Identification of a novel form of the α_3 integrin subunit: covalent association with transferrin receptor

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The $\alpha_3\beta_1$ integrin is a cell-surface receptor for laminin, entactin, collagen, fibronectin and epiligrin. On some prostatic-carcinoma cell lines that express the $\alpha_3\beta_1$ heterodimer we have identified a novel form of the α_3 subunit. Whereas the prototypic α_3 subunit has a molecular mass of ~ 155 kDa, we have isolated a ~ 225 kDa protein (p225) which is recognized by monoclonal antibodies to the α_3 subunit. Protein sequence analysis revealed that p225 consists of two polypeptides, namely integrin α_3 heavy chain (~ 130 kDa) disulphide-bonded to a monomer of the transferrin receptor (~ 95 kDa) instead of the typical α_3 light chain (~ 25 kDa). The p225 seems to be directly associated with β_1 subunit, since it was immunoprecipitable with anti-(β_1 subunit)

antibodies. The association of transferrin receptor and integrin α_3 was apparently not the result of spurious disulphide-bond formation occurring during the protein purification, as iodoacetamide and GSH did not block the formation of the complex. The transferrin receptor is normally a homodimer that is involved in the internalization of iron-bound transferrin into cells and can be expressed at relatively high levels in the cell lines which we have studied. The p225 is not found on all cell types examined to date and therefore it may represent a unique complex between the integrin α_3 subunit and the transferrin receptor, a covalent association which may play a role in the adherence and/or proliferation of some types of tumour cells.

INTRODUCTION

The integrins are a family of integral membrane glycoproteins involved in cellular adhesion to the extracellular matrix and, in some cases, intercellular adhesion. Integrins are heterodimeric proteins formed by the selective non-covalent association of one of 15 known α subunits with one of eight known β subunits (Hynes, 1987; Ruoslahti, 1991). These integrin heterodimers are responsible for specific interactions with extracellular-matrix components or cell-surface ligands; interactions which ultimately affect cellular adhesion, migration, growth and differentiation, partly via the transduction of biochemical signals into cells (Albeida et al., 1990; Hynes et al., 1991; Hynes, 1992; Schwartz, 1993).

Of the 15 known α subunits, eight (α_{3-9} , α_v and α_{11b}) are proteolytically cleaved into two polypeptide chains. This results in an extracellular heavy chain and a transmembrane light chain which are joined by a disulphide bond (Dedhar, 1990). The 155 kDa α_3 subunit is one such subunit, being composed of a 130 kDa extracellular heavy chain which is disulphide-bonded to a 25 kDa membrane-spanning light chain (Hemler, 1990; Takada et al., 1991). The cDNA for α_3 has been cloned (Takada et al., 1991), and this subunit forms a heterodimer with β_1 , resulting in what was first characterized as VLA-3, a receptor for fibronectin, laminin and collagen (Hemler, 1990; Takada et al., 1991). Subsequently, the $\alpha_3\beta_1$ integrin has been identified as a receptor for entactin (Dedhar et al., 1992b), epiligrin (Carter et al., 1991), $\alpha_3\beta_1$ (Symington et al., 1993) and, in some instances, for $\alpha_3\beta_1$ itself (Sriramarao et al., 1993).

Several integrin subunits have been shown to exist in structurally alternative forms or isoforms. In a cell-type-dependent manner, forms of the β_3 subunit (van Kuppevelt et al., 1989), the β_4 subunit (Tamura et al., 1990) and the α_6 subunit (Tamura et al., 1991; Cooper et al., 1991) have been identified as containing alternative cytoplasmic domains. In addition, the α_3 subunit has

been shown to have isoforms of its cytoplasmic domain (Tamura et al., 1991). In all of these cases the changes arise from alternative splicing of mRNA, and likely result in different cytoplasmic interactions, perhaps contributing to modification of cellular responses to ligation of the integrins involved. Recently, a novel, high-molecular-mass form of the integrin α_3 subunit was identified (Dedhar et al., 1992a). This protein, which has a molecular mass of 225 kDa, was isolated from DU 145 and PC-3 prostatic-carcinoma cell lines. Characterization of this protein indicated that it consists of the 130 kDa heavy chain of the 155 kDa α_3 integrin subunit which is disulphide-bonded to an unidentified 95 kDa polypeptide instead of the typical 25 kDa light chain.

