Biosynthesis, surface expression and function of the fibronectin receptor after rat liver cell transformation to tumorigenicity

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Zajdela hepatoma cells are poorly-adherent cells derived from an undifferentiated tumour and transplanted into rat. We compared the biosynthesis, structure and function of the fibronectin receptor in normal rat hepatocytes with that in Zajdela hepatoma cells. The rat hepatocyte fibronectin receptor has been isolated. It is composed of two subunits: α_5 (molecular mass 155 kDa) and β_1 (molecular mass 115 kDa). However, its biosynthesis has not yet been described. Using polyclonal antibodies raised against each of the subunits of the receptor, we observed that the α_5 -subunit was synthesized as a 155-kDa polypeptide in normal rat hepatocytes and Zajdela hepatoma cells. In contrast, the molecular mass of the β_1 -subunit was 130 kDa in Zajdela hepatoma cells versus 115 kDa in normal rat hepatocytes. Pulse-chase experiments showed that the apparent transition time from the 100-kDa β_1 -precursor to the 130-kDa mature form was abnormally prolonged in Zajdela hepatoma cells since the latter was not detected until 24 h, while the transition from the 100-kDa precursor to the 115-kDa mature form began within 3 h in normal rat hepatocytes. Digestion of both the normal rat hepatocytes and Zajdela hepatoma cells 100-kDa β_1 -precursors

with endo- β -N-acetylglucosaminidase H and peptide N-glycosidase yielded products from 100 kDa to 84 kDa and 82 kDa, respectively, as judged by SDS/PAGE, suggesting that the same polypeptide chain is synthesized in normal rat hepatocytes and in Zajdela hepatoma cells. Incubation of the mature normal rat hepatocyte β_1 -subunit with peptide N-glycosidase reduced its molecular mass from 115 kDa to 82 kDa, as judged by SDS/PAGE, while the molecular mass of the abnormal mature Zajdela hepatoma cell β_1 -subunit decreased from 130 to 110 kDa. Thus, in addition to alterations in the Asn-linked oligosaccharide processing, 'ascitic growth' induced other post-translational modifications in the Zajdela hepatoma cell β_1 -subunit. Furthermore, both the abnormal mature 130-kDa and precursor 100-kDa β_1 -subunits were detected on the surface of Zajdela hepatoma cells, associated with the α_5 -subunit. The relationship between these structural alterations in the fibronectin receptor and the impaired Zajdela hepatoma cell binding to soluble fibronectin or to a coated fibronectin matrix that was observed in this study is discussed.

INTRODUCTION

Integrins comprise a family of transmembrane glycoproteins that mediate cell adhesion to extracellular matrix and basement membranes, to other cells and to plasma proteins (reviewed in [1,2]). The basic integrin structure is a heterodimer of one 130–200-kDa α -subunit linked noncovalently to one 90–130-kDa β -subunit [1–3]. Initially, the integrins were divided into classes based on the three different β -subunits characterized [1]. However, additional β -subunits have been recently recognized (reviewed in [4,5]). Most integrins are members of the β_1 (CD29) or very-late-antigen subfamilies. VLA antigens are molecules that appear on lymphocytes several days after mitogen activation [6]. The β_1 -subfamily includes the fibronectin receptor (FNR), designated as $\alpha_5\beta_1$ in human cells [7], the collagen receptor [8] and the laminin receptor [9]. The β_2 -subfamily of integrins, also known as Leu-cell adhesion molecules (Leu-CAMs) or CD18 antigens, consists of three leukocyte-adhesion receptors, lymphocyte-function-associated antigen 1 (CD11a), Mac-1 (CD11b) and p150–95 complexes [10]. The β_3 -subfamily of integrins, also termed cytoadhesins, are represented by the vitronectin receptor (CD51) [11] and the CD41 extracellular matrix receptor, expressed on platelets, which serves as the receptor for fibronectin, von Willebrand's factor, fibrinogen and vitronectin [12].

The FNR, which was originally purified from a human osteosarcoma [13] and from human placenta cells [14], is one of

the best characterized integrins and has now been isolated from a variety of mammalian cells and tissues [13–17]. Both the α_5 - and β_1 -subunits of the human FNR have been cloned and their sequences have been deduced from complementary DNA [18,19].

In many cases, malignant transformation results in an abnormal localization of the FNR, a change in the level of FNR expression or a modification of the FNR structure [20–26]. For instance, Saga et al. [21] noted that some cultured transformed cells overexpress the FNR, whereas Plantefaber and Hynes [22] showed that others expressed greatly reduced levels of the FNR. Moreover, in transformed chicken cells, the FNR becomes phosphorylated on a cytoplasmic tyrosine residue [26]; this post-translational event affects the ability of the FNR to bind to cytoskeletal elements and to extracellular ligands [27,28].

Neoplastic transformation and tumour progression are also frequently accompanied by structural modifications in the carbohydrate moiety of glycoconjugates [29–32]. Nevertheless, relatively little is known about the relationship between malignant cell behaviour and changes in FNR glycosylation. To our knowledge, in one case, the weakly metastatic wheatgerm-agglutinin-resistant glycosylation mutant (Wa4bl), it was found that reduced cell adhesion to fibronectin is associated with altered N-glycosylation of the β_1 -FNR subunit [33]. In addition, recent studies using inhibitors of specific intracellular carbohydrate-processing enzymes have shown that oligosaccharide processing is important for the binding function

Abbreviations used: HyC, normal rat hepatocytes; ZHC, Zajdela hepatoma cells; FNR, fibronectin receptor; MEM, minimal essential medium; PBS, phosphate-buffered saline; PMSF, phenylmethanesulphonyl fluoride; FCS, foetal calf serum; endo H, endo- β -N-acetylglucosaminidase H; N-glycanase, peptide N-glycosidase.

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of the FNR, but not for receptor assembly or insertion into the plasma membrane [34].

In the present study, we investigated the biosynthesis and function of the FNR expressed in Zajdela hepatoma cells (ZHC), which are poorly-adherent cells derived from an undifferentiated tumour and transplanted into rats [35]. The characteristics of the cells grown *in vivo* and *in vitro* are well known [36,37]. ZHC have also been used extensively in studies on carbohydrate modifications in cell-structure glycoproteins expressed on liver cells after transformation to tumorigenicity and were found to exhibit dramatic changes in their *N*- and *O*-glycan patterns as compared to normal rat hepatocytes (HyC) [38]. The rat hepatocyte FNR has been identified only recently; it is composed of a 155-kDa α -subunit and a 115-kDa β -subunit which are held together by noncovalent interactions [17] whose maturation processes have not been described yet. Although the tumour cells were isolated from an ascitic rat hepatoma and the hepatocytes from normal liver, HyC/ZHC constitute a suitable model for studying FNR biosynthesis in normal and malignant rat hepatocytes and for determining how such post-translational changes can modulate the affinity of the FNR on tumour cells and contribute to their malignant phenotype.

