Identification of a keratin-associated protein that localizes to a membrane compartment

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We describe the characterization of an acidic glycoprotein (molecular mass ~ 85 kDa) that associates with keratin intermediate filaments of 'simple'-type epithelia. Using a number of anti-keratin monoclonal antibodies, the 85 kDa glycoprotein was identified by co-immunoprecipitation with keratin polypeptides 8 and 18 (K8/18) from the human colonic epithelial cell line HT29 and several other epithelial cell lines. This Keratin-Associated Protein (termed KAP85) was readily detected after *in vitro* galactosylation of K8/18 immunoprecipitates obtained from mitosis-arrested cells. Its solubilization and detection were dependent on the detergent used, and it was barely detected after *in vitro* galactosylation of asynchronously growing G0/G1-phase cells. Its poor *in vitro* galactosylation in G0/G1-phase cells is

likely a reflection of the lack of available terminal *N*-acetylglucosamine residues, since it can be labelled to a similar extent in G0/G1- and G2/M-phase cells using NaIO₄/NaB³H₄. Glycosidase digestion showed that KAP85 contains high mannose and complex oligosaccharides. Fractionation of total cellular K8/18 into soluble and cytoskeletal insoluble pools showed that KAP85 associates exclusively with the cytoskeletal K8/18 pool. Subcellular fractionation showed that KAP85 co-localizes with a plasma-membrane-enriched fraction that includes the transferrin receptor and KS-1 antigen. Our results demonstrate *in vitro* evidence of a membrane-associated glycoprotein (KAP85) which may serve as an attachment site for filamentous K8/18.

INTRODUCTION

Intermediate filaments (IF), microfilaments and microtubules comprise the three major cytoskeletal proteins found in most mammalian cells [1]. Within intermediate filaments, keratins are the most complex subgroup, and are composed of at least 21 different gene products [2–4], catalogued as keratin polypeptides 1–21 (K1–K21). A characteristic feature of keratins is their expression as pairs in a differentiation- and tissue-specific manner [1]. For example, single layered 'simple' epithelia, such as in the intestine, express predominantly K8 and K18; oesophageal epithelia express K4 and K13, corneal epithelia express K3 and K12, and so on.

There is ample biochemical, immunofluorescence and electron-microscopic evidence for the association of IF proteins with desmosomal and hemi-desmosomal structures of epithelial cells (see [5–8] for reviews). Furthermore, several intermediate-filament-associated proteins (IFAP) have been identified that may directly or indirectly associate with the inner aspect of the plasma membrane [9–10]. These plasma-membrane-associated IFAP include a 140 kDa desmosomal antigen [11] and ankyrin [12]. A recent report also suggested co-localization of the $\alpha_6\beta_4$ integrin and a cytokeratin fraction, although a direct or indirect biochemical association was not shown [13]. This integrin is also a component of the hemidesmosome and is thought to play a role in bridging hemidesmosomal plaques with keratin IF [14,15]. In addition, extensive work has been carried out in characterizing the proteins of the desmosomal complex. Of these proteins, desmocollin appears to be the cell-surface glycoprotein that is

involved in the desmosomal complex attachment to IF [16,17]. In contrast, little is known regarding other interactions of IF with the plasma membrane [9,10].

The large body of morphological data showing IF as a network that extends from the nucleus to the plasma membrane has made it tempting to propose that IF play an important role in mediating signal transfer between the exterior and interior of cells [18]. Although the mechanism and role of IF in facilitating information transfer between outside and inside cells are unknown, preliminary studies have suggested that IF proteins may be involved at some level in signal transduction. For example, K8/18 [19] and vimentin [20,21] appear to associate with protein kinase C, and vimentin transiently associates with cyclic GMP-dependent protein kinase upon neutrophil activation [22]. In addition, stimulation of cultured hepatocytes by epidermal growth factor resulted in increased phosphorylation of K8 in less than 30 min, coupled with IF rearrangement and subsequent entry of the cells into the S-phase of the cell cycle [23]. Therefore, a direct or indirect association of IF with cell-surface proteins may be predicted as a way to transfer information between the inner and outer cell environments.