In the present study we further characterize the novel 225 kDa protein and show that the 130 kDa α_3 heavy chain is disulphide-bonded to a 95 kDa monomer of the transferrin receptor. The transferrin receptor is a 184 kDa cell-surface receptor which is ubiquitously expressed and is up-regulated on rapidly proliferating cells (Larrick and Cresswell, 1979; Newman et al., 1982). It is a disulphide-bonded homodimer of 95 kDa subunits, each of which spans the plasma membrane, leaving a short N-terminal cytoplasmic domain (Newman et al., 1982; McClelland et al., 1984). The transferrin receptor binds the iron-carrying serum protein transferrin and participates in the internalization of iron and the recycling of transferrin (Newman et al., 1982; Dautry-Varsat et al., 1983). This process is an absolute requirement for cell proliferation. The expression of the p225 is cell type-dependent, and we suggest that expression of this 225 kDa protein at the cell surface may result in an altered cellular response to $\alpha_3\beta_1$ ligands and/or transferrin.

EXPERIMENTAL

Materials

Protein A-Sepharose and CNBr-activated Sepharose 4B were purchased from Sigma (St. Louis, MO, U.S.A.). Iodogen reagent

Abbreviations used: RIPA, radioimmunoprecipitation assay; mAb, monoclonal antibody.

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for iodination of cell-surface proteins was purchased from Pierce Chemicals (Rockford, IL, U.S.A.) and Na^{125}I from Amersham Corp. (Oakville, Ont., Canada). Chemicals for SDS/PAGE, including molecular-mass standards, were purchased from Bio-Rad (Mississauga, Ont., Canada). The mouse monoclonal antibodies against integrin α_3 (P1B5), α_2 (P1E6), α_5 (P1D6) and β_1 (A051) were purchased from Telios Pharmaceuticals (La Jolla, CA, U.S.A.). The mouse monoclonal antibody (mAb) J143 raised against human α_3 (Kantor et al., 1987) was provided by Dr. Albino (Memorial Sloan Kettering Cancer Center, New York, NY, U.S.A.). The anti-(integrin subunit α_6) mAb (G0H3) (Sonnenberg et al., 1988) was provided by Dr. A. Sonnenberg (Red Cross Laboratories, Amsterdam, The Netherlands) and anti-(integrin β_1 subunit) mAb (A1IB2) was a gift from Dr. Carolyn Damsky (University of California, San Francisco, CA, U.S.A.). The anti-(transferrin receptor) antibody (B3/25) was purchased from Boehringer Mannheim Biochemica (Laval, Que., Canada). V8 proteinase from *Staphylococcus aureus* was obtained from Sigma, and recombinant N-glycosidase F was purchased from Boehringer Mannheim (Laval).

Cells and growth conditions

Prostatic-carcinoma cell lines DU 145 and PC-3 were purchased from American Type Culture Collection (Rockford, MD, U.S.A.). Cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 7% heat-inactivated fetal-bovine serum (Gibco, Burlington, Ont., Canada).

Surface labelling and preparation of cell extracts

Cells were harvested from rapidly growing subconfluent (3×10^4 cells/cm²) cultures and resuspended in PBS containing 0.2 mM phenylmethanesulphonyl fluoride. The suspended cells were radiolabelled with Na^{125}I as described by Leblond et al. (1982). Briefly, Iodogen reagent was dissolved in chloroform and used to coat the inside of 20 ml glass vials. Cells (1×10^6 in 1 ml of PBS) were placed in each vial with 0.5 mCi of Na^{125}I and gently agitated at room temperature for 15 min. The cells were washed with 5 vol. of PBS to remove free iodine. The radiolabelled cells were extracted in a radioimmunoprecipitation assay (RIPA) lysis buffer consisting of PBS with 1.0% Triton X-100, 0.1% SDS and 0.5% sodium deoxycholate. Proteinase inhibitors used included 1 mM phenylmethanesulphonyl fluoride, 1 mM benzamidine hydrochloride and 10 $\mu\text{g}/\text{ml}$ each of antipain, aprotinin, pepstatin A and leupeptin. GSH (25 mM) or iodoacetamide (20 mM) were included during cell lysis and all subsequent protein purification procedures in an effort to minimize spurious disulphide-bond formation.