MATERIALS AND METHODS

Cells and cell culture

ZHC were originally obtained following dimethylaminoazobenzene injection into Sprague-Dawley rats [35]. They were maintained as an ascites by serial intraperitoneal transplantation of tumour cells (10^7 cells/animal) into 8-week-old male Sprague-Dawley rats, weighing 250 g (Charles River, Clon, France). For *in vitro* culture, cells were collected by abdominal puncture under sterile conditions 7 days after transplantation and washed four times in minimum essential medium (MEM) (Flow Laboratories, Irvine, U.K.). Centrifugation at 55 *g* for 5 min permitted the isolation of tumour cells with a minimal contamination by red blood cells. Cells were then cultured at 37 °C in CO₂/air at 95% humidity as previously described [38,39]. ZHC are very poorly differentiated cells which have lost several hepatic functions, and constitute a population of isolated round-shaped cells with simplified cytological organization [36,37].

HyC were isolated from Sprague-Dawley rats, using the collagenase-perfusion technique described by Seglen [40], and modified by Guguen-Guillouzo et al. [41]. Cells were incubated at 37 °C for 3 h as described previously [38,39]. The unattached cells were removed by changing the medium and incubating the cells for a further 3, 6 or 24 h period. For binding and cell-surface-labelling experiments freshly-isolated HyC were used.

Antibodies

Rabbit anti-human plasma fibronectin IgG was purchased from Dakopatts (Glostrup, Denmark). Rabbit polyclonal antisera raised against the α _s and β ₁-subunits of rat hepatocyte FNR were provided kindly by Dr S. Johansson (University of Uppsala, Sweden). The specificities of the anti- β ₁-subunit antibodies [42–44] and of the anti- α _s-subunit antibodies have been described previously [43].

Plasma membrane fractions

HyC and ZHC plasma membranes were prepared by the method of Nato et al. [45]. In outline, ZHC and HyC were suspended in

phosphate-buffered saline (PBS; 0.9 mM CaCl₂, 2.7 mM KCl, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂ · 6H₂O, 140 mM NaCl, 8.2 mM Na₂HPO₄ · 7H₂O), pH 7.4, containing 2 mM phenylmethane-sulphonyl fluoride (PMSF) and were disrupted by nitrogen cavitation using a nitrogen bomb (Poly-Labo Paul-Block, Strasbourg, France) under 8 MPa of pressure. The homogenates were then centrifuged successively at 1000 and 9000 *g*, at 4 °C for 20 min to remove cell fragments and nuclei, and mitochondria, respectively. Finally the supernatants were centrifuged at 100000 *g* at 4 °C for 60 min. The pellets constituted the enriched plasma membrane fractions. Protein contents were determined by the method of Bradford [46] and the enriched plasma membrane preparation was stored at –80 °C until it was used.

Electrophoresis and Immunoblotting

SDS/PAGE was performed using the method of Laemmli [47]. Immunoblotting was performed using the method of Burnette [48]. In outline, after SDS/PAGE, proteins from enriched plasma membrane fractions or cell lysates were electrophoretically-transferred overnight onto nitrocellulose using a Blot Transfer System (Bio-Rad Laboratories, Richmond, U.S.A.) according to the procedure recommended by the manufacturer. Then the nitrocellulose membranes were incubated for 60 min in PBS, pH 7.4, 0.05% Tween-20, 2% bovine serum albumin (BSA) containing anti-fibronectin antiserum (1:200 dilution), anti- α _s-subunit antibodies (1:50 dilution) or anti- β ₁-subunit antibodies (1:200 dilution) [49]. After four washes for 10 min in PBS containing 0.05% Tween-20, the nitrocellulose membranes were incubated either with horseradish peroxidase-conjugated anti-rabbit IgG (Dakopatts) or with ¹²⁵I-labelled protein A. In the former case, colour was developed by incubating the treated nitrocellulose sheets with 4-chloro-1-naphthol. In the latter case, the antigens recognized were visualized after autoradiography of the nitrocellulose membranes, using Hyperfilm-MP (Amersham, Bucks, U.K.).

Biosynthetic labelling of HyC and ZHC

Cells in 25 cm²-culture flasks were labelled for 60 min with 100 μ Ci of [³⁵S]methionine (800 Ci/mmol, Amersham) per ml of methionine-free medium (pulse period). The labelled medium was removed, the cells were washed twice (in the case of ZHC, by centrifugation at 55 *g*) with fresh MEM and further incubated in their respective culture media [38,39] containing unlabelled methionine (chase periods).

Immunoprecipitation experiments

Immunoprecipitation of the β ₁-subunits from metabolically-labelled cells was carried out at each time point of the chase as described by Bosch et al. [50]. A temperature of 4 °C was maintained throughout the procedure. In outline, aliquots of HyC and ZHC lysates (2×10^6 – 8×10^6 c.p.m.) were diluted in 800 μ l of PBS, pH 7.4, containing 2 mM PMSF, 0.5% Triton X-100, 0.5% SDS and 0.25% BSA, and were precleared with 100 μ l of a 10% (v/v) suspension of *Staphylococcus aureus* (immunoprecipitin) (Bethesda Research Laboratories, Gaithersburg, U.S.A.). After 60 min of incubation at 4 °C, samples were centrifuged at 900 *g* for 6 min at 4 °C. β ₁-subunits were immunoprecipitated by incubating the resulting supernatants with anti- β ₁-subunit antibodies (1:200 dilution). After a further 2 h incubation, antigen–antibody complexes were isolated by the addition of 50 μ l of immunoprecipitin, followed by centrifugation at 9000 *g* for 5 min at 4 °C. After several

washes, the pellets were resuspended in the appropriate buffer and the immune complexes were dissociated by heating at 90 °C for 5 min, followed by centrifugation at 9000 g for 2 min. The β_1 -chains were analysed by SDS/PAGE, and visualized after fluorography of the dried gel using Kodak X-Omat AR film (X AR-5) with an intensifying screen. Different durations of autoradiography were used (3–20 days).