We describe here the identification and partial characterization of a Keratin-Associated Protein (~ 85 kD; termed KAP85). This IFAP was identified by virtue of its co-immunoprecipitation with K8/18, and its intense *in vitro* labelling with [³H]galactose after K8/18 immunoprecipitates were obtained from G2/M-phase-arrested cells. Here we present *in vitro* evidence that KAP85 is a membrane-associated glycoprotein that has high mannose and complex oligosaccharides, and that it associates with the cytoskeletal, but not the soluble, K8/18 pool.

Abbreviations used: DNS, 1% deoxycholate/1% Nonidet P40/0.1% SDS; IF, intermediate filament(s); i.e.f., isoelectric focusing; K, keratin; KAP, Keratin-Associated Protein; MAb, monoclonal antibody; IFAP, IF-associated protein; endo H, endo- β -*N*-acetylglucosaminidase H.

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MATERIALS AND METHODS

Reagents and antibodies

Tissue-culture cell lines were purchased from the American Type Culture Collection (Rockville, MD, U.S.A.). Normal human colon tissue was obtained from residual surgical specimen under a protocol that was approved by the Human Subjects Committee at Stanford University. Radiochemicals used were: UDP [4,5-³H]galactose (32.1 Ci/mmol), D-[6-³H]glucosamine (40.4 Ci/mmol), NaB³H₄ (54 Ci/mmol), and were purchased from du Pont-New England Nuclear (Wilmington, DE, U.S.A.). Other reagents used were: bovine milk galactosyltransferase; goat anti-mouse IgG- and IgM-Sepharose conjugate; monoclonal antibodies (MAb) CK5 and 8.13 (Sigma); endoglycosidase H and ENTENSIFY autoradiography enhancer (New England Nuclear); and N-glycanase F (Boehringer Mannheim).

Other monoclonal antibodies used were: L2A1 [24], which recognizes a conformation-dependent form of human K8/18 (not shown); CR48, which recognizes mouse, rat and human K18 (not shown); CK5 and 8.13, which recognize human K18 and a broad spectrum of human keratins respectively. Additional MAb used were B3/25 and I4D4, which recognize the transferrin receptor [25] and the KS-1 antigen [26] respectively.

Cell culture and metabolic labelling

The tissue-culture cell lines HT29, Caco2, SK-CO1 (human colon), and HeLa (human cervix) were grown at 37 °C in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were used during asynchronous near-confluent growth (primarily G0/G1 cells, referred to as 'G cells') or were arrested at the G2/M stage of the cell cycle (M cells) using colcemid (0.5 µg/ml) or nocodazole (0.5 µg/ml) for 36 and 24 h respectively. Optimal enrichment of G2/M cells was obtained when the anti-mitotic agents were added to cells at 30–50% confluency. The percentage of G2/M cells was 88–93% and was determined by cell-cycle analysis of 70%-ethanol-fixed, then propidium iodide-stained, cells, exactly as described in [24]. Cells were metabolically labelled with [³H]glucosamine for 12 h in glucose-free RPMI 1640 medium containing 10% dialysed fetal-calf serum and 1 mM sodium pyruvate. In the case of M cells, the label was added for the last 12 h of the 36 h colcemid treatment.

Cell disruption and immunoprecipitation

Preparation of the soluble (S) and cytoskeletal pellet (P) fractions was carried out exactly as described [27]. Briefly, cells were washed twice with PBS, then disrupted in 1.5 ml using a cell disruption bomb (no. 4639; Parr Instrument Co., Moline, IL, U.S.A.) at 6.9 MPa (1000 lbf/in²) for 5 min, followed by ultracentrifugation (300 000 g; 90 min; 4 °C, SW50.1 rotor). The disruption buffer consisted of 10 mM Tris/HCl (pH 7.5), 1 mM MgCl₂, 130 mM KCl, 5 mM EDTA and 5 mM NaCl. Immunoprecipitation from the S and P fractions was carried out as described in [27]. The P fraction was solubilized in detergent 2 (see below) with homogenization using a Dounce homogenizer (45 min, 4 °C), followed by centrifugation to remove non-solubilized material (20 000 g; 15 or 60 min, which gave identical results). Immunoprecipitation from intact cells was carried out after solubilization (45 min, 4 °C) in 1% Nonidet P40 in PBS (detergent 1) or 1% deoxycholate/1% Nonidet P40/0.1% SDS (DNS) in PBS (detergent 2). Since detergent 2 also solubilizes the nuclear membrane, solubilized material was sheared using a 26-gauge needle. All solubilization and cell-disruption solutions

