Restin: a novel intermediate filament-associated protein highly expressed in the Reed–Sternberg cells of Hodgkin's disease

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We have identified a cDNA coding for a protein of 160 kDa which is expressed in *in vitro* cultured human peripheral blood monocytes. The predicted amino acid sequence contains an α -helical rod domain possessing features characteristic of intermediate filament proteins. However, the immunocytochemical staining pattern, abundance and solubility in Triton X-100/high salt buffers suggest that this protein is probably only associated with the intermediate filament network and represents a new type of intermediate filament associated protein. In a survey of normal, inflammatory and human tumour tissue samples, this protein, which we have named restin, was found to be highly expressed in Reed–Sternberg cells, the tumoral cells diagnostic for Hodgkin's disease. We suggest that restin overexpression may be a contributing factor in the progression of Hodgkin's disease.

Key words: Hodgkin's disease/intermediate filament associated protein/monocytes

Introduction

Intermediate filaments (IF) and IF-like proteins are prominent components of the cytoskeleton and, in contrast to the structural components of microfilaments (actin) and microtubules (tubulin), they form a complex family of proteins with diverse functional roles (for review see Steinert and Roop, 1988). The IF protein family comprises six distinct subclasses, all related on the basis of a structurally similar, central α -helical rod domain: type I and type II are the acidic and basic keratins respectively; type III includes vimentin, desmin, glial fibrillary acidic protein and peripherin; type IV includes the light, medium and heavy neurofilament proteins and α -internexin of the nervous system; type V are the lamins of the nuclear lamina. Nestin, an IF protein of neuroepithelial stem cells, has recently been shown to define a sixth type (Lendahl *et al.*, 1990).

Most IF proteins are restricted to certain cell types; for example, cytokeratins are found predominantly in epithelial cells (Franke *et al.*, 1978a), desmin in muscle cells (Gard *et al.*, 1979), glial fibrillary acidic protein in astrocytes

(Goldman *et al.*, 1978) and neurofilaments in neurons (Liem *et al.*, 1978). Additionally, other studies have demonstrated that IFs can be coexpressed in certain tissues (reviewed by Coggi *et al.*, 1989). As such, IF protein expression is not simply related to the histogenesis of the cell but is the result of complex interactions between the cell and its microenvironment (Grover *et al.*, 1983; Ben-Ze'ev, 1984; Semat *et al.*, 1986; von Koskull and Virtanen, 1987). Although the function of individual IF proteins has not been fully elucidated, they are thought to form a structural network which integrates cytoplasmic structures (Lazarides, 1980). Evidence is now emerging that IFs are actually dynamic structures which are equally important to cell function. For example, phosphorylation of the nuclear lamins and of the amino terminus of vimentin results in the dispersion of these IF networks and is essential to the process of mitosis (Dessev *et al.*, 1988; Chou *et al.*, 1989). The pattern of IF protein expression changes markedly during neuronal development, thus nestin is expressed in neuronal epithelial cells (Lendahl *et al.*, 1990) and is replaced by α -internexin following commitment to the neuronal pathway (Fliegner *et al.*, 1990). Alpha-internexin is in turn replaced in mature neurons by light, medium and heavy chain neurofilaments (for a review see Steinert and Liem, 1990). Hence the coordinated and sequential expression of IFs plays an important functional role in the development of the neuronal cell lineage.

In addition to the superfamily of IF proteins, a growing number of proteins have been demonstrated to be associated, and in some cases, coisolate with IF proteins and play a role in the supramolecular organization of cellular IF networks (Steinert and Roop, 1988). Although the molecular mechanisms involved in the interaction between IFs and IF associated proteins (IFAPs) are not fully understood, they have been classified into four different types (Steinert and Roop, 1988). (i) Type I is made up of low molecular weight (10–45 kDa) molecules, as typified by filaggrin, which bind IFs laterally into tight macrofilament aggregates; (ii) higher molecular weight type II IFAPs (100–300 kDa), including paranemin, synemin, trichohyalin and plectin, which crosslink IFs into loose networks; (iii) type III includes proteins involved in coupling the ends of IFs to the plasma membrane and the nuclear lamina, such as ankyrin, spectrin and desmoplakin; and (iv) a number of proteins for which the association with the IF network is still unknown, e.g. epinemin and β -internexin (for reviews see Steinert and Roop, 1988 and Foisner and Wiche, 1991).

Many studies have shown that IFs and IFAPs are important markers in the classification of tumours (Gabbiani *et al.*, 1981; Ramaekers *et al.*, 1981; Miettinen *et al.*, 1984; Bolen and McNutt, 1987) and antibodies to IF proteins have been used for the diagnosis of malignant disorders (Osborn and Weber, 1983). Thus IF proteins are important markers for cell lineage and/or differentiation and aid in classifying cells of unknown origin occurring in malignant tumours. Similarly IFAPs have also been implicated in disease states such as

keratinizing disorders of hair (Raphael *et al.*, 1984) and skin (Dale *et al.*, 1987).

As part of an ongoing investigation of monocyte/macrophage differentiation, we have identified a gene product of *in vitro* cultured human peripheral blood monocytes which is also highly expressed by the Reed–Sternberg cells of Hodgkin's disease. This protein shares a number of features with the IF protein superfamily but has a cellular distribution more characteristic of IFAPs. We present evidence that this protein represents a novel type of IFAP which may be involved in the aetiology of Hodgkin's disease. The protein has been named restin because of its specific expression in the Reed–Sternberg cell of Hodgkin's disease.