Enzymic deglycosylation of the FNR β_1 -subunits

For digestion with endo- β -N-acetylglucosaminidase H (endo H) (Genzyme, Boston, U.S.A.), the immune complexes were dissociated directly in 50 mM sodium citrate buffer, pH 5.0, containing 0.1 % SDS, as described above, and treated with 20 m-units/ml of endo H. After 16 h of incubation at 37 °C, samples were analysed by SDS/PAGE and visualized as described above. For peptide N-glycosidase (N-glycanase) digestion (Genzyme), the immune complexes were dissociated in 10 μ l of 0.5 % SDS by heating at 90 °C for 5 min, and deglycosylation was carried out overnight according to the manufacturer's suggestions. Samples were subjected to SDS/PAGE and proteins were visualized as described above.

Surface labelling of HyC and ZHC

ZHC and freshly-isolated HyC (10⁷ cells) were washed three times in PBS, pH 7.4, at 55 g, for 5 min at room temperature, and surface-labelled with 500 μ Ci of carrier-free Na¹²⁵I in 0.1 M NaOH (210 Ci/mmol; Oris Industrie SA, Gif-sur-Yvette, France) using the lactoperoxidase and glucose oxidase method described elsewhere [51]. Then the cells were solubilized in PBS, pH 7.4, containing 2 mM PMSF, 2 % Triton X-100 and pepstatin A (1 μ g/ml). After centrifugation at 15000 g for 15 min at 4 °C, the supernatants were immunoprecipitated using the anti- β_1 -subunit antibodies, as described above. Proteins were analysed by SDS/PAGE with and without endo H. The receptors present on the two cell populations were visualized after autoradiography of the dried gel, using Kodak X-Omat AR film (X AR-5).

Assay for cell attachment

Human plasma fibronectin (5–50 μ g/ml) (Boy SA, Reims, France) was used to coat 24-well tissue culture plates for 1 h at 22 °C in Ca²⁺- and Mg²⁺-free PBS, pH 7.4. The plates were treated with 0.1 % BSA for 30 min at 22 °C, to saturate the plastic sites still available. For long-term (3–24 h) adhesion experiments, assays were performed by adding 2 $\times$ 10⁵ cells in 1 ml of MEM without foetal calf serum (FCS); while for short-term adhesion assays (< 1 h), 10 mM Hepes buffer, pH 7.4, containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄ and 1 mM CaCl₂, was used [52]. After incubation at 37 °C, the plates were washed three times with PBS to remove non-adherent cells. Then 500 μ l of 2 % glutaraldehyde (Fluka, Buchs, Switzerland) in PBS were added. Finally, the cell number was determined *in situ* by scoring four different fields using an inverted Zeiss microscope ICM 405.

Binding of iodinated fibronectin to HyC and ZHC

Human plasma fibronectin was iodinated using Iodo-Gen (Pierce Chemical Co., Rockford, U.S.A.) and carrier-free Na¹²⁵I (Oris Industrie) [53]. Iodination of fibronectin (specific activity 1.5 $\times$ 10⁶ c.p.m./ μ g) did not alter its capacity to bind to the cell-surface [54]. The concentration of fibronectin was determined

according to Bradford [46]. Human plasma fibronectin (1 μ g/ml–18 μ g/ml) was allowed to bind to ZHC and HyC in suspension, in binding medium (MEM, 20 mM Hepes, pH 7.4, 2 % BSA) at 4 °C for 2 h, under continuous rotary agitation. Non-specific binding (5 % of the total added radioactivity) was determined in the presence of a 100-fold excess of unlabelled ligand. The radioactivity associated with the cell pellet was quantified as previously described [39] and counted in a Mini-gamma counter 127 (Pharmacia, Uppsala, Sweden). Experimental values were plotted using Scatchard's method [55]. The numbers of fibronectin-binding sites per cell (*N*) and the dissociation constants (*K_d*) were obtained from computer data using Munson and Rodbard's ligand program [56].

RESULTS

Identification of the FNR subunits

The rat hepatocyte FNR consists of one 155-kDa α_5 -subunit and one 115-kDa β_1 -subunit, held together by noncovalent interactions. After reduction, the 155-kDa subunit gave rise to two polypeptides of 145 kDa and 20 kDa, while the 115-kDa subunit shifted to 130 kDa [17]. Polyclonal antibodies directed against either the α_5 -subunit or the β_1 -subunit of the HyC FNR were used in immunoblotting experiments to identify the FNR in ZHC. Under non-reducing conditions, the anti- α_5 -subunit antibodies revealed the α_5 subunit of the receptor in HyC (Figure 1, lane 1) as well as in ZHC (Figure 1, lane 2).

Under non-reducing conditions, the anti- β_1 -subunit antibodies readily recognized the typical β_1 -subunit in HyC, as well as a minor 100-kDa protein (Figure 2, lane 1) and, in the case of

Figure 1 Detection of the α_5 -subunit in ZHC

HyC and ZHC lysates were prepared as described, and 20 μ g of protein was loaded on to each lane of a 10 % SDS/PAGE gel, under non-reducing conditions and processed for immunoblotting. The nitrocellulose membranes were incubated with the anti- α_5 -subunit antibodies (diluted 1:50) for 2 h at room temperature. After washing, the nitrocellulose membranes were further incubated with ¹²⁵I-labelled protein A. The antigens recognized were visualized by autoradiography of the nitrocellulose membranes, for 3 days. HyC (lane 1); ZHC (lane 2). The arrows indicate the migration of the α_5 -subunits.

Figure 2 Detection of the β_1 -subunit in ZHC

HyC and ZHC lysates were prepared as described, and 20 μ g of protein was loaded on to each lane of a 10% SDS/PAGE, under non-reducing conditions. After transfer onto nitrocellulose, the proteins were analysed for their reactivity with the anti- β_1 -subunit antibodies (diluted 1:200) and the nitrocellulose membranes were incubated further with ^{125} I-labelled protein A. The antigens recognized were detected as described in the legend to Figure 1. HyC (lane 1); ZHC (lane 2). The arrows indicate the migration of the individual β_1 -subunits.

ZHC, a minor 130-kDa band and a major 100-kDa band were identified (Figure 2, lane 2). However, unlike the anti- α_5 -subunit antibodies, the anti- β_1 -subunit antibodies are able to recognize precursor and mature β_1 -subunits in HyC and ZHC.