contained 0.1 mM phenylmethanesulphonyl fluoride (added fresh), 5 mM EDTA, 10 µM pepstatin, 10 µM leupeptin and 25 µg/ml aprotinin. The insoluble material was pelleted (16 000 g, 5 min) and 150 µl of solubilized lysate, 2 µl of MAb ascites (non-immune, L2A1, CR48, CK5 or 8.13) were incubated (45 min 4 °C with rocking). Immune complexes were collected using goat anti-mouse IgM (CR48 antibody) or goat anti-mouse IgG (L2A1, CK5, 8.13 and B3/25). Alternatively, MAb L2A1 or I4D4 conjugated to agarose beads were used to directly immunopurify K8/18 and the KS-1 antigen respectively [24].

Immunoprecipitates were analysed by SDS/PAGE [28] under non-reducing conditions unless otherwise indicated. ³H-containing gels were enhanced using ENTENSIFY, as recommended by the manufacturer. Isoelectric focusing (i.e.f.) was carried out using a 4:1 mix of ampholine pH 5–7 and pH 7–10 respectively [29].

In vitro galactosylation and NaB³H₄ labelling

Labelling of immunoprecipitates with [³H]galactose was carried out as described in [24]. Immunoprecipitates were routinely washed three times with PBS containing one half the concentration of the solubilization detergent(s) and 5 mM EDTA, then washed once with galactosylation buffer (0.4 ml of 20 mM MnCl₂/100 mM sodium cacodylate, pH 6.5). Galactosylation was done by using 0.6 µCi of UDP-[³H]galactose and 25 munits of galactosyltransferase (90 min, 37 °C) in 20 µl of galactosylation buffer. Labelled immunoprecipitates were washed twice, then analysed by SDS/PAGE, or used for i.e.f. or glycosidase digestion.

Labelling of cells with NaB³H₄ after NaIO₄ oxidation was carried out on intact cells directly on the dish or on scraped G cells or floater M cells in a manner similar to that described previously [30,31]. For this, cells were incubated (in suspension or on the dish) with 2 mM NaIO₄ in PBS (10 min, 22 °C, with shaking), followed by washing three times with PBS. NaB³H₄ was then added (2.5 mCi/ml in 200 µl for suspension G and M cells; 1.5 mCi in 1 ml for G cells directly on the dish) for 30 min (22 °C). Cells were then washed, solubilized in DNS and processed for immunoprecipitation.

Glycosidase digestion

Immunoprecipitates of K8/18 that were labelled with UDP-[³H]galactose were incubated with 10 µl of 1% SDS/25 mM EDTA/5 mM NaN₃ in sodium phosphate, pH 7.2, for 5 min. The supernatant containing released immune complexes (10 µl) was mixed with 7 µl of 10% Nonidet P40 and 1 µl (0.2 unit) of N-glycanase F [32] for 16 h (37 °C). Control immunoprecipitates were either stored at –20 °C or treated in a similar manner (37 °C) without the addition of enzyme. Digestion with endo-β-N-acetylglucosaminidase H (endo H) [33] (0.15 unit/ml) was carried out in 20 µl of 50 mM Tris/HCl, pH 7.5, for 16 h (37 °C). Control immunoprecipitates were also processed as for the N-glycanase F digestion.

