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IMMUNOAFFINITY PURIFICATION AND CHARACTERISATION OF p29—AN ESTROGEN RECEPTOR RELATED PROTEIN

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Summary—p29, a 29 kDa protein recognised by D5, a monoclonal antibody prepared against partially purified cytosolic estrogen receptor (ER), has been purified to homogeneity from ZR-75-1, a human breast cancer cell line. Ammonium sulphate fractionation followed by immunoaffinity chromatography on a three column system using Protein A–Sepharose coupled D5, produced purified p29. Silver stained SDS one-dimensional polyacrylamide gel electrophoresis (PAGE) and two-dimensional PAGE showed p29 to have been purified to homogeneity. Amino acid analysis showed no unusual characteristics. Partial N-terminal sequencing studies showed that purified p29 shared a 100% homology with the sequence of a pp89, murine cytomegaloviral protein.

INTRODUCTION

In attempts to raise monoclonal antibodies against an affinity purified soluble oestradiol receptor (ER) from human myometrium [1, 2], a 29 kDa protein, p29, which can associate with ER was discovered. p29, the protein recognised by D5, a monoclonal antibody, has been partially characterized in human myometrium and in the ZR-75-1 breast cancer cell line [3]. p29 is a cytoplasmic M_r 27–29 kDa serine phosphoprotein which does not bind steroid itself but will, under activating conditions, associate with ER [4, 5]. p29 will not react with other steroid receptors or binding proteins and is quantitatively and qualitatively related to ER [5]. Immunohistochemical assays for p29 in breast tumour tissues have also shown that p29 is at least as effective as ER in predicting clinical response of patients to hormone therapy [6, 7]. In normal endometrium p29 has also been shown, immunohistochemically, to be regulated during the menstrual cycle [8].

As a means of elucidating its function, the isolation and sequencing of p29 was attempted. We describe, in this paper, the development of a purification system for p29 from the ZR-75-1 breast cancer cell line and the partial characterization of the final purified protein.

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EXPERIMENTAL

Chemicals

Protein A–Sepharose was obtained from Pharmacia-LKB (Milton Keynes, Bucks, England). Affi Gel-10 was obtained from Bio-Rad (Watford, Herts, England). Ampholines (pH range 3–10 and 5–8) were obtained from LKB Producter, A.B. (Stockholm, Sweden). Acrylamide and bis-acrylamide were obtained from Kodak–Eastman (U.S.A.). Ammonium sulphate $[(NH_4)_2SO_4]$ was obtained from Schwartz–Mann Inc. (N.Y., U.S.A.). All other chemicals unless otherwise specified were obtained from Sigma Chemicals (Poole, Dorset, England).

Buffer A had the following composition: 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 2 mM DTT (4°C). Buffer B had the following composition: 0.1 M borate buffer (pH 8.2) containing 0.1 mM leupeptin, 1 μg/ml pepstatin, 100 μg/ml bacitracin, 0.5 mM PMSF (phenylmethylsulphonyl fluoride) and 0.2% sodium azide (4°C). Buffer C had the following composition: PBS/Tween 20 [0.05% (w/v)]. D5 antibody was produced essentially as described [6].

Protein determination

Protein concentration was determined using the Bio-Rad Protein Assay with BSA as standard.

Preparation of cytosols

Human myometrium obtained at hysterectomy was transported to the laboratory on dry ice and stored at $-70°C$ until required. Cytosol was produced by homogenisation (Polytron 2×2 s passes) in 5 volumes of buffer A.

ZR-75-1 cells were routinely cultured in E4 medium containing 5% FCS. Cultured cells were disrupted by hand using a Teflon–glass homogeniser in 10 volumes of buffer B.

High speed supernatants of myometrium of ZR-75-1 cells were produced by centrifugation (200,000 g_{av} for 60 min at 4°C).

Ammonium sulphate fractionation of cytosols

Cytosols were fractionated with ammonium sulphate by slowly stirring solid $(NH_4)_2SO_4$ into the cytosol over 15 min to obtain the desired saturation. After stirring for a further 30 min the mixture was centrifuged (30 min at 10,000 g_{av} at 4°C) and supernatants reprecipitated, if necessary. Precipitates were dissolved in 1/10th the original volume of buffer B and undissolved material was removed by centrifugation (100,000 g_{av} at 4°C for 30 min).