Results

cDNA clone

This study was initially undertaken in an effort to clone a macrophage migration inhibitory factor (MIF). The factor had been partially purified from cell supernatants of concanavalin A-stimulated peripheral blood mononuclear cells and was used to prepare monoclonal antibodies. One of these antibodies, 1C5, was found to deplete cell supernatants of MIF activity. It recognized an antigen on macrophages in inflammatory conditions and identified three major proteins of 8, 14 and 28 kDa in concanavalin A-stimulated mononuclear cell supernatants (Burmeister *et al.*, 1986). Both the 8 and 14 kDa species were purified and sequenced, and the cDNAs were cloned using an oligonucleotide strategy. They were shown to be calcium-binding proteins specific to the monocyte lineage, but did not have MIF activity (Odink *et al.*, 1987). The present study was an attempt to clone the cDNA coding for the protein bearing the 1C5 epitope. Instead of the expected 28 kDa protein, the cloned cDNA codes for an immunoreactive, cytoskeleton-associated protein of 160 kDa.

Antibody screening of cDNA libraries in λ gt11 made from linoleic acid-induced mouse peritoneal macrophages and human U937 histiocytic lymphoma cells identified one clone among $\sim 10^6$ recombinants. Both clones were ~ 2.5 kb long and were 96% homologous, therefore the human clone was investigated further. Using the 2.5 kb clone as a probe, a full length clone was isolated from a cDNA library made from concanavalin A-stimulated human peripheral blood mononuclear leukocytes yielding a cDNA of 6 kb in length. The cDNA sequences of several independently isolated clones were determined and shown to contain one long open reading frame of 1471 amino acid residues preceded by two in-frame stop codons at bases 89–91 and bases 119–121 upstream of the first methionine residue. S1 nuclease and primer extension analyses confirmed that the clone contained the 5' end of the cDNA (data not shown). The sequence contains 1.5 kb of 3'-non-coding sequence with two potential polyadenylation signals of the sequence AATAAA at positions 4464 and 5707 (Figure 1). The predicted 160 kDa protein lacks a hydrophobic stretch of amino acids at its N-terminus characteristic of a signal sequence suggesting that the protein is not secreted. Nine potential glycosylation sites with the consensus sequence Asn-X-Thr/Ser were identified. Northern blots of RNA prepared from elutriated human lymphocytes, peripheral blood monocytes and 4 day *in vitro* cultured peripheral blood monocytes were probed with the 6 kb cDNA clone (Figure 2). Only *in vitro* cultured

peripheral blood monocytes contained high levels of the corresponding mRNA. Longer exposures of the same blots did not reveal even low levels of mRNA in peripheral blood monocytes or lymphocytes, suggesting that the mRNA appeared only after 4 days in culture and that the signal was specific to *in vitro* cultured monocytes.

Structural features of restin

Sequence homology searches of the GenBank/EMBL databases with the TfastA program of the UWGCG software package did not reveal any significant homologies with known proteins. However, a number of very low homologies ($< 21\%$) were detected with proteins containing sequences of high α -helical character such as the myosins, IFs and tropomyosins. These regions of homology were confined to a segment of restin extending from residue 800 to residue 1150. Secondary structure analysis of restin with the Peptide Structure program of the UWGCG software package predicted a region of high α -helical content extending for ~ 825 residues from amino acid (aa) 350 to aa 1275. A highly negatively charged α -helical coil corresponding to the conserved IF rod domain is contained within this segment between residues 851 and 1145 (Figure 1). The rod domain of IF proteins consists of four coils, 1A and 1B, 2A and 2B, that have a characteristic heptad repeat, abcdefg, where positions a and d are occupied predominantly by hydrophobic residues. The coil domain of restin contains such a heptad motif characteristic of IF proteins. The homology between restin and other IF proteins is restricted mainly to the coil domains and in particular to the amino acid motifs localized in coil 1A and towards the ends of coils 1B and 2B (Figure 3). This region of restin was compared with the other types of IF protein using the Align program of the Protein Identification Resource. This program computes a score of relatedness for each pairwise comparison of sequences where the lowest significant score for two distantly related sequences is 2 and for very similar sequences is ≥ 12 (Schwartz and Dayhoff, 1978). The alignment scores show that restin is significantly but distantly related to the IF protein family (Table I). However, restin contains a number of deletions within the rod domain, one of which falls within the last heptad of coil 2B. This region is highly conserved between IF proteins and is thought to be important in the assembly of IF proteins into 10 nm filaments (Lazarides, 1980). The lack of the last heptad in restin suggests that restin may not form filaments by itself in the same way as do other members of the IF family. The α -helical nature of the rod domain of IF proteins is of paramount importance for the formation of filaments. Therefore, the homology of a large part of restin with the IF rod domain may indicate that restin interacts through this domain with other members of the IF family.