Finally, postulating that the 100-kDa proteins are the precursor forms of the β_1 -subunit, and the 115-kDa and 130-kDa proteins are the mature forms in HyC and ZHC, respectively, our results suggest that ZHC synthesizes an abnormally-large 130-kDa β_1 -subunit.

Biosynthesis of HyC and ZHC FNR β_1 -subunits

Since the β_1 -subunit of the receptor appeared to be modified in ZHC, we particularly focused our attention on its biosynthesis both in HyC and ZHC. The identity of the different β_1 -subunits was confirmed by pulse-chase experiments using [^{35} S]methionine as the tracer. Immediately after the pulse, only the 100-kDa proteins were recognized by immunoprecipitation in HyC (Figures 3 and 4, lane 1) and in ZHC lysates (Figure 3, lane 3; Figure 5, lane 1), whereas under reducing conditions, they shifted to 110-kDa components (Figure 3, lanes 2 and 4 for HyC and ZHC, respectively). In contrast, the maturation processes of the 100-kDa proteins were quite different in HyC and ZHC. In the case of HyC, after 3 h of chase, the 100 kDa protein began to be converted progressively into the typical mature 115-kDa β_1 -subunit with 100% completion within 24 h of the chase (Figure 3, lanes 5, 7 and 9; Figure 4, lanes 3, 5 and 7). As expected, the mature form migrated, under reducing conditions, as a 130-kDa protein (Figure 3, lanes 6, 8 and 10). In the case of ZHC, the 100 kDa protein was detected throughout the chase period (Figure 3, lanes 11, 13 and 15; Figure 5, lanes 3 and 5). However the decrease observed in the amount of the 100-kDa protein after 24 h of chase was correlated with the appearance of the 130-kDa protein, the size of which increased to 140 kDa upon reduction (Figure 3, lane 16). Surprisingly, the α_5 -subunit of the receptor did not co-precipitate with the β_1 -subunit; however, it did co-

Figure 3 Biosynthesis of the FNR β_1 -subunit in HyC and ZHC

HyC and ZHC (3×10^6 cells) were pulse-labelled (P) for 1 h with 100 μ Ci/ml of [^{35} S]methionine, subsequently chased for 3, 6 and 24 h and then solubilized. After centrifugation at 9000 *g*, the supernatants (2×10^6 c.p.m., for each sample) were immunoprecipitated with the anti- β_1 -subunit antibodies (diluted 1:200). Precipitates were analysed by SDS/PAGE (5–18% gradient gel) in the presence (HyC: lanes 2, 6, 8, 10; ZHC: lanes 4, 12, 14, 16) or absence of dithiothreitol (DTT) (HyC: lanes 1, 5, 7, 9; ZHC: lanes 3, 11, 13, 15), and fluorographed (20 days). Arrows indicate the positions of the immature and mature forms of the β_1 -subunits.

Figure 4 Endo H sensitivity of the FNR β_1 -subunit in HyC

HyC were pulse-labelled and subsequently chased as in the legend to Figure 3. Aliquots of cell lysates (8×10^6 c.p.m., for each sample) were immunoprecipitated with the anti- β_1 -subunit antibodies as described in Materials and methods. The precipitates were then incubated with (lanes 2, 4, 6, 8) or without (lanes 1, 3, 5, 7) endo H (20 munit/ml), analysed by SDS/PAGE on a 7.5% gel and fluorographed (5 days). Arrows indicate the position of the β_1 -subunits.

Figure 5 Endo H sensitivity of the FNR β_1 -subunit in ZHC

ZHC were pulse-labelled and subsequently chased (6 and 24 h) as in the legend to Figure 3. Aliquots of cell lysates (8×10^6 c.p.m., for each sample) were immunoprecipitated with the anti- β_1 -subunit antibodies (diluted 1:200). The precipitates were incubated with (lanes 2, 4, 6) or without (1, 3, 5) endo H (20 munit/ml), analysed by SDS/PAGE on a 7.5% gel and fluorographed (11 days). The arrows indicate the positions of the β_1 -subunits.

precipitate when it was expressed on the surface of HyC and ZHC (Figure 7; see below). Similar results were obtained by Oz et al. [33] and Heino et al. [57]. Heino et al. suggested that, in human lung fibroblasts, the β_1 -subunit of the FNR is synthesized in excess and at a faster rate than the α_5 -subunit. Thus, the same phenomenon could occur for both normal and malignant rat hepatocytes, although, in this case, the anti- β_1 subunit antibodies would co-precipitate α_5 -subunits only when they were associated with the β_1 -subunits. Alternatively, the rate of methionine

Figure 6 *N*-Glycanase sensitivity of the FNR β_1 -subunit in ZHC

The isolated β_1 -subunits obtained as described in the legend to Figure 5 (pulse and 24 h of chase) were treated with (lanes 2 and 4) or without (lanes 1 and 3) *N*-glycanase (10 munit/ml) as described in Materials and methods, analysed by SDS/PAGE (7.5% gel) and fluorographed (12 days). Arrows indicate the migration positions of the precursor and atypical forms of the β_1 -subunits.

incorporation by the α_5 - and the β_1 -subunits of the receptor could differ.

Finally, taken as a whole, our results showed that: (1) there was a direct precursor-product relationship between the 100/115-kDa and 100/130-kDa proteins in HyC and ZHC, respectively; (2) the apparent transition time from precursor to mature form was exceptionally long in ZHC (up to 24 h); (3) the heterogeneous appearance of the 130-kDa broad band (Figure 3, lane 15) did not reflect different folding of the ZHC β_1 -subunits since, under reducing conditions, this 130-kDa β -subunit migrated still as a broad band (Figure 3, lane 16).

Glycosylation of FNR β_1 -subunits in HyC and ZHC

Although the precise number of oligosaccharide chains present on the FNR subunits has not been determined yet, both subunits have been shown to contain asparagine-linked carbohydrates [33,34]. Therefore we examined whether the variations in the size of the mature ZHC β_1 -subunit were due to major differences in *N*-glycosylation or were in the polypeptide chain. For this purpose, the β_1 -chains, immunoprecipitated from the metabolically-labelled cells used above, were subjected to glycosidase digestion.