Preparation of affinity matrices

Protein A–Sepharose–D5 matrix was produced using the method of Schneider et al. [9]. Protein A–Sepharose CL-4B (Pharmacia) was mixed with antibody in Buffer B for 60 min at room temperature with gentle shaking. Beads were then washed with excess buffer and 0.2 M triethanolamine (pH 8.2) and resuspended in 20 volumes of dimethyl pimelimidate dihydrochloride freshly prepared in 0.2 M triethanolamine with the pH readjusted to pH 8.2. After agitation at room temperature (45 min), the reaction was stopped by centrifugation (500 g_{av} for 1 min) and the gel resuspended in an equal volume of ethanolamine (pH 8.2) of the same molarity as the dimethyl pimelimidate dihydrochloride. After 5 min, the cross-linked beads were washed 3 times with buffer B and stored at 4°C until required.

D5 was coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturers instructions. The matrix was therefore washed with isopropyl alcohol and H_2O on a sintered glass funnel. Affi-Gel 10 and D5 antibody in coupling buffer (0.1 M $NaHCO_3$, pH 8.0) at 4°C were then incubated on a rocking table at room temperature for 1 h. Gel and antibody were then centrifuged (1500 g_{av} for 15 min), the supernatant removed and gel washed twice with coupling buffer (2 ml). The gel was then incubated with 1 M ethanolamine (pH 8.0) in coupling buffer (500 μl) for 1 h at room temperature. After washing with coupling buffer, the gel was stored at 4°C.

Preparation of 3 column system

For the purification of p29 with Protein A-Sepharose–D5, a 3 column system was used similar to that adopted by Logeat et al. [10] for the purification of progesterone receptor. For the isolation of p29 the 3 column system consisted of: column 1, Protein A–Sepharose alone in order to adsorb out any proteins non-specifically binding to the matrix alone: column 2, a non-specific control IgG_1, $H17E_2$ [11] (an antibody against human alkaline phosphatase obtained from H. Durbin, ICRF) linked to Protein A–Sepharose in order to adsorb out any proteins non-specifically binding to IgG_1 immunoglobulins and column 3, the Protein A–Sepharose–D5 linked column for p29 isolation.

Application of samples

Columns (Pharmacia C10 columns) were set up and after a 0–35% $(NH_4)_2SO_4$ fraction of ZR-75-1 cytosol (5 ml) was passed through all 3 columns, column 3 was disconnected from columns 1 and 2. To remove non-specifically bound protein column 3 was washed with the following high and low salt and/or high and low pH buffers; (1) 50 ml of 10 mM Tris–HCl (pH 8.3) containing 50 mM NaCl, (2) 90 ml of 10 mM Tris–HCl (pH 7.4), (3) 40 ml of 10 mM Tris–HCl (pH 7.4) containing 0.3 M NaCl, (4) 20 ml of 10 mM Tris–HCl (pH 7.4) containing 1 M NaCl, (5) 40 ml of 10 mM Tris–HCl (pH 6.0), (6) 40 ml of 10 mM Tris–HCl (pH 9.0).

Elution of p29

p29 was reverse eluted from the matrix with 0.05 M diethylamine (pH 11.5). Fractions (1.5 ml) were collected throughout the purification and affinity eluate fractions collected into a 1/10th volume of 0.5 M NaH_2PO_4.

Quantitation of p29

Enzyme-linked immunosorbent assays (ELISA) were performed as previously described [6]. Briefly, aliquots of samples were

incubated for 90 min in 200 μl PBS in Immulon 1 (Dynatech Ltd, Billinshurst, England microtitre plates. After 3 washes in distilled water, 200 μl D5 (0.75 μg/ml) were added to each well and incubated at 37°C for 2 h. After 3 further distilled water washes, 200 μl of peroxidase-labelled sheep anti-mouse immunoglobulins (DAKO immunoglobulins, Denmark) were added to each well (1/1000 dilution in buffer C) and incubated for 1 h at 37°C. Wells were washed 3 times with distilled water and incubated with 200 μl of peroxidase substrate (50 mg of *O*-phenylene diamine hydrochloride in 100 ml of 0.1 M sodium phosphate–citric acid buffer (pH 5.0) containing 20 μl hydrogen peroxide (100 vol, Fisons Ltd, Loughborough, England). After 20 min the reaction was stopped with 50 μl of 0.5 M sulfuric acid and the plates were read at 450 nm. Control samples were as described above with the omission of D5 from the appropriate step. Calibration curves were prepared using a myometrial cytosol standardised to an arbitrary number of units of p29.