Immunoprecipitations

Immunoprecipitations of extracts of ^{125}I -radiolabelled cell-surface protein were carried out using 1×10^6 c.p.m./immunoprecipitation, the appropriate antibodies and Protein A-Sepharose as described previously (Dedhar et al., 1989). The antigen-antibody complexes were dissociated by boiling in sample buffer (200 mM Tris/HCl, pH 6.8, containing 3% SDS, 10% glycerol and 0.001% Bromophenol Blue). Samples were analysed by electrophoresis in non-reducing SDS/6.0%-polyacrylamide gels, followed by autoradiography.

Immunoaffinity chromatography and isolation of proteins

Detergent extracts from ^{125}I -surface-labelled DU 145 cells were incubated on anti- α_3 IgG-Sepharose columns, which were pre-

pared by coupling 100 μg of anti- α_3 mAb (P1B5) to 700 μl of pre-swollen CNBr-activated Sepharose 4B. The unbound material was removed by washing the column with 30 ml of RIPA lysis buffer and then 20 ml of 10 mM sodium phosphate buffer, pH 8.0. The proteins bound to the column were eluted with 4 ml of 100 mM sodium phosphate buffer, pH 11.5, collected, and neutralized with 800 μl of 1 M sodium phosphate buffer, pH 4.2. The eluted material was concentrated by centrifugation at 6250 rev./min and 4 °C in a Centricon 30 microconcentrator (Amicon) and then electrophoresed on SDS-containing 6.0% polyacrylamide gels under non-reducing conditions. The gels were dried and exposed to X-ray film at -70 °C. To isolate specific proteins, gels were dried overnight at room temperature prior to autoradiography. Protein bands were excised from the gel, placed in dialysis tubing and electroeluted overnight at 18 V in 12.5 mM Tris/96 mM glycine/0.5% SDS. The electroeluted material was concentrated using a Centricon 30 apparatus as described above.

Cell-free protein labelling, deglycosylation and proteinase digestion

In order to ensure that purified proteins contained sufficient ^{125}I label for V8-proteinase mapping, it was necessary to relabel these proteins. Proteins isolated by SDS/PAGE were further labelled with ^{125}I using chloramine-T (Sigma) by methods based on those first described by Greenwood et al. (1963). Briefly, concentrated protein electroeluate was adjusted to 500 μl in PBS with 0.1% SDS, 20 mM Tris, pH 7.5, and 0.05 mg/ml chloramine-T (final concns.). Na^{125}I (0.3 mCi) was then added to the protein solution in glass vials and the mixture was gently shaken for 10 min at room temperature. The reaction was quenched with sodium metabisulphite (Sigma), (0.5 mg/ml final concn.) and excess label removed by passage through two successive PD10 Sephadex G-25M columns (Pharmacia, Baie D'Urfe, Que., Canada) equilibrated with 0.1% SDS/20 mM Tris, pH 7.5, containing the proteinase inhibitors described above. The purified labelled protein sample was then concentrated using a Centricon 30 apparatus and deglycosylated with N-glycosidase F according to the protocol provided by the suppliers. Samples were analysed by SDS/PAGE under reducing conditions, followed by autoradiography. Protein bands obtained from the reduction and deglycosylation step were excised from the gel and the gel slices were loaded into wells of SDS-containing 12.5%- or 15%-polyacrylamide gels. The wells were then filled with 1 $\mu\text{g}/\text{ml}$ V8 proteinase in sample buffer [62.5 mM Tris (pH 6.8)/2.3% SDS/10% glycerol] containing 1 mM EDTA. When the dye front reached the end of the stacking gel, electrophoresis was interrupted for 30 min to allow digestion of the proteins. After electrophoresis, the gel was dried under vacuum at 75 °C and exposed to X-ray film.