Restin has charged N- and C-terminal domains flanking the α -helical tract containing the IF homology. The N-terminal domain (residues 1–350) is rich in serine (12%), threonine (8%) and alanine (10%), has an overall positive charge and contains a 24 amino acid serine-rich motif which is also present in the N-termini of type IV and some type III IF proteins (Figure 4). This serine/threonine-rich area within the region N-terminal to the IF homology domain suggests that this area could be a hot spot for phosphorylation. Phosphorylation has been shown to play an important role in IF and IFAP function. Several potential phosphorylation sites were detected in the amino acid

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Fig. 2. Northern blot analysis of restin mRNA expression. Total RNA from (A) 4 day *in vitro* cultured human blood monocytes (10 μ g), (B) elutriated peripheral blood monocytes (20 μ g) and (C) lymphocytes (30 μ g) were separated on 0.8% formaldehyde-agarose gels, transferred to nylon membranes and probed with restin cDNA. Panel A was exposed for 24 h, panels B and C for 3 days. In a control experiment (below) the same RNA samples were probed with eukaryotic initiation factor eIF-4A cDNA.

sequence of restin. Motifs recognized by the Ca^{2+} /calmodulin protein kinases (RXXS/T; Kemp and Pearson, 1990) occur 11 times and 24 potential phosphorylation sites for casein kinase (EXXS or S(P)XXS; Kemp and Pearson, 1990) were found. Restin also contains a potential site for phosphorylation by the cell cycle kinase cdc28 (XKTPX; Langan, 1978). Most of these potential phosphorylation sites are found within or N-terminal to the rod domain. The N-terminal region also contains an imperfect repeat of 52 amino acid residues (Figure 1). In contrast, the C-terminal domain (residues 1200–1427) is negatively charged, contains a high number of negatively charged amino acids (22%) and is serine- and threonine-rich (15%).

Immunocytochemistry

To determine if restin is associated with the cytoskeleton and can form filamentous structures as suggested by the structural analyses of the predicted protein, a CHO cell line was permanently transformed with restin cDNA and the intracellular distribution of restin visualized with affinity purified rabbit polyclonal antibodies raised to the 70 kDa C-terminus of the molecule. CHO cells are known to produce vimentin constitutively, hence, anti-vimentin antibodies were used as an endogenous control to confirm that the staining pattern resembled that of an IF protein (Cabrál *et al.*, 1981). To distinguish between microtubules, microfilaments and intermediate filaments, a series of double-label immuno-

Fig. 3. Alignment of the α -helical rod domains of IF proteins. The predicted amino acid residues 852–1145 of restin were manually aligned with the conserved core domains (as discussed by Steinert and Roop, 1988) of other IF proteins: human keratin 18 (HK1; Oshima *et al.*, 1986), mouse epidermal type II keratin (MK2; Steinert *et al.*, 1985), mouse glial fibrillary acidic protein (GFA; Lewis *et al.*, 1984), rat middle molecular weight neurofilament protein (RNF; Napolitano *et al.*, 1987), chicken lamin B2 (CLB2; Vorburger *et al.*, 1989) and nestin (NES; Lendahl *et al.*, 1990). The subdivisions into coil and linker regions are delimited by solid vertical bars. Amino acids occupying the 'a' and 'd' positions of the heptad repeats are marked with closed circles underneath the sequences according to the scheme of Geisler and Weber (1983). Dots are inserted to optimize alignment.

fluorescence experiments were performed using antibodies directed against vimentin and restin. Antibodies to restin exhibited a typical IF protein staining pattern, specifically, perinuclear staining combined with a wave-like pattern of filamentous staining radiating towards and underlying the plasma membrane in some cells (Figure 5A). This figure also shows that the antibodies were specific for restin as not all cells were stained, whereas vimentin did stain all cells, thus confirming that the anti-restin antibodies did not crossreact with another IF protein. Anti-vimentin antibodies also gave pronounced perinuclear staining although the fibres did not extend as far into the cytoplasm as those for restin in these cells. However, distinct differences were seen between the staining pattern for restin and the known patterns for microfilaments and microtubules following co-staining with anti-actin and anti-tubulin antibodies as detailed by other investigators (Hynes and Destree, 1978; Franke *et al.*, 1978a, 1979). Microfilaments formed straight ordered arrays of fibres whereas microtubules were more evenly distributed in the cell and did not have a distinct perinuclear organization (data not shown; Hynes and Destree, 1978). Treatment with cytochalasin B dissociated the microfilament bundles which then appear as densely staining clumps when stained with anti-actin antibodies (data not shown; Hynes and Destree, 1978). Restin and vimentin staining patterns are distorted by the change of cell shape following microfilament dissociation but the organization of fibres is still evident (Figure 5B). Staining of colchicine-treated cultures with anti-restin and anti-vimentin antibodies showed an altered

distribution of fibres, which were clumped into bundles or coils concentrated around the perinuclear area with some bundles coiling into the cytoplasmic area (Figure 5C). The tubulin staining pattern was more diffuse with some perinuclear concentration as has been described by other workers (data not shown; Hynes and Destree, 1978; Franke *et al.*, 1979). Although the staining pattern due to the overexpression of restin in the transformed cell line demonstrated that restin could form IF-type structures, it was important to determine if this staining pattern was found in non-transformed cells.