Endo H sensitivity of the β_1 -subunits in HyC and ZHC

Endo H removes high-mannose but not complex N-linked oligosaccharides from glycoproteins. As shown in Figures 4 (HyC) (lanes 1–6) and 5 (ZHC) (lanes 1–6), the size of the 100 kDa precursors decreased to 84 kDa after incubation with endo H, which could indicate that the same number of high-mannose oligosaccharides was removed from the precursor forms synthesized in both HyC and ZHC. In contrast, the mature 115 kDa (HyC) (Figure 4, lanes 3–8) and 130 kDa (ZHC) (Figure

Figure 7 Cell-surface expression of the FNR on HyC and ZHC

Detergent-soluble extracts of surface-iodinated HyC (lane 1) and ZHC (lanes 2 and 3) (2×10^7 cells) were immunoprecipitated with the anti- β_1 -subunit antibodies as described in Materials and methods. The immunoprecipitates were incubated with (lane 3) and without (lanes 1 and 2) endo H (30 m-unit/ml), analysed by SDS/PAGE (7.5% gel), and fluorographed for 3 days (HyC) or 5 days (ZHC). The molecular masses of the proteins run in parallel are indicated.

5, lanes 5 and 6) β_1 -subunits remained insensitive to the endo H treatment, suggesting at least that, in both HyC and ZHC, the oligosaccharides had been converted into the complex form.

N-Glycanase sensitivity of the β_1 -subunits in HyC and ZHC

Treatment with *N*-glucanase of the 100-kDa precursor forms of the β_1 -subunit immunoprecipitated from both HyC (data not shown) and ZHC (Figure 6, lanes 1–4), as well as of the 115-kDa protein from HyC (data not shown), yielded an 82-kDa product. In contrast, when the mature atypical 130-kDa β_1 -chain, immunoprecipitated from ZHC, was incubated with *N*-glycanase, its size decreased only to 110 kDa (Figure 6, lanes 3 and 4).

This finding confirmed that the polypeptide backbones of the β_1 -subunits were similar in HyC and ZHC, and that, in addition to effects on the *N*-glycosylation processes, other post-translational modifications had occurred in the ZHC β_1 -chain.

Expression of the atypical β_1 -subunit on the surface of ZHC

To study the influence of this altered glycosylation on the cell-surface expression of the FNR on ZHC, HyC and ZHC were subjected to lactoperoxidase-catalysed iodination [51]. The FNR specifically isolated by immunoprecipitation with the anti- β_1 -subunit antibodies was analysed by SDS/PAGE before and after endo H digestion. Under non-reducing conditions the 155-kDa α_5 - and the 115-kDa β_1 -subunits of the receptor were detected at the surface of HyC (Figure 7, lane 1) and they were endo H-insensitive (data not shown). For ZHC, a broad 100–155-kDa band was detected by the anti- β_1 -subunit antibodies (Figure 7, lane 2). After endo H digestion the more slowly migrating protein remained unchanged, while a more rapidly migrating 84-kDa band was seen (Figure 7, lane 3). Thus the atypical 130-kDa mature form of the β_1 -subunit was inserted into the plasma membrane of ZHC. Likewise, the β_1 -precursor form was able to reach the surface of ZHC. This finding could suggest, at least,

(a)

(b)

Figure 8 Adhesion assay of HyC and ZHC to a fibronectin matrix

Tissue culture plates (24-well) were coated with different concentrations of human plasma fibronectin (5–50 $\mu\text{g}/\text{ml}$) and then treated with BSA to saturate the available plastic sites as described in Materials and methods. HyC and ZHC (2×10^5 cells/ml), in MEM without FCS, were allowed to adhere for 3 h at 37 °C. The plates were washed with PBS and the number of adherent cells was determined *in situ* using an inverted Zeiss microscope ICM 405. HyC (**a**) and ZHC (**b**) after 3 h of adhesion. Bars: 30 μm .

that association occurs between the α_5 - and the abnormal mature and/or precursor β_1 -subunits in ZHC.

Biological and biochemical properties of ZHC

ZHC adhesion

Unlike HyC, ZHC do not adhere when cultured on plastic culture dishes [36–39]. Since the addition of high concentrations of exogenous fibronectin to their culture media can restore the adhesive capacity of many tumour cells partially or totally [58,59], we examined ZHC attachment to a fibronectin substrate. Regardless of the fibronectin concentration used (5–50 $\mu\text{g}/\text{ml}$)

Figure 9 Detection of endogenous fibronectin on HyC and ZHC

HyC and ZHC plasma membrane proteins (40 μg) were separated by SDS/PAGE on a 5–18% gradient gel in the presence of dithiothreitol. After transfer onto nitrocellulose, the proteins were analysed for their reactivity with rabbit anti-human plasma fibronectin antibodies (diluted 1:100), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:400). Specific proteins were revealed using 4-chloro-1-naphthol. ZHC (lane 1); HyC (lane 2). The arrow indicates the position of the fibronectin subunit.

and of the duration of the adhesion assay (30 min–24 h), few ZHC (Figure 8b; 3 h of adhesion) were attached to the culture dishes compared with HyC (Figure 8a; 3 h of adhesion). After 24 h of adhesion, only 4% of the ZHC seeded were attached as against 80% for HyC. Thus the adhesive capacity of ZHC was not restored by fibronectin, even in the presence of a large excess.

ZHC binding properties

(1) Endogenous cell surface fibronectin on ZHC. The deposition of fibronectin into the extracellular matrix is perturbed in malignant cells; some of them either totally lack fibronectin or have greatly reduced amounts [60,61]. Therefore, we compared the amounts of endogenous fibronectin associated with the membranes of HyC and ZHC. Antibodies raised against human plasma fibronectin were used on immunoblots of plasma membrane proteins. As shown in Figure 9, under reducing conditions a 220-kDa protein similar to that of dissociated fibronectin subunits [61] was recognized by the antibodies on both cell types, in a lower amount in ZHC (lane 1) than in HyC (lane 2). Taking into account that the experiments were carried out with the same quantity of plasma membrane proteins and that freshly-isolated HyC [40] and ZHC [37] have similar diameters, our data showed that fibronectin was poorly retained on the ZHC surface.