Amino acid sequence analysis

SDS/PAGE purified samples of both the 155 kDa α_3 and the 225 kDa α_3 -related protein were electroeluted, reduced and separated by SDS/6.0%-PAGE (to obtain the 100 kDa heavy chains and the 95 kDa light chain). The proteins were then transferred to nitrocellulose (Schleicher and Schuell, Keene, NH, U.S.A.) and digested *in situ* with endoprotease Lys-C (Boehringer Mannheim) as previously described (Aebbersold et al., 1987). The resulting peptides were isolated using an Applied Biosystems model 130 microbore h.p.l.c. instrument equipped with a RP300 L8 Brownlee cartridge. Peptides represented by prominent elution peaks were then loaded on to the hydrophobic half of a

Hewlett-Packard biphasic sequences column and sequenced on a G1000S Hewlett-Packard protein-sequencing system.

RESULTS

Isolation and characterization of p225

To analyse and identify the p225, it was isolated either by immunoprecipitation or immunoaffinity chromatography using an anti- α_3 mAb (P1B5). DU 145 or PC-3 prostatic-carcinoma cells were surface-labelled with ^{125}I , lysed with RIPA buffer and the lysate was then cleared by centrifugation. The labelled extract was then applied to an anti- α_3 mAb-Sepharose affinity matrix and incubated overnight at 4 °C. After washing with RIPA followed by 10 mM phosphate buffer, pH 8.0, the bound proteins were eluted with 100 mM phosphate buffer, pH 11.5. The eluted proteins were then analysed by SDS/PAGE. Alternatively, labelled extracts were immunoprecipitated using subunit-specific integrin antibodies. Figure 1, lane 2, shows the major proteins that were isolated by the α_3 antibody P1B5. The main bands represent the 155 kDa α_3 subunit, the 116 kDa β_1 subunit and the 225 kDa protein (p225). Figure 1 also shows the two peptides (heavy and light chains) which result from reduction and deglycosylation of the 225 kDa protein (lane 4). On reduction and deglycosylation, p225 yielded a 110 kDa heavy chain and an approx. 78 kDa light chain (referred to here as 'p95' in accordance with its relative molecular mass in its glycosylated form). In comparison, lane 3 of Figure 1 shows the result of reduction and deglycosylation of the 155 kDa α_3 protein. In lane 3, only the 110 kDa deglycosylated heavy chain of α_3 is shown. The 25 kDa light chain typically associated with α_3 heavy chain to form a 155 kDa subunit has been demonstrated previously (Dedhar et al., 1992a) and is not shown here. Unlike the 155 kDa α_3 , p225 did not yield any detectable 25 kDa light chain on reduction (results not shown; see Dedhar et al., 1992a). The 110 kDa peptide from p225 has previously been reported to be identical with the 110 kDa heavy chain of the 155 kDa α_3 by V8-proteinase mapping and N-terminal amino acid analysis (Dedhar et al., 1992a). V8-proteinase digests of these two heavy chains are shown again in Figure 4 (below).

Figure 1 Isolation and characterization of the peptide chain structure of p225

Extracts of ^{125}I -surface-labelled DU 145 prostatic-carcinoma cells were immunoprecipitated with non-specific mouse IgG (lane 1) or anti- α_3 antibody P1B5 (lane 2) and then separated by SDS/6.0%-PAGE. Individual protein bands from lane 2 were then excised and electroeluted from the acrylamide, labelled again with ^{125}I , deglycosylated, and reduced. Deglycosylation and reduction of the 155 kDa α_3 subunit produced a 110 kDa peptide (lane 3), while the 225 kDa protein yielded a 110 kDa peptide and an ~78 kDa peptide, designated p95 (lane 4). Abbreviation: M, molecular mass.