To study the intracellular distribution of restin in a non-transformed cell type, human peripheral blood monocytes were cultured for up to 8 days *in vitro* and stained with polyclonal anti-restin antibodies (Figure 6). Strong cytoplasmic staining was seen in the cultured monocyte/macrophages (corroborating cloning and Northern data). In contrast to the staining pattern seen in the transfected cell line where the protein is overexpressed, the restin staining pattern in cultured monocytes had a distinct punctate appearance, with a tight clustering around the nucleus, stretching out into the cytoplasm (Figure 6). This pattern appeared to follow the vimentin staining pattern to some extent although the restin staining pattern extended further to the periphery of these cells and seemed to underlie the plasma membrane (Figure 6). Close examination of a number of similar indirect immunofluorescent staining experiments of *in vitro* cultured monocytes indicated that the punctate staining was more concentrated towards the cytoplasmic or nuclear 'interfaces' of the vimentin network, although this supposition would have to be confirmed by immunoelectronmicroscopy. This staining pattern is more reminiscent of an IFAP such as plectin (Wiche, 1989) or gyronemin (Brown and Binder, 1990). Thus, the data clearly indicate that restin is associated with the cytoskeleton and is probably part of the IF network.

In a parallel series of experiments, the *in situ* expression pattern of restin was analysed in a range of normal, inflammatory and human tumour tissue samples. Little or no positive staining was seen in normal human tissue samples. The most striking finding was the presence of large amounts of restin in the Reed–Sternberg cells, a characteristic cell type present in tumorally transformed lymph nodes and diagnostic for Hodgkin's disease (Figure 7). In the Reed–Sternberg cells, restin (as determined by immunoperoxidase staining) was localized in the cytoplasm and correlated strongly with the appearance of Hodgkin's cells in Hodgkin's disease lymph nodes [full details of these data will be published separately (J.Delabie, in preparation)]. Restin was not detected in the surrounding normal tissue, inflammatory cells or in a number of other tumoral conditions, which otherwise resemble Hodgkin's disease histologically, underlining the value of restin as a diagnostic marker for Hodgkin's disease. To confirm and extend these findings, a Hodgkin's disease-derived cell line, HS445, was stained with the polyclonal anti-restin antibodies; strong cytoplasmic staining was seen, indicating that restin expression is indeed linked to Hodgkin's disease (Figure 8).

We next investigated the association of restin with the cytoskeleton in cultured monocytes and the HS445 cell line. It has been well documented that IF proteins are enriched in Triton X-100-insoluble fractions of cell lysates (Hynes and Destree, 1978; Franke *et al.*, 1979). This property has been exploited in the purification of a number of IFs and

Table I. Align scores for the IF homology domain of restin compared with members of the previously described IF protein subtypes

	HK1	MK2	GFA	RNF	CLB2	NES	RESTIN
HK1	/	8	19	22	12	9	3
MK2		/	29	21	10	8	5
GFA			/	40	14	15	5
RNF				/	11	12	4
CLB2					/	7	4
NES						/	4
RESTIN							/

The intermediate filaments were as described in Figure 3. Align scores were generated using the align program of the Protein Identification Resource.

Fig. 4. Homology between the serine-rich motif of restin and types III and IV IF proteins. The sequences were manually aligned. Mvim, mouse vimentin (Wood *et al.*, 1989); Rnf, rat middle molecular weight neurofilament protein (Napolitano *et al.*, 1987); Hvim, human vimentin (Ferrari *et al.*, 1986); Mnf, mouse middle molecular weight neurofilament protein (Levy *et al.*, 1987); Nex, α -internexin (Fliegener *et al.*, 1990); Hnf, human middle molecular weight neurofilament protein (Myers *et al.*, 1987); Per, rat peripherin (Thompson and Ziff, 1989); con, consensus sequence. Identities at four or more positions are boxed.

A

B

C

Fig. 5. Intracellular localization of restin and vimentin in CHO cells transformed with the restin cDNA using indirect double-label immunofluorescence. (A), untreated cells double-stained for restin (green) and vimentin (red). (B), cytochalasin B-treated cells double stained as in A. (C), colcemid-treated cells double stained as in A (magnification $\times 450$).

IFAPs. Triton-insoluble fractions of cultured monocytes and HS445 cells were prepared and analysed by Western blotting. As controls, the distribution of actin and vimentin were also determined. As expected, both vimentin and actin were enriched in the Triton X-100-insoluble fraction (Hynes and Destree, 1978; White *et al.*, 1982; Figure 9). The anti-restin

monoclonal antibody identified a single band of ~ 160 kDa molecular weight (thus confirming the predicted size from the cDNA; Figure 9) although restin was found predominantly in the Triton X-100-soluble fraction. Thus restin did not remain associated with IFs in the presence of non-ionic detergent and high salt concentrations. Longer

Fig. 6. Intracellular localization of restin and vimentin in 8 day *in vitro* cultured human monocytes by indirect double-label immunofluorescence. Monocytes were stained with polyclonal anti-restin (**left**) and monoclonal anti-vimentin antibodies (**right**) followed by fluorescein-conjugated donkey anti-rabbit IgG and Texas red labelled sheep anti-mouse IgG (magnification $\times 720$).

development of Western blots with the colour reagent demonstrated the presence of a minor quantity of restin in the Triton X-100-insoluble fraction (Figure 9). Restin was also very susceptible to proteolysis in these extracts unless a mixture of protease inhibitors were included in the extraction buffer and there was much less restin than vimentin. Hence these results suggest that restin is not an integral part of the IF network and is probably not abundant enough in non-transformed cells to form an IF-type network. Moreover, these results indicate, together with the immunocytochemistry data, that restin is not tightly associated with the IF network. Based upon this evidence and the amino acid sequence homologies we conclude that restin probably represents a new type of IFAP.