Gel electrophoresis

Proteins were separated by one-dimensional SDS–PAGE at room temperature (15% polyacrylamide) according to the method of Laemmli [12], using a Hoeffer SE 400 apparatus. Gels (160 × 140 × 0.75 mm) were run at 20 mA for 1 h and 40 mA for a further 3 h. During the purification of p29, 2 μg of cytosolic protein and $(NH_4)_2SO_4$ protein fractions and excess purified sample as was possible, were loaded onto the gel. Gels were routinely stained for protein using silver staining.

Two-dimensional gels were run according to a method modified from O'Farrell [13, 14]. Briefly glass tubes (dimensions 8 × 120 mm with i.d. 3 mm) were filled with gel (5% acrylamide with a 4:1 mixture of 3.5–7.0 and 5.0–7.0 ampholines, LKB) and samples, mixed with 1:1 SDS sample buffer, laid on top. The samples were overlayed with H_2O: sample buffer (2:3) and then overlaid with H_3PO_4 (0.02 M). Gels were run with acid (0.02 M H_3PO_4) at the anode and base (0.02 M NaOH) at the cathode for 1500–2000 Vh. Gels were then extruded, equilibrated for 1–2 h at room temperature in SDS sample buffer and placed on the top of SDS slab gels (15% acrylamide) poured without a stacking gel and overlaid with a 1:1 mixture of agar (2%): sample buffer. Gels were then run as SDS slab gels and stained for protein using silver staining.

Silver staining of SDS gels

Gels were stained using a method modified from Morrisey [15] whereby gels were incubated at room temperature in 50% methanol/10% acetic acid for 30 min, 5% methanol/7% acetic acid for 30 min, 10% glutaraldehyde for 30 min and washed overnight in H_2O. After incubation in 5 μg/ml dithiotheitol in H_2O for 30 min and 0.1% $AgNO_3$ for 30 min, the gel was rinsed in H_2O and developer (0.3 M sodium carbonate with 0.05% formaldehyde) and incubated in developer until the required intensity of bands was reached. The reaction was stopped by the addition of 2.3 M citric acid for 10 min. Gels were then rinsed in H_2O and stored in 0.03% sodium carbonate.

Immunoblotting

Detection of p29 on nitrocellulose blots with D5, was carried out as previously described [3]. Briefly, electrophorectic transfer of proteins from gels to nitrocellulose paper (BA85: Schleicher and Schull, GmbH; D-3354: Dassel, W. Germany) was performed at 8 V/cm (4°C) in a "Trans-blot" cell (Bio-Rad). After blotting and prior to incubation with D5, the blot was washed for 2 h at room temperature with buffer C. The washed blot was incubated with D5 at 37°C for 1.5 h followed by 3 × 10 min washes in buffer C at room temperature. After washing, blots were incubated (37°C, 1 h) with 1/200 dilution of horseradish peroxidase-labelled rabbit anti-mouse immunoglobulins (DAKO) in buffer C. After washing again, blots were incubated in substrate reagent (3-chlor-1-naphol, 0.06% w/v in 20% methanol–PBS containing 60 μl of H_2O_2 [100 vol]) at 37°C for 15–20 min until colour development occurred.

Amino acid analysis and sequencing

Purified p29 was thawed slowly on ice, pooled, transferred into dialysis membrane (size 2–18/32", Medicell, London, England) and dialysed against 500 volumes of H_2O overnight. The resulting dialysate was then freeze dried and analysed using a Beckman 6300 Amino Acid Analyser. Briefly, 30–60 μg of p29 were taken up in 0.1% trifloroacetic acid (100 μl). 5 μl of this was freeze-dried in a picotube (Millipore Waters), placed into a picovial with 200 μl 6 M HCl (constant boiling, amino acid analyser grade) and 20 μl butane dithiol. The vial was flushed 3 times with N_2, sealed under vacuum and incubated at 110°C for 20–24 h. After removal of the tube from the vial, the liquid

was lyophilised, resuspended in Na–S buffer (Beckman) and injected onto a Beckman 6300 Amino Acid Analyser. The hydrolysed amino acids were separated on a high performance, mixed bed, ion exchange column by buffers which separate acidic, basic and neutral amino acids. The resulting separated amino acids were detected by reaction with ninhydrin and absorbance at 570 nm (440 nm for proline). All amino acids have characteristic retention times on the ion exchange column. The resulting chromatogram was quantified by a Waters 840 system.