(2) Measurement of fibronectin-binding parameters for ZHC. The binding capacity of the FNR expressed on HyC and ZHC was analysed by using ^{125}I -labelled fibronectin rather than the 105-kDa cell-binding fibronectin domain coupled to Sepharose. Indeed, the former allowed us to determine precisely the apparent dissociation constant (K_d) and the average number of binding sites per cell (N). The kinetics of ^{125}I -labelled fibronectin binding to the two cell types indicated that maximal specific binding was reached at 4 °C within 2 h (data not shown). When HyC (Figure 10a) and ZHC (Figure 10b) were incubated with different

Figure 10 Binding studies of labelled fibronectin to HyC and ZHC

Cells (3×10^6 cells/ml) were incubated with increasing concentrations of ^{125} I-labelled fibronectin for 2 h at 4 °C. The ratio bound/free (B/F) was plotted as a function of the fibronectin bound to the cells. The inserts show fibronectin binding to HyC (a) and ZHC (b) as a function of the fibronectin concentration. Experiments performed in the presence of 100 μ g of laminin are indicated by $\blacktriangle$. HyC (a) and ZHC (b).

amounts of labelled fibronectin, with and without unlabelled ligand, saturation curves of binding were obtained for both HyC (Figure 10a, insert) and ZHC (Figure 10b, insert). Furthermore, fibronectin binding was not prevented by the addition of laminin (Figure 10, inserts). This latter observation agrees very well with those of Johansson [62] and Bissel et al. [63] and indicated that, as for HyC, the binding of fibronectin and laminin to ZHC involved distinct surface receptors. The Scatchard plots of the binding data indicated a single class of receptor for HyC (Figure 10a) and ZHC (Figure 10b). The K_d appeared to be only slightly different for the two cell populations: 2.0×10^{-8} M and 2.7×10^{-8} M for HyC and ZHC, respectively. In contrast, the N

value differed greatly and was considerably decreased in ZHC, being 8868 per cell, compared with 120000 per cell in HyC.

DISCUSSION

Our comparative study on the structure and function of FNR in normal rat hepatocytes and ascitic Zajdela rat hepatoma cells showed that the α_s -subunit of the receptor was similar in the two cell types, whereas the kinetics of β_1 -subunit biosynthesis and glycosylation processes were altered. In a recent investigation using a mutant mouse melanoma cell-line (Wa461) with abnormal glycosylation, Oz et al. [33] found that the N-linked carbohydrate

structure of the FNR β -chain was altered. However, our present results differ in several aspects from those of Oz et al. [33].

First, we found that the intracellular processing of the ZHC FNR is associated with the appearance of an abnormally large β -chain. In immunoblotting and pulse-chase experiments, the anti- β_1 -subunit antibodies recognized an abnormal 130 kDa β_1 -chain in ZHC as against a 115-kDa chain in HyC. In their study, Oz et al. [33] observed a decrease in the molecular mass of the β_1 -subunit synthesized by the mutant Wa4b1 cells. In addition, we found that the maturation kinetics of the atypical ZHC FNR β_1 -chain was exceptionally prolonged. Indeed, the transition from the precursor to the mature form began within 3 h in HyC, and was analogous to previous reports in other cell types [34,57,64], whereas it took up to 24 h to start in ZHC. The biosynthetic pathway of N-linked carbohydrate side chains of glycoproteins is well understood [65]. In outline, a precursor molecule containing a high proportion of mannose is synthesized on the lipid carrier dolichol and then transferred co-translationally to the growing protein chain. Once covalently bound, the high-mannose precursor can undergo maturation to a complex oligosaccharide chain. Using inhibitors of specific intracellular carbohydrate-processing enzymes, Spiro et al. [66] have shown that specific stereochemical conformations are essential for the maturation of high-mannose chains present on the vitronectin receptor. Therefore the delay in the conversion of high-mannose chains into the complex forms could result from an unusual folding of the β -precursor form in ZHC. This conformational defect could be too subtle to be detected in SDS/PAGE. Possible mechanisms to explain an improper folding of the ZHC β_1 -chain include: (1) One of the α -glucosidases or the $\alpha(1,2)$ mannosidase could be defective and the failure to remove the corresponding glucosyl residues could affect the transfer along the rough endoplasmic reticulum. Such a defect was observed in the case of the vitronectin receptor [66]. In addition, it has been suggested that the glucosyl residues may also be acting as 'holding' signals in the rough endoplasmic reticulum [67]. Regarding endo H and N-glycanase digestions, the 100-kDa β_1 -precursors in HyC and ZHC behaved almost identically. Thus, a total derepression of genes coding for these enzymes in ZHC could be excluded. (2) A polypeptide β_1 -chain with a particular structure could be synthesized in ZHC, inducing an unusual folding. (3) The altered compartmentalization resulting from malignant transformation could also account for the structural differences observed in the same polypeptide chain expressed by both normal and tumour cells. Ultrastructural examination of ZHC shows a simplified cytological organization compared to that of normal HyC; in particular, the rough endoplasmic reticulum and the Golgi apparatus are markedly reduced in ZHC [36,37].

Second, we found that the α_5 -subunit and both the mature 130-kDa and the precursor 100-kDa β_1 -chains are expressed at the surface of ZHC. This finding also differs from that obtained by Oz et al. [33], who could not identify the precursor form of the β_1 -chain on the surface of Wa4b1 cells. However, some of our data are in agreement with the results of Akiyama et al. [34] and Spiro et al. [66]. Indeed, like these authors, we found that complete maturation of carbohydrates was not required for integrin complex assembly or cell surface expression. In fact carbohydrates did not play a major role in the assembly or integration of the FNR into the plasma membrane.

Third, we found that, following liver cell transformation to tumorigenicity, post-translational events occurred in the ZHC FNR β_1 -chain. Indeed, incubation of the HyC and ZHC β_1 -precursors with endo H and N-glycanase decreased their size from 100 kDa to 84 and 82 kDa, respectively, as judged by SDS/PAGE, indicating that these precursor forms are

deglycosylated in the same way. N-Glycanase digestion cleaved the normal 115-kDa mature β_1 -subunit to 82 kDa in HyC, while the abnormal mature chain in ZHC was reduced from 130 kDa to 110 kDa. One characteristic feature of ZHC is the presence of enlarged glycoproteins bearing both N- and O-linked glycan chains on its cell surface [38]. Furthermore, when lectin-binding experiments were performed, both HyC and ZHC interacted with ricin [39], a toxic lectin which binds to cell-surface glycoconjugates exhibiting non-reducing terminal galactose [68]. On the other hand, only ZHC bound peanut agglutinin [69], whose most effective inhibitors are the disaccharide Gal $\beta(1 \rightarrow 3)$ GalNAc and desialylated O-linked glycoproteins [70]. In this regard, the difference in size between the 115 kDa and 130 kDa FNR β_1 -subunits could result from the addition of extra O-linked glycan chains onto ZHC.