Discussion

Structural features

We have cloned a cDNA encoding a protein of 160 kDa which is highly expressed in the Reed–Sternberg cells of Hodgkin's disease. Secondary structure analysis of the encoded protein predicts a region of high α -helical content containing a rod domain with some structural features exhibited by the IF protein family. Immunocytochemistry indicates that restin is associated with the cytoskeleton but is solubilized by buffers containing non-ionic detergent. These observations strongly suggest that this polypeptide is not an IF protein but rather an IFAP.

The α -helical rod domain of restin exhibits low but significant homology to the conserved rod domain of other IF proteins. In IF proteins this domain is composed of two α -helical coil regions, the precise nature of which is defined by a heptad repeat (Steinert and Roop, 1988). Hypervariable N- and C-terminal non-helical domains specific to the functions of individual IF proteins reside outside the central rod domain. The regions of highest homology between restin and the IF coil domain are restricted to coil 1A and the ends of coils 1B and 2B with a high number of conservative amino acid changes between restin and the other groups of IFs. The coil region of restin lacks helix-breaking proline residues and differs in some respects from the canonical features described by Steinert and Roop (1988). Coil 1A of restin

Fig. 7. Reed–Sternberg cells in a case of Hodgkin's disease (nodular sclerosing subtype). Restin is expressed in the cytoplasm of Reed–Sternberg cells. Lymphocytes and other inflammatory cells in the background do not stain (magnification: $\times 100$; inset: $\times 475$).

contains four-heptad repeats which are maintained into linker L1 and continue to the end of coil 1B. This pattern is reminiscent of the structure described for the human medium neurofilament protein (Myers *et al.*, 1987). Coil 2 of restin maintains the heptad repeat pattern and is similar to the same region in nestin (Lendahl *et al.*, 1990) in that it has a very short L2 linker. The alignment scores (Table I) suggest that restin is distantly related to the IF protein family. However, restin differs significantly from IF proteins in having a deletion of five amino acid residues near the beginning of coil 2B and also lacks the penultimate heptad repeat which is common to all other IF proteins, although the heptad repeat structure and the α -helical nature is maintained outside the region of homology. Albers and Fuchs (1989) have elegantly demonstrated that even small deletions in the rod domain inhibit filament formation, i.e. an intact rod domain is essential for filament formation. This feature of the restin rod domain probably excludes restin from being considered as an IF protein, but it is still possible that restin interacts with the IF network via its rod domain. Additionally, the IF homology domain of restin is positioned within the C-

Fig. 8. Immunostaining of restin in a Hodgkin's disease-derived cell line HS445. Normal rabbit IgG (**upper panel**) and polyclonal anti-restin antibodies (**lower panel**) followed by alkaline phosphatase conjugated donkey anti-rabbit IgG (magnification $\times 420$).

terminal part of a region of high α -helical content extending over ~ 800 amino acids. Such a large coil region is unusual for IF proteins, although the human medium neurofilament protein is also predicted to contain extended α -helical regions (Myers *et al.*, 1987).

Outside the central coil region are distinct N- and C-terminal non-helical domains. The N-terminal domain has an overall basic character, is rich in glycine, proline, serine and threonine residues and could assume a very flexible structure. All of these properties are exhibited by types III and IV IF proteins (Myers *et al.*, 1987; Conway and Parry, 1988). In addition, restin contains a serine-rich, 22 residue motif located in the N-terminal domain which is also conserved among some types III and IV IF proteins (Figure 4). This serine-rich motif may have an important function in the interaction of the N-terminal domain with other cytoskeletal components, as has been demonstrated for vimentin and desmin. Both vimentin and desmin are known to interact specifically with ankyrin of erythrocyte plasma membranes via their N-terminal head domains (Georgatos and Marchesi, 1985; Georgatos *et al.*, 1985; Georgatos and Blobel, 1987). The serine-rich motif may be essential for this function. Most of the potential phosphorylation sites for restin are clustered in the N-terminal region surrounding the serine-rich motif which may be a hot spot for kinase action. Phosphorylation of IFs (reviewed by Robson, 1989) and the IFAP plectin (Wiche *et al.*, 1991) are important events in the reorganization of the cytoskeleton. In contrast, the C-

Fig. 9. Immunoblotting of cells extracted with Triton X-100. Triton X-100-soluble (S) and Triton X-100-insoluble (C) proteins from the Hodgkin's disease cell line HS445 (**A**) and human 8 day *in vitro* cultured human monocytes (**B**) were resolved by SDS-PAGE and transferred to Immobilon PVDF membranes and then incubated with (1) monoclonal anti-vimentin, (2) monoclonal anti-restin or (3) polyclonal anti-actin antibodies.

terminal domain is rich in charged residues and overall is negatively charged. Thus, the overall architecture of the restin protein with its central rod domain, basic N-terminus and acidic C-terminus is similar to that of other IF proteins (see Steinert and Roop, 1988).