Subcellular fractionation

A crude subcellular fractionation was carried out using differential centrifugation as described in [34]. Cells were homogenized at 1.035 MPa (150 lbf/in²) (5 min, 4 °C) which disrupts > 99% of cells while leaving the nuclei intact (as determined by Trypan Blue staining and electron microscopy; results not shown). Fractions were collected as follows: nuclei (450 g, 15 min), mitochondria (4000 g, 15 min), plasma membrane (20 000 g,

30 min), endoplasmic reticulum and Golgi (125000 g, 1 h). Each pellet fraction was removed and solubilized in DNS, then used for immunoprecipitation.

RESULTS

Identification of KAP85

During our studies of K8/18 glycosylation in asynchronously growing interphase HT29 cells (primarily G0/G1 stage of the cell cycle; 'G cells') and nocodazole- or colcemid-arrested G2/M cells ('M cells') [24], we noted an 85 kDa glycoprotein that co-immunoprecipitated with K8/18 using anti-K8/18 MAb L2A1. As shown in Figure 1 (detergent 2), immunoprecipitation of K8/18 from M cells followed by *in vitro* galactosylation using

Figure 1 KAP85 is co-immunoprecipitated with K8/18 and is detected by *in vitro* galactosylation

Asynchronous (G) and mitosis-arrested (M) HT29 cells were solubilized in detergent 1 containing 1% Nonidet P40 in PBS or detergent 2 (containing 1% Nonidet P40, 1% deoxycholate and 0.1% SDS in PBS), as described in the Materials and methods section. Immunoprecipitation was performed by using anti-K8/18 MAb L2A1 coupled to agarose. Immunoprecipitates were then galactosylated *in vitro* using 0.6 μ Ci of UDP-[3 H]galactose, 25 units of galactosyltransferase in 20 mM MnCl_2 /100 mM sodium cacodylate, pH 6.5, at 37 °C for 90 min. Closed arrowheads correspond to the antibody band which migrates near the 200 kDa marker under non-reducing conditions. The radiograph was obtained from the Coomassie Blue-stained gel shown.

Figure 2 Two-dimensional gel analysis of galactosylated KAP85

A K8/18-KAP85 co-immunoprecipitate obtained from M cells was labelled with UDP-[3 H]galactose, followed by i.e.f. and SDS/PAGE. KAP85 showed a pI of ~ 5.2 (major spot) and 5.4 (minor spot) and is therefore more acidic than K18 (pI ~ 5.6 –5.8). The autoradiograph (a) was obtained from the Coomassie Blue-stained gel (b). The left of each panel (indicated by $\square$) shows an identical immunoprecipitate to that used for two-dimensional gel analysis, except that it was analysed only in the SDS/PAGE dimension.

UDP-[3 H]galactose and galactosyltransferase (which specifically attaches a galactose to accessible terminal *N*-acetylglucosamine residues) resulted in the pronounced labelling of an 85 kDa protein. This protein was barely detected, as determined by galactosylation, when K8/18 was immunoprecipitated from G0/G1 cells (Figure 1, detergent 2). The detergent used was very important in determining the quantity of solubilized KAP85. For example, the detergent 2 (1% deoxycholate/1% Nonidet P40/0.1% SDS) was far more efficient in solubilizing KAP85 than the detergent 1 (containing only 1% Nonidet P40). It was noteworthy that although the immunoprecipitate from the M lysate of detergent 1 revealed higher K8/18 levels by Coomassie Blue stain as compared with the detergent 2 immunoprecipitate, detergent 2 conditions resulted in much higher levels of KAP85, as shown in the radiograph of Figure 1. Two additional bands, indicated by numbered arrows in Figure 1, were also occasionally noted and were not characterized.

I.e.f. followed by SDS/PAGE showed that KAP85 is an acidic protein (pI ~ 5.2 ; Figure 2). It is unclear whether the two isoelectric species represent different levels of sialic acid content, sulphation, phosphorylation or represent two different proteins that co-migrate on one-dimensional SDS/PAGE. There was no detectable labelling of KAP85 after cells were labelled with orthophosphate or sulphate followed by immunoprecipitation of

Figure 3 KAP85 co-immunoprecipitates with K8/18 using anti-keratin antibodies of different specificities

Immunoprecipitates were prepared from detergent-solubilized M cells (detergent 2 as in Figure 1) using a panel of anti-keratin antibodies as described in the Materials and methods section. Immunoprecipitates were then galactosylated using UDP-[^3H]galactose followed by SDS/PAGE. Lane b shows a higher intensity of KAP85, which may be related to enhanced accessibility of terminal *N*-acetylglucosamine residues on KAP85 after CR48 antibody binding. Key to lanes: a, control ascites; b, CR48; c, CK5; d, L2A1; e, 8.13.