The causes of the multiple and profound changes in glycosylation during malignant transformation of cells are still hypothetical. They may be linked to the expression or repression of mRNA inducing the biosynthesis of particular, slightly different polypeptide chains and/or post-transcriptional changes in protein glycosylation. The derepression of gene(s) coding for glycosyltransferases as a result of malignant transformation could also account for these minor structural differences occurring on the same polypeptide chain expressed by both normal and transformed cells. Indeed, important changes in glycosyltransferases during cell transformation have been reported [71], but the conclusions drawn have often been contradictory.

Previous data from *in vivo* and *in vitro* studies showed that the β_1 -subunits of integrins are preferentially modified upon malignant transformation, in inheritable diseases [72] or in cells with altered glycosylation [33]. Our results are in agreement with these observations, since we found that only the β_1 -subunit and not the α_5 -subunit of the FNR was altered in ZHC.

The final interesting aspect of this study was the observation that the adhesive and binding properties of ZHC were affected. Scatchard analyses of HyC binding to fibronectin gave an apparent K_d of 2.0×10^{-8} M and an N value of 120 000 sites per cell. In contrast, ZHC apparently have non-functional FNRs, since their N value (8868) was decreased. However, this binding was specific for ZHC since, unlike laminin, unlabelled fibronectin did compete with ^{125} I-fibronectin. In previous reports Saga et al. [21] and Dedhar et al. [24] noted an increase in FNR expression in Rous sarcoma virus-transformed fibroblasts and in cultured osteosarcoma cells, respectively. On the other hand, Plantefaber and Hynes [22] reported that reduction in fibronectin binding was correlated with the loss of the FNR from the surface of Chinese hamster ovary cells. Our data are in agreement with the results of Plantefaber and Hynes [22], since we observed a dramatic decrease in the ZHC N value, and also with the results of Matyas et al. [54], who showed that the amount of ^{125} I-fibronectin bound to plasma membrane vesicles prepared from metastatic and non-metastatic hepatomas was reduced compared to that bound to similar vesicles prepared from normal liver. The HyC and ZHC K_d values were slightly different. This difference could be due in part to: (1) an association between 155 kDa α_5 -subunits and residual normal 115 kDa β_1 -subunits in ZHC, even though we never found 115 kDa proteins in ZHC lysates, regardless of the chase period; (2) an association between α_5 -subunits and immature β_1 -subunits. Indeed, when glycosylation inhibitors were used, both the α_5 - and β_1 -subunits of the FNR were altered and the FNR complex was inactive [34]; (3) an association between α_5 -subunits and abnormal mature β_1 -subunits; (4) an association between α_5 and abnormal mature β_1 -subunits. Indeed, ZHC were found to express a higher level of

α_5 -subunits than HyC (M. Decastel, unpublished results); or (5) binding through cell-surface proteoglycans, which are also important in cell adhesion [73]. Furthermore, ZHC were found also to express a higher level of proteoglycans than HyC, particularly with highly-branched lactosaminoglycan side chains (C. Goulut-Chassaing, unpublished results). Despite the lack of a clear-cut explanation, our observation raises the question of the exact function(s) of the carbohydrate chains on each subunit of the FNR.