Immunochemical properties

To test the hypothesis that restin may be an IF protein, the restin cDNA was transformed into CHO cells. Over-expression of restin in this way demonstrated that it could form filamentous structures. Intermediate filaments exhibit a characteristic pattern of staining typified by pronounced perinuclear staining with a wave-like pattern of filaments extending towards, and sometimes underlying, the plasma membrane (Hynes and Destree, 1978; Franke *et al.*, 1978b). The intracellular distribution of restin in CHO cells parallels that of vimentin and presents a pattern typical of IF proteins. Treatment with the drugs colcemid and cytochalasin B demonstrated that restin is probably not associated with either microtubules or microfilaments. To analyse the intracellular staining pattern of restin in non-transformed cells, 8 day old *in vitro* cultured peripheral blood monocytes were stained with the anti-restin antibodies and were shown to have a pronounced punctate pattern which on close examination appeared to follow the pattern for vimentin in these cells. Further biochemical analysis revealed that restin did not partition into the Triton X-100-insoluble fraction as found for all IF proteins and most IFAPs (Steinert and Roop, 1988; Foisner and Wiche, 1991). It has been well documented that IF proteins (Hynes and Destree, 1978; Franke *et al.*, 1979) and a number of IFAPs are enriched in Triton X-100-insoluble fractions of cell lysates (Foisner and Wiche, 1991).

Utilizing such an approach we showed that restin, in contrast to actin and vimentin, was enriched in the Triton-soluble fraction. This observation indicates that restin is only associated with the cytoskeleton.

The central rod structure and globular end domains of restin are features of IFs and IFAPs. For the reasons described above, restin is probably not a member of the IF family although it does have a number of IF-like features. However, the immunostaining pattern and solubilization properties in Triton X-100 suggest that, like the IFAPs, restin is only associated with the IF network. The IFAPs have been divided into four different size classes (Steinert and Roop, 1988). Although no significant homologies have been found between restin and previously described IFAPs (Foisner and Wiche, 1991), the enrichment of restin in the Triton X-100-soluble fraction of cell extracts is a property shared with gyronemin, an IFAP of 240 kDa which is localized along vimentin networks in a periodic manner and has a punctate immunostaining pattern (Brown and Binder, 1990). Taken together, the intracellular staining pattern of restin, its solubilization by Triton X-100/high salt buffers and relative abundance, as compared with vimentin, on Western blots strongly suggest that restin is a new type of IFAP which may associate with the IF network via its rod domain.

Restin and Hodgkin's disease

Recent data have altered the classical view of the IF proteins as static moieties serving a purely mechanical role in the maintenance of cell shape and preservation of the physical integrity of the cytoskeleton (Lazarides, 1980). IF proteins are now considered not only to be carefully regulated during cell differentiation, but even to be instrumental in ensuring the correct direction of development as observed for cells of the neuronal lineage (Steinert and Roop, 1990). Thus, IF proteins are important markers of cell lineage and differentiation state and, moreover, are useful in the analysis of tumour cells which are known to retain and express the same IF complement as their parent cells (Osborn and Weber, 1983; Miettinen *et al.*, 1984; Bolen and McNutt, 1987). Little is known about the involvement of IFAPs in the development of disease, although they have been implicated in keratinizing disorders of hair (Raphael *et al.*, 1984) and skin (Dale *et al.*, 1987).

It has long been recognized that during the process of differentiation to the macrophage phenotype, monocytes undergo a number of changes in gene expression, including structural reorganization of the cytoskeleton. Extensive cytoskeletal rearrangement is correlated with the monocyte/macrophage transition and is characterized, in the case of vimentin, by a change from a perinuclear ring or juxtanuclear mass in the monocyte to a distinct fibrillar network in the differentiating macrophage (Lehto *et al.*, 1982). Reorganization of the cytoskeletal framework undoubtedly plays a role in the specialized functions of the macrophage. Hass *et al.* (1990) have shown that U937 cells, when induced to differentiate to macrophages by TPA, undergo surface attachment and alterations in cell surface antigens as well as enhanced expression of vimentin, lamin A and lamin C, suggesting a functional relationship between the expression of these IF proteins and the differentiation process. Interestingly, a gene involved in the progression of colon tumours from highly proliferative lesions to adenomas, mutated in colorectal cancer (MCC; Kinzler *et al.*, 1991), has been cloned and shown to code for a

predicted protein with globular end domains and central coiled coil regions similar in size to nuclear lamins (McKeon *et al.*, 1986; Bourne, 1991a). A near neighbour gene on chromosome 5q21, adenomatous polyposis coli (APC; Groden *et al.*, 1991; Joslyn *et al.*, 1991; Nishisho *et al.*, 1991) is also involved in the appearance of sporadic colorectal tumours. APC codes for a large protein containing a domain with a coiled coil structure which is postulated to form complexes with other proteins such as MCC (Bourne, 1991b). Bourne (1991a,b) speculated that genetic disruption of the possible cytoskeletal structures formed by MCC and/or APC and other proteins might somehow derepress a mitogenic signal due to the influence of MCC and APC on cell shape. It is of particular interest that restin expression is restricted to the Reed–Sternberg cell of Hodgkin's disease since the origin and nature of this enigmatic tumour cell and the disease to which it is so characteristic is still debated (Jones, 1987). To date, no other IF protein or IFAP has been shown to be expressed at significant levels in Reed–Sternberg cells, except for vimentin in some cases of Hodgkin's disease (Carbone *et al.*, 1990). It is interesting to speculate that in a manner similar to MCC, overexpression of restin and alteration of the cytoskeletal architecture may contribute to the Reed–Sternberg cell phenotype. How restin usurps the function of IF proteins expressed in the cells of normal lymph nodes and sustains the abnormal Reed–Sternberg cell phenotype is a subject for further study.