Figure 4 KAP85 is associated with the insoluble fraction of K8/18

G and M cells were disrupted using nitrogen cavitation [6.9 MPa (1000 lbf/in 2) pressure]. Soluble (S) and insoluble pellet (P) fractions were prepared by ultracentrifugation (300 000 g; 90 min). Immunoprecipitates were then obtained from the P fraction after homogenization with detergent and from the S fraction using anti-K8/18 MAb L2A1. (a) *In vitro*-galactosylated immunoprecipitates of K8/18. (b) HT29 cells were metabolically labelled with [^3H]glucosamine followed by immunoprecipitation of K8/18 from the S and P fractions, then SDS/PAGE analysis. Arrowheads indicate an unidentified 98 kDa glycoprotein that preferentially associates with soluble K8/18. Exposure was 3 and 19 days for (a) and (b) respectively.

K8/18 (not shown). This suggests that the two isoforms of KAP85 may be due to differences in sialic acid, which is supported by labelling with $\text{NaIO}_4/\text{NaB}^3\text{H}_4$ (shown in Figure 6 below). The level of galactosylation of K18 is greater than K8 (Figures 1 and 2), as shown previously [24].

KAP85 was also co-immunoprecipitated with K8/18 using three other MAbs that recognize different epitopes on K8/18 (Figure 3). This indicates that the revelation of KAP85 after immunoprecipitation of K8/18 using MAb L2A1 (Figures 1 and 2) is not due to cross-reactivity of the antibody with KAP85, but rather is related to a K8/18–KAP85 direct or indirect association. Of note, KAP85 was also detected in association with K8/18

immunoprecipitates obtained from several epithelial cell lines (SK-CO-1, Caco2, and HeLa) and from normal human colonic surgical tissues (results not shown).

KAP85 associates with the cytoskeletal fraction of K8/18

We recently showed that a number of colonic epithelial cell lines contain a substantial soluble pool representing ~5% of total cellular keratin [27]. The functional significance of a K8/18 soluble pool and what the solubility 'factor' is, be it a post-translational modification, an inherent biophysical property of the keratins within their microenvironment or an associated protein, are unknown.

We tested whether KAP85 associates preferentially with the soluble or the cytoskeletal K8/18 pool. As shown in Figure 4(a), KAP85 associates with the insoluble keratin pool (i.e. cytoskeletal fraction) of G and M cells as determined after *in vitro* galactosylation of K8/18 immunoprecipitates. An additional protein which we have not characterized (molecular mass ~98 kDa) associates with K8/18 immunoprecipitates obtained from the soluble fraction (indicated by arrowheads in Figure 4). Further support for the presence of KAP85 in HT29 cells was obtained by *in vivo* labelling of G and M cells with [^3H]glucosamine. As shown in Figure 4(b), KAP85 coimmunoprecipitated with K8/18 that was isolated from the P, but not the S, fraction of [^3H]glucosamine-labelled M cells. A faint 98 kDa *in vivo* ^3H -labelled band could also be seen with the S fraction, which likely corresponds with the same molecular-mass band seen by *in vitro* labelling (Figure 4a). The labelling efficiency of K8/18 and KAP85 by [^3H]glucosamine in M cells was far less than in G cells (Figure 4b), which was also reflected by trichloroacetic acid-precipitable radioactivity of total cellular labelled protein (results not shown).

