, Yersinia pseudotuberculosis, uses the integrin β1 chains as receptors for penetration into mammalian cells, via an integrin-binding domain located in its outer membrane protein invasin (Isberg & Leong, 1990; Leong et al., 1990a, b). RGD and RGD core-containing peptides (RGDS, GRGDS and antisense SDGR) were found to be all active in inhibiting Ad2 binding to HeLa cells. At a molarity of 20 mM, RGD and related peptides RGDS, GRGDS and SDGR reduced Ad2 adsorption to the cell surface by a factor of two- to threefold (Fig. 3a). A similar degree of virus attachment inhibition (65 to 70%) was obtained with RGD peptides and FMDV (Fox et al.,
1989). These values were significantly lower than the level of reduction of viral progeny yield (92 to 97%) observed at the end of the Ad2 infectious cycle in the presence of RGD peptides (Table 3). This apparent discrepancy was not due to some peptide-induced cytoxic effect, which in turn could inhibit the virus replication. In control experiments where peptides were added to Ad2-infected cells at 3, 8 or 16 h after Ad2 infection, no reduction of virus yields was detected (Table 3). Our results are in fact reminiscent of the complexity of surface phenomena observed during the adsorption of herpes simplex virus (HSV) to heparan sulphate proteoglycans of plasma membrane receptors (Shieh et al., 1992). In HSV, envelope glycoproteins gB and gD are dispensable for virus binding but are required for virus penetration, whereas the spike glycoprotein gC plays a role in both adsorption and infectivity (Herold et al., 1991). In particular, gC-negative virions of HSV showed a specific infectivity of 5 to 10% that of wild-type virions, whereas their adsorption activity was only reduced to 20% that of wild-type virions (Herold et al., 1991). In the light of these HSV data, our observations on the peptide inhibitory effect of Ad2 adsorption and penetration would suggest that proteins of the integrin family are mainly involved in cellular events taking place after the initial binding of Ad2 to the cell receptor. The recent finding that integrins can circulate and participate in the endocytotic cycle (Bretscher, 1992a, b) would support this hypothesis. In terms of the virus entry mechanism, binding to an integrin receptor would predispose the Ad2 virions to entering the cell via the receptor-mediated endocytosis pathway.

Binding data from competition reactions between RGD-containing peptides and Ad2 virions showed that their apparent affinity constants for the Ad2 receptor site ranged from 0.75 x 10⁸ to 2.2 x 10⁹ M⁻¹, with a number of peptide-recognizing sites ranging from 15 x 10³ to 20 x 10³ per cell for RGDS, GRGDS and SDGR, and from 50 x 10³ to 90 x 10³ per cell for the core peptide RGD (Fig. 3b). When compared with the 10⁶ receptors per cell previously estimated for Ad2 fibres on human cells (Philipson et al., 1968), with the number of 5 x 10⁸ to 10 x 10⁹ receptors per cell for Ad2 virions, and with the affinity constant value of 9 x 10⁴ M⁻¹ to 10 x 10⁶ M⁻¹ for the whole receptor site (Defer et al., 1990; Persson et al., 1985), our data with competing peptides suggested that one single Ad2 receptor site recognizes multiple complementary sequences on the Ad2 capsid. Peptide motifs representing certain consensus domains of adhesion proteins can indeed be found in the sequences of two vertex proteins of the Ad2 capsid (Roberts et al., 1986), i.e. the fibre, which is responsible for cellular attachment, and the penton base, which has a cell-detaching effect (Boulanger & Lonberg-Holm, 1981; Pettersson, 1984).

The sequence SDGK, which is closely related to the SDGR antisense peptide and competes the most efficiently with Ad2 for cellular attachment (Fig. 3a), is present within the shaft domain of Ad2 fibre, at amino acid 153. RGD'T, which is homologous to RGDS (Fig. 3a), is found at amino acid 340 in the Ad2 penton base. RGD'T is also present in collagen I, and the RGD tripeptide core in fibronectins. Peptide LDVD, identified as a LDV segment within the CS1 site of human fibronectin and resembling the EDVD sequence in the CS5 site (Garcia-Pardo et al., 1990; Guan & Hynes, 1990; Humphries, 1990; Shimuzu & Shaw, 1991; Woods et al., 1988; Yamada, 1989), is also encountered in Ad2 fibre at position 268, and in Ad2 penton base at position 287. Site-directed mutagenesis will be necessary to confirm the role of these putative cell-binding domains of the fibre and penton on Ad2 adsorption and penetration. Three-dimensional models of the Ad2 penton base and fibre, when available, should also give valuable informations on the accessibility of these sequences to cellular receptors.

We are grateful to Bernard Gay for photographic reproductions, Claudine Bernard for cell cultures, Patrice Codogno for his sample of purified laminin and Marie Staquet for helpful discussions. We also thank Pierre Lemay who initiated this study. This work was supported by the Institut National et de la Santé et de la Recherche Médicale (CIF 92-03), the Ministère de l’Education Nationale (DRED), the Ministère de la Recherche (ATP-Virologie Fondamentale), the Fondation pour la Recherche Médicale, the Association pour la Recherche sur le Cancer, and the Groupement des Entreprises Françaises dans la Lutte contre le Cancer.

References


(Received 4 December 1992; Accepted 17 March 1993)