N-terminal amino acid sequence was produced on an Applied Biosystems 475A Peptide Sequencer. The remaining 95 μl was freeze-dried and taken up in 20–30 μl 70% formic acid/10% methanol. The sample was dried onto a precycled polybrene treated GF disc and placed into an Applied Biosystems 475A Peptide Sequencer. The sequencer uses Edman degradation to cyclically remove one amino acid from the N-terminus of a peptide. Phenylthiohydantoin amino acids are identified on a Biosystems 120 amino acid analyser by their absorbance at 270 nm and characteristic retention time.

RESULTS

Development of purification system

Cytosols. Human myometrium was initially selected as the tissue source for fundamental studies on p29 purification as it was the original source of the immunogen used to produce D5 and because of its availability. However, further studies demonstrated the presence of actin in myometrial cytosols which copurified with p29. To overcome this problem, cytosols from ZR-75-1 human breast cancer cells, which contain much less actin and are a rich source of p29, were used in the final purification.

Ammonium sulphate fractionation. Enrichment of cytosolic p29 from both myometrium and ZR-75-1 cells was attempted using $(NH_4)_2SO_4$ fractionation. Initial studies showed that saturations of 0–25% and 25–35% selectively precipitated p29 from tissue and cell cytosols but the product from ZR-75-1 cytosol contained minimal actin "contaminant". Subsequent precipitations of p29 were performed using a 0–35% saturation of $(NH_4)_2SO_4$ of a ZR-75-1 cytosol.

Immunoaffinity chromatography comparison of Protein A–Sepharose and Affi Gel-10 matrices. An initial study of the immunoaffinity purification of p29 using D5 antibody was undertaken comparing Protein A–Sepharose, an adsorbent specific for the Fc fragment of IgG antibodies with Affi Gel-10, a general non-specific adsorbent with a 10-carbon neutral spacer arm which combines spontaneously with free primary alkyl or aryl amino groups of any ligand. The amount of D5 coupled was monitored by the disappearance of protein from the antibody solution before and after incubation with the matrix. Myometrial cytosol containing p29 was then incubated with matrix (2 h at 4°C), packed into a column, washed with buffer and eluted with 0.05 M diethylamine for the Protein A–Sepharose (pH 11.5) or 50 mM citrate buffer (pH 3.0) for the Affi Gel-10.

Results with the two matrices showed that although the Affi Gel-10–D5 bound more protein (40%) and more p29 (77%) than Protein A–Sepharose–D5 (21 and 50% respectively), the product from the Protein A–Sepharose–D5 gave a higher specific activity (1000 units/mg D5) than the Affi Gel-10-D5 matrix (580 units/mg D5). The recovery of p29 from the Protein A–Sepharose–D5 matrix was also higher ($\sim$80%) than that of the Affi Gel-10 matrix (4%) (unpublished data). The Protein A–Sepharose-D5 matrix was selected for further methodological development using D5 coupled at a concentration of 6.5 mg/ml Protein A–Sepharose.

Elution of matrix-bound p29

Optimisation studies for the pH elution of adsorbed, $(NH_4)_2SO_4$ fractionated p29 from the Protein A–Sepharose–D5 matrix were performed initially with myometrial cytosols. Maximum recovery of p29 (80–100%) from the immunoaffinity adsorbent occurred at a pH above 11.0 and below 3.0 [16]. In further experiments, elution of p29 was effected with 0.05 M diethylamine at a pH of 11.5.

Final purification

Experiments in the development of the three column system of purification of p29 used columns of approximately the same size and diameter as those of Logeat *et al.* (see Experimental and Ref. [10]).

The isolation of p29 was finally accomplished by a two-step procedure using $(NH_4)_2SO_4$ fractionation followed by chromatography on a three column affinity system using Protein

Table 1. Data from a three column system of p29 purification from ZR-75-1 cells

Sample	Volume (ml)	Soluble protein (mg/ml)	Specific activity (units p29/mg protein)	Purification factor (fold)	Recovery protein (%)	Recovery p29 (%)
Cytosol	8	3.1	54	1	100	100
0–35% Ammonium sulphate fraction	1	6.0	205	4	24	91
Affinity eluate	12.8	0.009	3888	72	0.625	36

A–Sepharose. Homogenates (1:5 w/v) of ZR-75-1 breast cancer cells (7–23 g) were centrifuged at 100,000 g_{av} and the resulting cytosols fractionated with 0–35% w/v of $(NH_4)_2SO_4$. Pellets were reconstituted in 1/10th the original volume in borate buffer and recentrifuged at 100,000 g_{av} for 30 min to remove aggregated material. The mean recovery of p29 in the reconstituted ammonium sulphate pellets from 6 experiments was 63%, the range of values being 34–91%. This step produced a 2–4-fold purification.