TfR	288	Phe-Pro-Ile-Val-Asn-Ala-Glu-Leu-Ser-Phe-Phe-Gly-His-Ala-His	302
Peptide 1 (p95)		Phe-Pro-Ile-Val-Asn-Ala-Glu-Leu-Ser-Phe-Phe-Gly-His-Ala-His	
TfR	694	Glu-Ser-Pro-Phe-Arg-His-Val-Phe-Trp-Gly-Ser-Gly-Ser-His-Thr	708
Peptide 2 (p95)		Glu-Ser-Pro-Phe-Arg-His-Val-Phe-Trp-Gly-Ser-Gly-Ser-His-Thr	
TfR	721	Gln-Asn-Asn-Gly-Ala-Phe-Asn-Glu-Thr-Leu	730
Peptide 3 (p95)		Gln-Asn-Asn-Gly-Ala-Phe-Asn-Glu-Thr-Leu	

Figure 2 Amino-acid-sequence analysis of the p95 light chain of p225

Gel-purified p225, isolated by anti- α_3 affinity chromatography was separated into its constituent heavy chain and light chain (p95) by SDS/PAGE. The proteins were then transferred to nitrocellulose, stained with Ponceau-S and the p95 was excised, digested and used in amino acid analysis as described in the Experimental section. Three non-contiguous peptide fragments from p95 are shown aligned below the sequence-identical segments of the transferrin receptor (TfR).

Figure 3 Subunit structure of the transferrin receptor

^{125}I -surface-labelled DU 145 prostatic-carcinoma cells were extracted and immunoprecipitated with the anti-(transferrin receptor) antibody B3/25 and the immunoprecipitates were analysed by SDS/6.5%-PAGE. The homodimeric transferrin-receptor protein of molecular mass (M) ~184 kDa is shown in lane 1, while the deglycosylated and reduced monomer is shown in lane 2.

Amino acid sequence of the p225 light chain

To obtain amino-acid-sequence information for the 95 kDa polypeptide chain of p225, the 95 kDa chain was isolated from affinity-purified p225. Amino acid sequences from three proteolytically derived peptides were obtained as described in the Experimental section and are shown in Figure 2. Peptide fragments from two independent preparations of the 95 kDa protein were sequenced and all showed complete sequence identity with peptide segments of the transferrin receptor. Figure 2 shows an alignment of the three sequences obtained from protein sequencing with corresponding sequences of the human transferrin receptor as derived from cDNA (McClelland et al., 1984). Proteolytic fragments of the heavy chain of p225 (110 kDa band of Figure 1, lane 4) were also sequenced and found to be identical with internal segments of the integrin α_3 heavy chain (results not shown).

We were next interested in determining whether any of the transferrin receptor expressed by the prostatic-carcinoma cells being studied was complexed to $\alpha_3\beta_1$. Extracts of ^{125}I -surface-labelled DU 145 or PC-3 cells were prepared as described above and immunoprecipitated with anti-(transferrin receptor) mAb (B3/25). In Figure 3, lane 1, the major protein isolated by B3/25, representing the transferrin-receptor homodimer of ~184 kDa, is shown. Overexposure of film to the gel has confirmed that

Figure 4 V8-proteinase peptide maps of the heavy and light chains of p225

The ^{125}I -labelled, deglycosylated and reduced polypeptide chains obtained from p225 were digested with V8 proteinase prior to separation by SDS/12%-PAGE. The heavy chain of p225 (lane 2) is compared with a V8-proteinase digest of the heavy chain of the 155 kDa α_3 subunit (lane 1). A map of the light chain, i.e. p95, of the 225 kDa protein (lane 4) is compared with a V8-proteinase digest of the monomeric subunit of the transferrin receptor (lane 3). Abbreviation: *M*, molecular mass.

there is no detectable $\alpha_3\beta_1$ in the transferrin-receptor immunoprecipitation. Lane 2 of Figure 3 shows the transferrin receptor after gel purification of the homodimer followed by chloramine- τ labelling, deglycosylation and reduction. The result is a deglycosylated monomer of the transferrin receptor with a relative molecular mass of ~ 78 kDa, the same molecular mass as that obtained by deglycosylation of the p95 light chain of p225 (Figure 1, lane 4).