KAP85 contains high-mannose and complex oligosaccharides

The glycoprotein nature of KAP85 was confirmed further using the glycosidases N-glycanase F, which cleaves complex and high-mannose oligosaccharides [32] and endo H, which cleaves high-mannose oligosaccharides [33]. As shown in Figure 5(a) (lane a), treatment of K8/18–KAP85 immunoprecipitates (labelled *in vitro* with [^3H]galactose) with N-glycanase F resulted in disappearance of the KAP85 band, consistent with removal of ^3H -labelled N-linked sugars. Similarly, treatment of K8/18–KAP85 immunoprecipitates with endo H decreased the apparent molecular-mass of KAP85 to ~83 kDa, consistent with the presence of high-mannose sugars (Figure 5b, lane d). KAP85 does not contain any interchain disulphide bonds and its protein nature was verified by its susceptibility to trypsin digestion (results not shown). It is not a secreted protein on the basis of its absence from culture medium as determined by adding K8/18 to concentrated culture medium followed by immunoprecipitation and galactosylation (results not shown).

KAP85-like protein can be labelled directly on the cell surface and is detected in G and M cells

The detection of KAP85 was facilitated by the marked increase in glycosylation of this protein during mitotic arrest (compare the *in vivo* and *in vitro* labelling of KAP85 in the P fraction of M as against G cells; Figure 4). Several possibilities may account for this: (1) the expression level of the protein increases with mitotic arrest; (2) the protein is present in G and M cells, but its glycosylation is altered during mitotic arrest; (3) the distribution of KAP85 is altered such that mitotic arrest results in redistribution of the protein and association with K8/18. To help

Figure 5 KAP85 contains high-mannose and complex-type oligosaccharides

K8/18-KAP85 immunoprecipitates were ^3H -labelled by galactosylation then treated with 0.2 units of N-glycanase F (NGase F, **a**, lane a) and 0.15 unit/ml endo H (Endo H, **b**, lane d) at 37°C for 16 h. Samples in lanes b and e were incubated as for samples in lanes a and d, but without the enzymes. Samples in lanes c and f were stored at -20°C after galactosylation. The Coomassie Blue stain shows the amount of K8/18 in the immunoprecipitate that was resolved by SDS/PAGE. Samples in (**a**) and (**b**) were analysed on 10% and 6% acrylamide gels respectively. The bands indicated by the upper closed arrowheads in (**a**) and (**b**) correspond to the position of the antibody used for immunoprecipitation. The band indicated by the lower closed arrowhead in (**a**) corresponds to deglycosylated KAP85.

distinguish between these possibilities, we compared the $\text{NaB}^3\text{H}_4/\text{NaIO}_4$ cell-surface labelling of intact G cells (labelled directly on the tissue-culture dish) with labelling of scraped-off G and M cells. This method introduces the ^3H -label into NaIO_4 -oxidized sialic acids preferentially [30,31]. As shown in Figure 6, an 85 kDa band is seen after labelling intact G cells on the tissue-culture dish then immunoprecipitating K8/18, and a faint 85 kDa band is also seen after immunoprecipitation from floater M cells and scraped-off G cells. The floater M cells and scraped-off G cells also show intense labelling of K8/18 and several other unidentified associated glycoproteins secondary to leakiness of manipulated cells, as we have recently shown [35]. As a control for cell-surface labelling, an adenocarcinoma/epithelial cell-surface marker termed KS-1 [36,37] was used for immunoprecipitation (Figure 6, lanes 2; molecular mass ~ 40 kDa). The fainter labelling of KAP85 in M and scraped-off G cells parallels the decreased labelling of the KS-1 antigen as compared with labelling of KAP85 and KS-1 in intact G cells, and is commensurate with increased labelling of intracellular proteins (e.g. K8/18). The 85 kDa protein identified after NaB^3H_4 labelling co-migrates with KAP85 labelled by *in vitro* galactosylation (Figure 6, lane 3). This suggests that KAP85 is present in both G and M cells, and that the easy detection by *in vitro* galactosylation is related to an overall increase in terminal GlcNAc residues, as we have noted for a number of proteins in M cells (results not shown). We have not been able to conclusively identify KAP85 by cell-surface radioiodination, biotinylation or [^{35}S]methionine labelling of G and M HT29 cells, because several non-specific bands are seen when these labelling techniques are used (results not shown).