The reconstituted $(NH_4)_2SO_4$ fractionated samples were dialysed overnight against excess borate buffer and applied to the three column system at flow rates of 11–25 ml/h. Assays of column effluents for total protein indicated that 30–88% of the applied protein was recovered from the system. After disconnecting the third column (the Protein A–Sepharose–D5 column), the column was washed with the 6 buffers listed in Experimental at flow rates of 80–114 ml/h. Recovery of bound p29 was achieved by 0.05 M diethylamine (pH 11.5). Eluates were immediately neutralised by the addition of 1/10th volume of 0.5 M NaH_2PO_4.

A summary of a purification of p29 using the above protocol is shown in Table 1. The overall recovery of p29 was 36%, the total recovery of protein 0.625% and the purification obtained 72-fold. Using this methodology, sufficient pure p29 was routinely isolated from ZR-75-1 cells for protein sequencing and biochemical characterization studies. Further attempts were made to scale up the developed procedure by factors of 9–12-fold and, although yielding pure p29, were less successful, resuting in lower overall recoveries ranging from 6–14% of the cytosolic p29 (data not shown).

Characterization of immunoaffinity purified p29

Affinity eluate fractions were subjected to one-dimensional SDS–PAGE and silver stained to detect any contaminating proteins (Fig. 1). Silver staining detected only one band in the immunoaffinity purified p29 (Gel A, track 3). Fractions were also Western blotted to detect p29 specifically with D5 (Gel B, track 3). The immunoaffinity purified p29 was therefore still recognised by the antibody D5. Silver stained two-dimensional PAGE of the purified p29 preparation also demonstrated the presence of a single protein species with a pI of about 6.6 (Fig. 2). p29 was therefore judged to be pure by the above criteria. Molecular weight, pI and the ability to be recognised by D5 confirmed the identity of the purified protein as p29.

The amino acid analysis of p29 is not unusual (Fig. 3A), no cysteine residues are seen as they are not detected by the amino acid analyser. No tryptophan is seen as it is destroyed during the hydrolysis, glutamic acid and glutamine are quoted together as glutamate and aspartic acid and asparagine are quoted together as aspartate. p29 is low in the aromatic amino acids, tyrosine and phenylalanine but does not show any other unusual characteristics.

Analysis of the N-terminus determined a sequence of 15 amino acids (Fig. 3B). A database search found a 100% homology between this

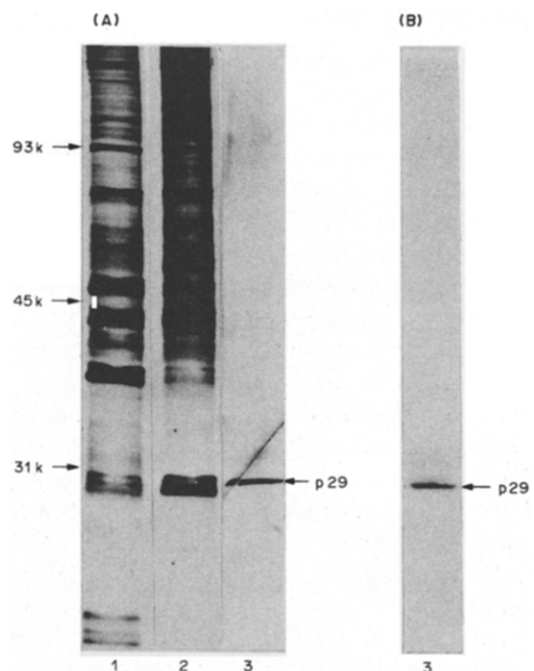

Fig. 1. SDS–PAGE of fractions from an immunoaffinity purification of p29 from ZR-75-1 cells. (A) Silver stained (B) Western blot-probed with D5 antibody. Fraction 1, ZR-75-1 cytosol (2 μg), fraction 2, ZR-75-1 0–35% ammonium sulphate (2 μg) and fraction 3, purified p29 (8 ng).