Peptide mapping of the heavy and light chains of p225

To confirm the results of the amino acid analyses, the 95 kDa light chain of p225 was labelled with chloramine- τ , reduced with β -mercaptoethanol and deglycosylated. The resultant peptide, seen in Figure 1, lane 4, was then digested with V8 proteinase. The V8-proteinase map of p95 is shown in Figure 4, lane 4, and this map can be compared with Figure 4, lane 3, a V8-proteinase map of the deglycosylated monomer of transferrin receptor which was prepared as described above (protein band of Figure 3, lane 2). The resulting maps are very similar, which is in agreement with the amino-acid-sequence data and supports the identification of the 95 kDa light chain of p225 as a monomeric subunit of the transferrin receptor. Also in Figure 4 a V8-proteinase digest of the 110 kDa heavy chain of the 155 kDa α_3 (Figure 4, lane 1) is shown next to a V8-proteinase digest of the 110 kDa heavy chain of p225 (lane 2), reconfirming the identity of the 110 kDa component of p225 as α_3 heavy chain.

In vivo association of transferrin receptor with Integrin α_3

During our purification of the p225, we wanted to minimize the possibility of any 'artifactual' interaction between the α_3 heavy chain and the transferrin receptor that could result in the spurious formation of disulphide bonds. To this end, experiments to isolate and characterize the p225 as described above were repeated with 25 mM GSH or 20 mM iodoacetamide present at the time of cell harvesting and subsequently during all stages of receptor purification. GSH is known to maintain the status of free thiol groups (Cotgreave et al., 1990) and iodoacetamide irreversibly alkylates thiol groups (Dawson et al., 1986). Thus the presence of either of these compounds effectively reduces the opportunity for

Figure 5 *In vivo* association of transferrin receptor and the integrin α_3

Extracts of ^{125}I -surface-labelled DU 145 cells were prepared in the absence (lanes 1, 2, 3 and 4) and in the presence (lanes 5, 6, 7, 8 and 9) of 20 mM iodoacetamide. The labelled extracts were then immunoprecipitated with various antibodies and the immunoprecipitated proteins were separated by SDS/6.0%-PAGE. Lanes 1 and 5, non-specific mouse IgG; lanes 2 and 6, anti- α_2 P1E6; lanes 3 and 7, anti- α_3 P1B5; lanes 4 and 8, anti- β_1 A051; lane 9, anti-(transferrin receptor) B3/25. The arrow at right shows the position of p225. Abbreviation: *M*, molecular mass.

the formation of extraneous disulphide bonds. As seen in Figure 5, lanes 3, 4, 7 and 8, the presence of 20 mM iodoacetamide had no effect on the purification of p225. Similar results were obtained with 25 mM GSH alone (results not shown). In addition, Figure 5 also shows that p225 is present in the immunoprecipitates of integrin β_1 subunit antibody A051 (lanes 4 and 8), but not in those of integrin α_2 antibody P1E6 (lanes 2 and 6) or non-specific mouse IgG (lanes 1 and 5). Lane 9 of Figure 5, like lane 1 of Figure 3, shows the immunoprecipitation of transferrin receptor by mAb B3/25. Higher-resolution gels have confirmed that no protein co-migrating with p225 is detectable in transferrin-receptor immunoprecipitations. This may be a reflection of the fact that, relative to transferrin receptor on these cells, p225 is a minor component.