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REFERENCES

- Hynes, R. O. (1987) *Cell* **48**, 549–554
- Ruoslahti, E. and Pierschbacher, M. D. (1987) *Science* **238**, 491–497
- Buck, C. A. and Horwitz, A. F. (1987) *Annu. Rev. Cell Biol.* **3**, 179–205
- Ruoslahti, E. and Giancotti, F. G. (1989) *Cancer Cells* **4**, 119–126
- Schreiner, C., Bauer, J., Margolis, M. and Juliano, R. L. (1991) *Clin. Expl. Metastasis* **9**, 163–178
- Hemler, M. E., Jacobson, J. G. and Strominger, J. L. (1985) *J. Biol. Chem.* **260**, 15246–15252
- Hemler, M. E., Crouse, C., Takada, Y. and Sonnenberg, A. (1988) *J. Biol. Chem.* **263**, 7660–7665
- Wayner, E. A. and Carter, W. G. (1987) *J. Cell Biol.* **105**, 1873–1884
- Sonnenberg, A., Moderman, P. W. and Hogervorst, F. (1988) *Nature (London)* **336**, 487–489
- Springer, T. A., Dustin, M. L., Kishimoto, T. K. and Morlin, S. (1987) *Annu. Rev. Immunol.* **5**, 223–252
- Pytela, R., Pierschbacher, M. D. and Ruoslahti, E. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5766–5770
- Phillips, D. R., Charo, I. F., Parise, L. V. and Fitzgerald, L. A. (1988) *Blood* **71**, 831–843
- Pytela, R., Pierschbacher, M. D., Ginsberg, M. H., Plow, E. F. and Ruoslahti, E. (1986) *Science* **231**, 1559–1562
- Pytela, R., Pierschbacher, M. D. and Ruoslahti, E. (1985) *Cell* **40**, 191–198
- Brown, P. J. and Juliano, R. L. (1986) *J. Cell Biol.* **103**, 1595–1603
- Akiyama, S. K. and Yamada, K. M. (1987) *J. Biol. Chem.* **262**, 17536–17542
- Johansson, S., Forsberg, E. and Lundgren, B. (1987) *J. Biol. Chem.* **262**, 7819–7824
- Argaves, W. S., Pytela, R., Suzuki, S., Millan, J. L., Pierschbacher, M. D. and Ruoslahti, E. (1986) *J. Biol. Chem.* **261**, 12922–12924
- Fitzgerald, L. A., Poncz, M., Steiner, B., Rall, S. C., Jr., Bennet, J. S. and Phillips, D. R. (1987) *Biochemistry* **26**, 8158–8165
- Kennel, S. J., Foote, L. J. and Flynn, K. M. (1986) *Cancer Res.* **46**, 707–712
- Saga, S., Chen, W. T. and Yamada, K. M. (1988) *Cancer Res.* **48**, 5510–5513
- Plantefaber, L. C. and Hynes, R. O. (1989) *Cell* **56**, 281–290
- Yläne, J. and Virtanen, I. (1989) *Int. J. Cancer* **43**, 1126–1136
- Dedhar, S., Argaves, W. S., Suzuki, S., Ruoslahti, E. and Pierschbacher, M. D. (1987) *J. Cell Biol.* **105**, 1175–1182
- Schreiner, C. L., Bauer, J. S., Danilov, Y. N., Hussein, S., Sczekan, M. M. and Juliano, R. L. (1989) *J. Cell Biol.* **109**, 3157–3167
- Hirst, R., Horwitz, A., Buck, C. and Rohrschneider, L. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6470–6474
- Buck, C. A. and Horwitz, A. F. (1987) *J. Cell Sci. Suppl.* **8**, 231–250
- Tapley, P., Horwitz, A., Buck, C., Duggan, K. and Rohrschneider, L. (1989) *Oncogene* **4**, 325–333
- Hakomori, S. and Kanagi, R. (1983) *J. Natl. Cancer Inst.* **71**, 231–251
- Smets, L. A. and Van Beek, W. P. (1984) *Biochim. Biophys. Acta* **738**, 237–249
- Yamamoto, K., Tsuji, T., Tarutani, O. and Osawa, T. (1984) *Eur. J. Biochem.* **143**, 133–144
- Debray, H., Orin, Z., Delannoy, P., Montreuil, J., Dus, D., Radzikowski, C., Christensen, B. and Kieler, J. (1986) *Int. J. Cancer* **37**, 607–611
- Oz, K. O., Campbell, A. and Tao, T.-W. (1989) *Int. J. Cancer* **44**, 343–347
- Akiyama, S. K., Yamada, S. S. and Yamada, K. M. (1989) *J. Biol. Chem.* **264**, 18011–18018
- Zajdela, F. (1968) *Arch. Mal. Appar. Dig. Mal. Nutr.* **52**, 1213–1217
- Staedel, C. C. and Beck, J.-P. (1978) *Cell Differ.* **7**, 61–71
- Flaig, C. (1984) Thèse de Doctorat D'Etat No. d'ordre: 1781, Strasbourg, France
- Chaumeton, B., Saunier, B., Nato, F., Goulut, C. and Bourrillon, R. (1987) *J. Cell. Biochem.* **34**, 269–281
- Decastel, M., Haentjens, G., Aubery, M. and Goussault, Y. (1989) *Exp. Cell Res.* **180**, 399–408
- Seglen, P. O. (1973) *Exp. Cell Res.* **82**, 391–398
- Guguen-Guillouzo, C., Szajnert, M. F., Glaire, D., Gregori, C. and Shapira, F. (1981) *In Vitro* **17**, 369–377
- Bottger, B. A., Hedin, U., Johansson, S. and Thyberg, J. (1989) *Differentiation (Berlin)* **41**, 158–167
- Stamatoglou, S. C., Sullivan, K. H., Johansson, S., Bayley, P. M., Burdett, I. D. J. and Hughes, R. (1990) *J. Cell Sci.* **97**, 595–606
- Hedin, U., Bottger, B. A., Luthman, J., Johansson, S. and Thyberg, J. (1989) *Dev. Biol.* **133**, 489–501
- Nato, F., Goulut, C., Bourrillon, R., Van Halbeck, H. and Vilegenthart, J. F. G. (1986) *Eur. J. Biochem.* **159**, 303–308
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Burnette, W. N. (1981) *Annu. Rev. Cell Biol.* **3**, 179–205
- Hawkes, R., Niday, E. and Gordon, J. (1982) *Anal. Biochem.* **119**, 142–147
- Bosch, R., Geuze, H. J., du Maine, A. P. M. and Strous, G. J. (1986) *Eur. J. Biochem.* **160**, 49–54
- Hynes, R. O. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3492–3496
- Johansson, S., Kjellen, L., Höök, M. and Timpl, R. (1981) *J. Cell Biol.* **90**, 260–264
- Fraker, P. J. and Speck, J. C., Jr (1978) *Biochem. Biophys. Res. Commun.* **80**, 849–857
- Matyas, G. R., Evens, D. C., Radinski, R. and Morré, D. J. (1986) *Exp. Cell Res.* **162**, 296–318
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Munson, P. J. and Rodbard, R. (1980) *Anal. Biochem.* **107**, 220–225
- Heino, J., Ignatz, R. A., Hemler, M. E., Crouse, C. and Massagué, J. (1989) *J. Biol. Chem.* **264**, 380–388
- Yamada, K. M., Yamada, S. S. and Pastan, I. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1217–1221
- Ali, I. U., Mautner, V., Lanza, R. and Hynes, R. O. (1977) *Cell* **11**, 115–126
- Hynes, R. O. (1976) *Biochim. Biophys. Acta* **458**, 73–107
- Hynes, R. O. and Yamada, K. M. (1982) *J. Cell Biol.* **95**, 369–377
- Johansson, S. (1985) *J. Biol. Chem.* **260**, 1557–1561
- Bissel, D. M., Stamatoglou, S. C., Nermut, M. V. and Hughes, R. C. (1986) *Eur. J. Cell Biol.* **40**, 72–78
- Jaspers, M., de Strooper, B., Spaepen, M., Van Leuven, F., David, G., Van den Berghe, H. and Cassiman, J. J. (1988) *FEBS Lett.* **231**, 402–406
- Kornfeld, R. and Kornfeld, S. (1985) *Annu. Rev. Biochem.* **78**, 369–379
- Spiro, R. C., Laufer, D. M., Perry, S. K. and Harper, J. R. (1989) *J. Cell. Biochem.* **41**, 37–45
- Copeland, C. S., Zimmer, K.-P., Wagner, K. R., Healy, G. A., Mellman, I. and Helenius, A. (1988) *Cell* **53**, 197–209
- Olsnes, S., Refnes, K. and Pihl, A. (1974) *Nature (London)* **249**, 627–629
- Goulut-Chassaing, C., Decastel, M., Tran, A. T., Tabary, F. and Bourrillon, R. (1992) *Biochimie* **514**, 101–108
- Dahr, W., Uhlenbruck, G. and Bird, G. W. G. (1975) *Vox Sang.* **28**, 133–148
- Yamada, K. M. and Pouyssegur, J. (1978) *Biochimie* **60**, 1221–1233
- Kishimoto, T. K., Hollander, N., Roberts, T. M., Anderson, D. C. and Springer, T. A. (1987) *Cell* **50**, 193–202
- Ruoslahti, E. (1989) *J. Biol. Chem.* **264**, 13369–13372