Materials and methods

cDNA cloning

Commercial cDNA libraries of the human U937 histiocytic lymphoma cell line and linoleic acid-induced mouse peritoneal macrophages in the vector λ gt11 (Clontech) were screened with a monocyte/macrophage reactive antibody, 1C5 (Burmeister *et al.*, 1986), using standard techniques. A partial clone from each library was identified, sequenced and found to encode the final 2000 bp of restin and 500 bp of the 3' non-coding region. A full length clone was isolated from a concanavalin A-stimulated human peripheral blood mononuclear leukocyte cDNA library constructed in λ gt11 as described (Huynh *et al.*, 1985). Several independently isolated clones were subcloned into pBluescript (Stratagene) or pUCKO (Odink *et al.*, 1987) and sequenced on both strands using standard techniques (Sambrook *et al.*, 1989).

RNA analysis

RNA was isolated by homogenization of cells in 4 M guanidinium thiocyanate, 0.5% sarkosyl, 0.1 M mercaptoethanol, 25 mM sodium citrate pH 7 and acid/phenol extraction (Chomczynski and Sacchi, 1987). Total RNA samples were electrophoresed through 0.8% agarose–formaldehyde gels and blotted onto Zetaprobe membranes (Bio-Rad) according to the protocol of the manufacturer. Hybridizations were performed using the entire coding region of the restin cDNA in 40% formamide, $5\times$ SSC, 1% SDS, 200 μ g/ml salmon sperm DNA, 50 mM sodium phosphate buffer pH 7.0 for 16 h at 42°C. All washes were in $0.2\times$ SSC/1% SDS at 60°C. Control hybridization for the amount of RNA was carried out by probing a constitutively expressed mRNA, the eukaryotic protein synthesis initiation factor gene *eIF-4A* (Nielsen *et al.*, 1985).

Antibody production and purification

A cDNA insert coding for the C-terminal 70 kDa (p70) of restin was subcloned into the vector pPLmu-bio (Biogen) under the control of the λ PL promoter. p70 peptide was isolated following temperature shift of the *Escherichia coli* culture and lysis in 8 M guanidinium hydrochloride. After dialysis, the cleared extracts were chromatographed on a DEAE–Trisacryl M column, concentrated by ultrafiltration and purified by reversed phase HPLC in acetonitrile/TFA/H₂O. Purified p70 was used to produce rabbit polyclonal antibodies. Anti-p70 (restin) antibodies were purified by precipitation with ammonium sulphate and the specific anti-restin IgG was isolated by immunoaffinity chromatography using p70 coupled to Affigel-10 (Bio-Rad).

Monoclonal antibodies to p70 were raised according to established protocols (Köhler and Milstein, 1975; Brügger and Sorg, 1983). Four

hybridomas were identified, recloned (mAbs 5-3E7,d7, 16-2F4, 16-2E6, 16-3E2; subclass: IgG1- κ) and used to produce large quantities of monoclonal antibodies. The antibodies were affinity-purified from supernatant using protein A-agarose (Bio-Rad). The four monoclonal antibodies showed an identical immunohistochemical staining pattern to the affinity-purified polyclonal anti-restin antibodies.

Restin expression in permanently transformed cells

To generate a cell line permanently expressing the restin protein, a cDNA fragment containing the entire coding region was cloned in between the promoter of the major immediate-early gene of murine cytomegalovirus (nucleotides -490/+39; Dorsch-Häsler et al., 1985) and a *Bam*HI-*Sal*I fragment of pU β (Weber and Schaffner, 1985) containing the second intron and polyadenylation site of the rabbit β -globin gene inserted clockwise in between the *Hind*III and *Sal*I sites of the plasmid pSV0d (Mellon et al., 1981). This plasmid was cotransfected with pND2 in dihydrofolate reductase-minus CHO cells and amplified by selection in increasing concentrations of methotrexate (Sigma) as described previously (Asselbergs et al., 1986). High producer clones of restin were identified by immunostaining and Western blotting with anti-p70 antibodies according to established procedures (Suter et al., 1983; Towbin et al., 1979).