DISCUSSION

In the present paper we have provided data which demonstrate that a 225 kDa protein complex containing the α_3 integrin heavy chain (Dedhar et al., 1992a) arises from a covalent interaction between the 95 kDa subunit of transferrin receptor and α_3 heavy chain. This complex was first observed by its immunoprecipitation from ^{125}I -surface-labelled cell extracts of DU145 and PC-3 prostatic-carcinoma cell lines using the anti- α_3 monoclonal antibodies J143 or P1B5 (Dedhar et al., 1992a). In that initial study, the high-molecular-mass form of α_3 was characterized as a 225 kDa cell-surface protein that was associated with the α_3 and β_1 heterodimer. The 225 kDa subunit was shown to consist of a typical 130 kDa α_3 heavy chain, to which was disulphide-bonded an unidentified 95 kDa light chain instead of the typical α_3 light chain of 25 kDa (Dedhar et al., 1992a). The evidence for this structure of p225 presented by Dedhar et al. (1992a) included the following results: (i) p225 co-immunoprecipitated with α_3 and β_1 using anti- α_3 monoclonal antibodies; (ii) reduction of p225 with β -mercaptoethanol generated two polypeptides of 130 kDa and 95 kDa, compared with the two polypeptides of 130 kDa and 25 kDa produced by reduction of the 155 kDa typical α_3 subunit; (iii) deglycosylation of the 130 kDa polypeptides from either source resulted in polypeptides of 110 kDa; (iv) V8-proteinase-generated peptide maps and N-terminal amino acid sequences of the 110 kDa

Figure 6 Model of p225 structure at cell surface

Schematic diagrams of the transferrin-receptor (TfR) homodimer and the $\alpha_3\beta_1$ integrin heterodimer are shown to the left. Biochemical evidence, both reviewed and presented here, suggest that the schematic diagram on the right may represent the structure of the p225 in association with β_1 .

polypeptides from either the p225 or the 155 kDa α_3 were identical.

In the present study we have further analysed the 95 kDa portion of the p225 and found that it is very similar to, if not identical with, the 95 kDa subunit of the human transferrin receptor. There are primarily two pieces of evidence on which to base this conclusion. First, the p225 was purified and reduced into its constituent 130 kDa and 95 kDa polypeptides. Proteolytically derived fragments of the 95 kDa polypeptide were subjected to protein sequencing. Of three non-contiguous internal peptides sequenced (two 15-residue and one 10-residue), all showed 100% sequence identity with segments of the human transferrin receptor (Figure 2), as reported by McClelland et al. (1984). Secondly, the peptide map generated by V8-proteinase digestion of the ^{125}I -labelled 95 kDa portion of p225 was in close agreement with the peptide map generated by similar treatment of the 95 kDa subunit of human transferrin receptor (Figure 4).

In order to ensure that the observed association between α_3 integrin heavy chain and transferrin receptor was not a result of spurious disulphide-bond formation during our purification procedures, we have shown that the presence of 25 mM GSH or 20 mM iodoacetamide during all purification steps had no effect on the isolation of p225 (Figure 5). Both of these compounds are known to prevent disulphide-bond formation between free thiol groups (Dawson et al., 1986; Cotgreave et al., 1990), and the results of these experiments suggest that the association between α_3 and transferrin receptor occurs *in vivo*. Co-immunofluorescence of $\alpha_3\beta_1$ and transferrin receptor on anti- α_3 -antibody-clustered cells also demonstrated the co-localization of some transferrin receptor molecules with $\alpha_3\beta_1$ (results not shown). Additionally, we have established that immunoprecipitation of ^{125}I -labelled Du145 cell extracts with the β_1 monoclonal antibodies AIIB2 (results not shown) and A051 also results in the detection of a 225 kDa protein (Figure 5). This, along with the fact that the 225 kDa protein is expressed at the cell surface, suggests that the p225 forms a heterodimer with β_1 or at least associates with $\alpha_3\beta_1$ at the cell surface. Thus Figure 6 shows models of the transferrin receptor, the $\alpha_3\beta_1$ integrin and a possible structure of the 225 kDa α_3 subunit expressed in association with β_1 at the cell surface.