Figure 6 KAP85 is labelled on the cell surface using $\text{NaIO}_4/\text{NaB}^3\text{H}_4$, and is present in both G and M cells

HT29 cells (G and M) were treated with 2 mM NaIO_4 . Oxidation was done in suspension for scraped-off G cells or floater M cells, or directly on the tissue-culture dish without manipulation (intact G) as described in the Materials and methods section. NaB^3H_4 was then added to the suspension cells (200 μl , 2.5 mCi/ml) or on the dish (1 ml, 1.5 mCi/ml) for 30 min at 22°C . After washing, cells were solubilized followed by immunoprecipitation and SDS/PAGE. Immunoprecipitates (i.p.) were obtained using antibodies to: lanes 1, K8/18; lanes 2, KS-1 cell-surface marker; lane 3, K8/18 that was galactosylated *in vitro* using UDP- ^3H galactose and analysed on the same gel to show the migration position of KAP85. Exposure was for 3 days (lane 3) and 32 days (lanes 1 and 2).

Figure 7 Subcellular fractionation localizes KAP85 to a plasma-membrane-enriched fraction

Subcellular fractions were obtained by differential centrifugation of M cells that were disrupted by nitrogen cavitation [1.035 MPa (150 lbf/in 2) 5 min]: N (nuclei, 450 g, 15 min), M (mitochondria, 4000 g, 15 min), P (plasma membrane, 20000 g, 30 min) and E (endoplasmic reticulum/Golgi, 125000 g, 60 min). After each spin, pellets were solubilized followed by immunoprecipitation of K8/18 (using MAb L2A1), the transferrin receptor (using MAb B3/25) and KS-1 antigen (using MAb I4D4). (**a**), (**b**) and (**c**) (i) show autoradiographs of galactosylated immunoprecipitates after SDS/PAGE. The Coomassie Blue stain in (**c**) shows K8/18 immunoprecipitated from each subcellular fraction using MAb L2A1. Immunoprecipitates in (**a**) were analysed by SDS/PAGE under reducing conditions, which accounts for the galactosylated antibody heavy-chain band at ~ 50 kDa (indicated by arrowhead). The band indicated by arrowhead in (**b**) has molecular mass (M) of 78 kDa and was not characterized.

Co-localization of KAP85 with the plasma-membrane fraction

The co-localization of KAP85 with the plasma-membrane fraction was determined by immunoprecipitation of K8/18 from subcellular fractions of M cells. As shown in Figure 7, KAP85 co-localized with the plasma-membrane fraction (labelled 'P' in Figure 7c), similar to what would be expected for the cell-surface transferrin receptor (immunoprecipitated with MAb B3/25; Figure 7a) and the KS-1 antigen (immunoprecipitated with MAb

I4D4; Figure 7b). Although the relative amount of K8/18 associated with the plasma-membrane fraction, as determined by Coomassie Blue-stained immunoprecipitated material, was the smallest, it was this fraction that had the highest associated KAP85 [compare the Coomassie Blue stain of Figure 7c(ii) with the radiograph in Figure 7c(i)].

DISCUSSION

The major finding of the present study is the identification of a unique membrane glycoprotein (KAP85) that associates with K8/18. The KAP85-K8/18 association involves the cytoskeletal, but not the soluble, IF pool. This is based on finding an exclusive association of KAP85 with the insoluble keratin pool obtained after cell disruption (Figure 4). Furthermore, harsh detergent solubilization conditions which include anionic detergents were required to obtain adequate levels of KAP85 (Figure 1). Evidence that KAP85 is a membrane-associated protein was suggested by: (1) co-localization with the plasma-membrane fraction, which was also enriched for the two well-characterized cell-surface proteins transferrin receptor and KS-1 antigen (Figure 7); (2) the presence of high-mannose and complex N-linked oligosaccharides (Figure 5); (3) revelation of a K8/18-associated cell-surface protein with a similar molecular mass to KAP85 after $\text{NaIO}_4/\text{NaBH}_4$ labelling of intact cells directly on the tissue-culture dish (Figure 6). A cell-surface topology for KAP85 is consistent with our observations; however, we cannot exclude an intracellular membrane compartment distribution. To that end, although trypsin treatment of [^3H]galactosylated KAP85-K8/18 immunoprecipitates abolished the ^3H label, pre-treatment of cells with trypsin or papain before immunoprecipitation of K8/18-KAP85 did not affect the level of KAP85 labelling (results not shown). This may be explained by inaccessibility of KAP85 to the proteinases on the cell surface (KS-1 antigen is also not affected by the proteinase treatment; results not shown) or by the presence of KAP85 (which is associated with K8/18) in an intracellular membrane compartment.