Immunostaining and cytology

Double-label immunofluorescence experiments with restin and vimentin antibodies were essentially as described by Lendahl et al. (1990) with the following modifications. A CHO clone producing high amounts of restin was grown to confluency in Lab-Tek tissue culture 8-chamber slides (Miles), rinsed in PBS (without Ca^{2+} and Mg^{2+}), presoaked in microtubule stabilizing buffer (MTSB: 2 M glycerol, 1 mM MgSO_4 , 2 mM EGTA, 0.1 M PIPES) for 2 min and then fixed for 5 min in 4% paraformaldehyde (EM Sciences), 0.5% NP40 in MTSB for 5 min. After rinsing with PBS-Tween 20 (0.05% v/v) and a 30 min incubation with blocking solution (BS: 20% horse serum, 0.05% Tween 20 in PBS) the cells were incubated with anti-restin polyclonal antibodies (4 $\mu\text{g}/\text{ml}$) and monoclonal mouse anti-vimentin (Dako 1:5) in BS for 30 min at 37°C. To visualize microfilaments or microtubules, the cells were incubated with 0.2 mM rhodamine-conjugated phalloidin (Molecular probes) or with monoclonal anti- β -tubulin antibodies (Amersham; 1:200) respectively. The cells were then treated with species-specific Texas red-labelled sheep anti-mouse IgG and fluorescein-labelled donkey anti-rabbit IgG (both diluted 1:25 in BS; Amersham) for 30 min at 37°C. After rinsing with PBS and mounting, the cells were examined on a Zeiss Universal microscope using epifluorescence optics and photographed (Kodak Ektachrome 400 ASA). In the cytoskeleton disruption experiments, the transformed cells were grown as above and treated with either colcemid (Gibco) at 0.6 $\mu\text{g}/\text{ml}$ for 4 h or with cytochalasin B (Calbiochem) at 4.8 $\mu\text{g}/\text{ml}$ for 2 h followed by fixation and staining as described (Botteri et al., 1990). Human peripheral blood monocytes were cultured as described for up to 8 days *in vitro* on Lab-Tek multi-chamber slides, then fixed and processed for immunofluorescent staining as above except that the FITC conjugate was diluted 1:10. Immunostaining of Hodgkin's disease derived cell lines was performed with polyclonal anti-restin antibodies (1 $\mu\text{g}/\text{ml}$) on fixed cells using a modified single cell assay procedure (Brüggen and Sorg, 1983; Suter et al., 1983). Nuclei were stained with haematoxylin.

Hodgkin's disease tissue samples (nodular sclerosing subtype) were prepared as 4 μm cryostat sections. The polyclonal anti-restin antibodies were used at a concentration of 5 $\mu\text{g}/\text{ml}$. For immunodetection, an alkaline phosphatase ABC complex (Dako) was used. The colour reaction was developed with fast red and naphthol-ASMX-phosphate (Dako).

Cytoskeletal protein preparation and Western blotting

Cytoskeletal proteins were isolated as an insoluble protein complex in 1% Triton X-100 (White et al., 1982). Cells were washed twice with PBS and pelleted. An equal volume of 2 $\times$ lysis buffer (2% Triton X-100, 20 mM KCl, 280 mM NaCl 20 mM EGTA, 20 mM Tris-HCl pH 7.6 containing the following protease inhibitors; 20 mM benzamide HCl, 100 mM *e*-aminocaproic acid, 40 mM iodoacetamide, 2 mg/ml leupeptin, 400 mg/ml aprotinin, 400 mg/ml soybean trypsin inhibitor and 2 mM phenylmethylsulphonylfluoride) was added and the cells homogenized. Samples were incubated for 10 min on ice, then centrifuged for 10 min in an Eppendorf microfuge at 4°C. The supernatant was transferred to a new tube and SDS and DTT added to final concentrations of 5% and 100 mM respectively, then heated at 95°C for 10 min. The pellet was resuspended in 500 μl solubilization buffer (10% SDS, 50 mM DTT, 20% glycerol, 62.5 mM Tris-HCl pH 6.8) by heating at 95°C for 15 min. Protein concentrations were determined by the method of Lowry et al. (1951). Each lysate supernatant (50 μg) was loaded onto a 7.5% SDS gel, electrophoresed and transferred to Immobilon PVDF membranes (Millipore) with a Milliblot

SDE transfer system (Millipore) following standard procedures (Towbin et al., 1979). After transfer the membranes were incubated for 15 min in TBS pH 7.5, 5% dried milk powder at room temperature and then 12–18 h at 4°C with monoclonal anti-restin antibody at a concentration of 5 $\mu\text{g}/\text{ml}$ in the same buffer. Following three 15 min washes in TBS pH 7.5, 0.05% Tween 20, membranes were incubated for 2 h at 4°C with either an affinity-purified goat anti-rabbit IgG alkaline phosphatase conjugate or goat anti-mouse IgG alkaline phosphatase conjugate (Bio-Rad) diluted 1:3000 in TBS pH 7.5, 5% dried milk powder. Membranes were then washed three times in TBS pH 7.5, 0.05% Tween 20 and once in 150 mM NaCl followed by development in 100 mM Tris pH 9.5, 100 mM NaCl, 100 mM MgCl_2 , 150 $\mu\text{g}/\text{ml}$ nitroblue tetrazolium, 75 $\mu\text{g}/\text{ml}$ 5-bromo-4-chloro-3-indolyl phosphate at room temperature.

Cell culture techniques

Human peripheral blood monocytes were obtained by elutriation of blood samples from healthy volunteers and cultured in DMEM (4.5 mg/ml glucose; Gibco) with 10% human male AB serum (Sigma) at a density of $3 \times 10^5/\text{cm}^2$. Medium was changed every 3 days. Hodgkin's disease derived cell line, HS445 (ATCC no. HTB146) was cultured in RPMI medium 1640 supplemented with 10% (v/v) fetal calf serum, glutamine, penicillin-streptomycin and grown to subconfluency before use. The DHFR-deficient CHO cell line was maintained and propagated as described by Urlaub and Chasin (1980) in α medium supplemented with nucleosides and 5% (v/v) fetal calf serum. Cells were cloned by limiting dilution plating in selective medium.

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