The transferrin receptor is typically a cell-surface complex (CD 71) comprised of two identical 95 kDa subunits which are disulphide-bonded to form an approx. 184 kDa homodimer (Newman et al., 1982). Unlike the integrin subunits, the subunits

of the transferrin receptor lack N-terminal signal sequences and are oriented with extracellular C-termini and intracellular N-termini (McClelland et al., 1984). The transferrin receptor binds the iron-carrying serum protein transferrin for the purposes of internalizing iron and recycling transferrin (Jandl and Katz, 1963; Newman et al., 1982; Dautry-Varsat et al., 1983). This receptor is expressed on all known cell types, is up-regulated on rapidly proliferating cells and its function is essential for the growth of cells *in vitro* (Newman et al., 1982; Trowbridge and Lopez, 1982; Musgrove et al., 1984).

The topological, albeit superficial, similarities between the transferrin receptor and the α_3 integrin subunit are intriguing sources of speculation regarding the origins of p225. Both transferrin receptor subunits and α_3 subunits are transmembrane proteins which traverse the plasma membrane once, leaving short cytoplasmic domains and relatively large extracellular domains (Takada et al., 1991; McClelland et al., 1984). Also, both types of subunits partake in one extracellular disulphide bond (Schneider et al., 1981; Hemler, 1990; Takada et al., 1991). These similarities allow for the possibility that, in the processes of post-translational disulphide-bond formation and peptide cleavage, a proportion of the α_3 subunit heavy chain becomes disulphide-bonded to a subunit of transferrin receptor. An interaction such as this is certainly feasible, considering the high levels of transferrin receptor expressed on the DU 145 and PC-3 prostatic-carcinoma cells studied here (Keer et al., 1990). Accordingly, we have observed that the level of p225 expressed by the prostatic cell lines we have studied is higher on low-density rapidly proliferating cultures than it is on higher-density slowly growing cultures (Dedhar et al., 1992a; M. Coppolino and S. Dedhar, unpublished work). This parallels the pattern of expression of transferrin receptor characterized on several cell types (Newman et al., 1982; Musgrove et al., 1984; Neckers and Trepel, 1986).

At this point it is not clear which ligands p225 recognizes. Indeed, the marked change to the structure of an integrin described here may result in its binding to ligands not previously characterized as integrin substrates. Moreover, beyond the substrates that p225 may interact with, there are potentially modified intracellular events which may be associated with such an altered transmembrane protein. It is possible that a p225/ β_1 heterodimer will not form the same cytosolic associations as $\alpha_3\beta_1$. Thus, even though the p225 is a small proportion of the total α_3 expressed on the cell lines examined (Dedhar et al., 1992a), its function may affect different intracellular events or be amplified along different signalling pathways than the normal $\alpha_3\beta_1$. Furthermore, the transferrin-receptor portion of p225 may effect its intracellular activity. For instance, if the 95 kDa portion of the p225 is a complete transferrin-receptor monomer, then it would contain the transferrin-receptor internalization signal YTRF (Collawn et al., 1993), a sequence not present in the α_3 integrin. A p225/ β_1 heterodimer could then be subjected to the cycling mechanisms of the transferrin receptor instead of normal integrin metabolism.

Variant forms of several integrin subunits, including α_3 , have previously been described (van Kuppevelt et al., 1989; Tamura et al., 1990, 1991; Cooper et al., 1991). In all of these cases, variations in the integrin subunits occur in the cytoplasmic domains, and the mechanism by which these alternative forms are believed to be generated is through the alternative splicing of mRNA. We have reported here on the expression of a novel α_3 integrin subunit which may arise by post-translational mechanisms and which depends upon a covalent association with the transferrin receptor. This results in a 225 kDa form of the α_3 subunit that has its 25 kDa light chain replaced by a 95 kDa

monomer of the transferrin receptor. The novel 225 kDa α_3 subunit may have significantly altered extracellular features as well as modified intracellular domains. With much recent research being done on the role that integrins play in the transduction of signals into cells, it will be of interest to determine what sort of functions the p225 subunit performs. It is possible that this novel complex between two seemingly disparate proteins, while resulting in a subtle change at the cell surface, could result in significantly altered intracellular events.

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