The molecular size and glycosylation of KAP85 suggests that it is a unique protein that does not correspond to any of the identified desmosomal or hemidesmosomal components. On the basis of size criteria alone, plakoglobin (85 kDa) is an attractive candidate for being KAP85. However, plakoglobin has not been reported to be a glycoprotein [8], and immunoprecipitates of plakoglobin were not galactosylated *in vitro* (results not shown).

It remains to be determined whether KAP85 associates with K8/18 directly or indirectly. Since several other faintly galactosylated glycoproteins associate with K8/18 immunoprecipitates (Figure 4, with KAP85 being the most prominent band), an indirect association of KAP85 with K8/18 is possible. We addressed K8/18-KAP85 association by testing the binding of [^{125}I - or [^{35}S]methionine-labelled K8/18 to a KAP85-like protein in a Western-blot fashion. The blots contained total cell lysates or immunoprecipitates of K8/18 from G and M cells. Our blotting results did not reproducibly show binding of radio-labelled K8/18 to a KAP-85 like protein (results not shown). This may be due to denaturation of KAP85 during SDS/PAGE or to the need for other components to allow binding of K8/18 to KAP85. Therefore our data cannot distinguish between a direct or indirect binding of K8/18 to KAP85.

Evidence for a physiological *in vivo* association of KAP85 with K8/18 will require immune electron microscopy using antibodies to KAP85. Although K8/18 association with KAP85 may be an *in vitro* phenomenon, there is some experimental evidence that suggests a specific association. For example, subcellular fractionation reproducibly partitions a small fraction of the total keratin

into a pool which associates with most of the KAP85 and the plasma-membrane fraction. In addition, detergent solubilization with DNS followed by a high-speed spin (20000 g, 60 min) maintains KAP85/K18 in a solubilized form (results not shown), indicating that KAP85 is not simply trapped in a partially solubilized aggregate.

The importance of the interaction of the plasma membrane with cytoskeletal proteins is being realized as more membrane-cytoskeletal associations are identified and their regulation is studied. Most of the studies to date have focused on interaction of cell-surface receptors with microfilaments and their cytoplasmic binding proteins. Examples include the intercellular adhesion molecule 1 [38], the lymphocyte-specific protein LSP1 [39] and the epidermal-growth-factor receptor [40]. It is tempting to speculate that intermediate filaments also interact directly or indirectly with one or more cell-surface proteins and/or with intracellular organelles. For example, B-cells stimulated with anti-Ig, but not with lipopolysaccharide, underwent reorganization of vimentin into extensive filamentous arrays [41]. Evidence also exists for a direct interaction of intermediate filaments with mitochondria [42]. In addition, the recent identification of a yeast IF-like protein that plays an essential role in mitochondrial and nuclear inheritance also supports an IF-intracellular-organelle association [43].

The functional significance of KAP85-K8/18 association remains to be determined. The membrane association of KAP85 and absence from the soluble K8/18 pool suggests that it may act as an anchor (either directly or as part of a complex) for a fraction of cytoskeletal K8/18. If indeed KAP85 is a cell-surface protein, then interaction with intermediate filaments may regulate its function. The protein amount of KAP85 that associates with K8/18 is unknown, but is substoichiometric to the amount of K8/18 immunoprecipitated from the plasma-membrane-enriched fraction and cannot be seen on Coomassie Blue or silver staining (results not shown). Generation of antibodies to KAP85 should facilitate the identification and further characterization of this glycoprotein.

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