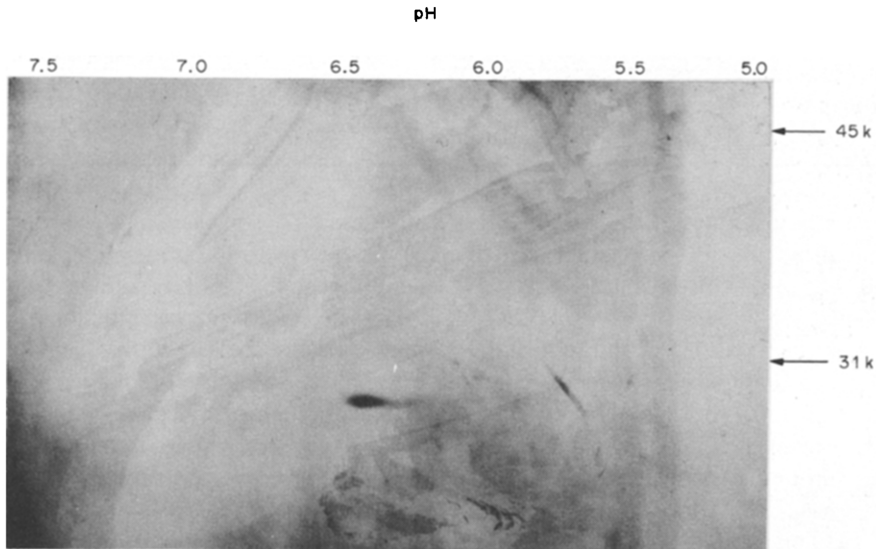

Fig. 2. Silver stained two-dimensional electrophoresis of purified p29.

sequence and a portion of internal sequence from an 89 kDa murine cytomegaloviral phosphoprotein (pp89) at amino acids 88–101 [17], although no significant nucleotide sequence homology between genes coding for murine and human proteins has been found.

(A)

AMINO ACID	NUMBER PER MOLECULE
Asp	35
Thr	6
Ser	37
Glx	37
Pro	4
Gly	30
Ala	35
Cys	0
Val	22
Met	8
Ile	10
Leu	26
Tyr	2
Phe	4
His	12
Lys	12
Arg	13

(B)

x-Asn-Asp-Asn-Ser-Asp-Met-Ala-Ile-Lys-His-Ala-Ala-Val-Arg

Fig. 3. Amino acid analysis (A) and N-terminal sequence analysis (B) of purified p29. Experimental details as text.

Use of purified p29 as a standard

Using p29 purified from ZR-75-1 cells, pure p29 was used to standardise the ELISA and an IRMA previously established in the laboratory. Prior to this a 0–25% $(NH_4)_2SO_4$ fraction of myometrial cytosol had been used as the p29 reference in both these assays. Once standardised with pure p29, the amount of p29 in the myometrial reference could be determined to allow accurate assessment of absolute units (pmol/g) p29 rather than relative units in assayed samples. By sequential dilution of a pure sample of p29 a standard curve was constructed and the assay calibrated (data not shown). A cut-off point of 20 pmol p29/ml was used to determine whether samples were positive ($\geqslant$20 pmol p20/ml) or negative ($<$20 pmol p29/ml) for p29 when patient breast tumour cytosols were assayed [7]. The use of p29 as a reference standard in this assay established that there was approximately 2.46 pmols p29/mg DNA in ZR-75-1 cells and approximately 1.6 pmols p29/mg DNA in human myometrium. It was also shown that an approximately 1000-fold excess of p29 over ER is present in ZR-75-1 cells on a molecule:molecule basis. The large amount of p29:ER equates with the low purification factor necessary to purify p29 from ZR-75-1 cells.

DISCUSSION

The method of p29 purification described in this paper represents the most practical method of obtaining pure p29 based on its affinity for the primate specific antibody D5. Purifications of p29 to only 100-fold homogeneity highlight the high concentrations of p29 in the ZR-75-1 cell line.

The biological function of p29 remains to be established. The significance of the N-terminus sequence homology with murine cytomegaloviral protein pp89 is unclear. pp89 is one of the first proteins expressed in cells after infection or reactivation from latency. After transient expression or stable transfer of the pp89 gene into fibroblasts, functional analysis confirms pp89 activates heterologous promoters [17, 18]. Ongoing attempts at cDNA cloning and more complete sequencing of p29 should further resolve questions of identity and function for this protein.

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