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Fungal fimbriae are composed of collagen

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Fungal fimbriae are surface appendages that were first described on the haploid cells of the smut fungus, *Microbotryum violaceum*. They are long (1–20 μm), narrow (7 nm) flexuous structures that have been implicated in cellular functions such as mating and pathogenesis. Since the initial description, numerous fungi from all five phyla have been shown to produce fimbriae on their extracellular surfaces. The present study analyses the protein component of *M.violaceum* fimbriae. The N-terminus and three internal amino acid sequences were determined. All four show a strong similarity to sequences which are characteristic of the collagen gene family. Enzymatic digests and immunochemical analyses support this finding. Based on these results, it is suggested that the proteinaceous subunits of fimbriae should be termed fungal collagens. Previously, collagen has been found only among members of the kingdom Animalia where it is the principal component of the animal extracellular matrix and is the most abundant animal protein. The unexpected finding of collagen in the members of the Mycota suggests that it may have evolved from a common ancestor that existed before the divergence of fungi and animals. Further, native fungal fimbriae can function as a mammalian extracellular matrix component. They can act as a substratum which permits animal cells to adhere, spread, and proliferate in a manner similar to animal collagens. The implications of this finding to both phylogeny and pathology are discussed.

Keywords: collagen/fimbriae/fungus/*Microbotryum*/*Ustilago*

Introduction

Cell-to-cell interactions are fundamental to the processes of fungal growth and development. In particular, cell-to-cell adhesions occur during mating and pathogenesis. The outermost fungal cell wall surface functions as an interface during both of these interactions, and thus it must contain key factors involved in cell-to-cell communication.

Many fungi produce flexible, long (1–20 μm), narrow (7 nm), unbranched appendages which appear similar to pili or fimbriae found on the surface of prokaryotic cells. These structures, termed fungal fimbriae, were first observed on the surface of haploid yeast-like cells of the anther smut *Microbotryum violaceum* (= *Ustilago violacea*) by Poon and Day (1974). Since their original description, fungal fimbriae have been shown to be widespread among the Mycota (Gardiner *et al.*, 1981, 1982; Benhamou and Ouellette, 1987; Castle *et al.*, 1992; Rghei *et al.*, 1992; Celerin *et al.*, 1995). In addition, it has been shown that at least in some fungi more than one type of fimbriae occur on the cell surface (Xu and Day, 1992).

In *M.violaceum*, fungal fimbriae appear to be involved in cell-to-cell communication during mating before pathogenesis. Both mechanical and enzymatic removal of fimbriae from the haploid cells delays mating until fimbrial regeneration occurs (Poon and Day, 1975). In addition, mating is almost completely blocked by coating fimbriae with anti-fimbrial protein antiserum (Castle *et al.*, 1996). Both of these studies establish that native, attached fimbriae are required during the early events in mating (i.e. formation of conjugation tubes) between compatible $\mathbf{a}_1$ and $\mathbf{a}_2$ mating types. Since only haploid cells with fimbriae will mate and a diploid mycelium is a requirement for parasitic growth in a host plant, mating is essential for pathogenesis.

Fungal fimbriae have also been implicated as factors involved in pathogenic adhesion. Rghei *et al.* (1992) suggested that fimbriae are used in the initial interactions between a parasitic fungus and its host. Yu *et al.* (1994b) found that fimbriae from *Candida albicans* can mediate the adhesion observed between the fungus and receptors on human buccal epithelial cells. Combined, the mating and pathogenic adhesion studies indicate that fimbriae are multifarious.

Earlier studies of the fimbriae from *M.violaceum* revealed that they self-assemble solely from 74 kDa glycoproteinaceous subunits (Gardiner and Day, 1985; Castle *et al.*, 1992). Recently, Celerin *et al.* (1994) used isopycnic gradient centrifugation to purify fimbriae to homogeneity. The latter study showed that fungal fimbriae contain a nucleic acid component, fimbrial-RNA (f-RNA), in addition to the glycoproteinaceous subunits. Nonetheless, no studies have described extensively the protein component of fungal fimbriae. The present work presents an analysis of the fimbrial aglycone from *M.violaceum* and examines the phylogenetic implications of the fimbrial proteins similarity to a known protein family, the collagens.

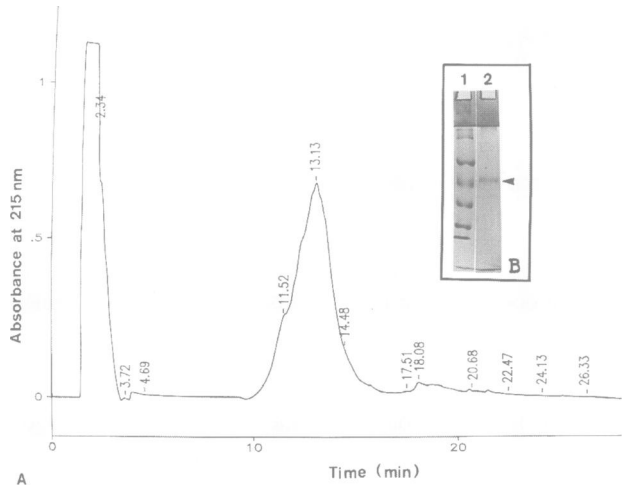

Fig. 1. Analysis by RP–HPLC and SDS–PAGE of the reduced fimbrial aglycone. (**A**) The RP–HPLC chromatogram of deglycosylated and irreversibly reduced fimbrial polypeptide. A prominent peak is evident at 13.13 min. (**B**) Material in the 13.13 min peak was collected and analyzed further by SDS–PAGE and N-terminal sequencing (Table I). The SDS–PAGE was stained with Coomassie blue R-250. Lane 1, protein standards (Bio Rad; 97, 68, 43, 32, 21 and 14 kDa); lane 2, contents of 13.13-min RP–HPLC peak.

Results

Purification and amino acid sequence of fimbrial protein

The fimbrial polypeptide was purified by reverse-phase high-pressure liquid chromatography (RP–HPLC). The chromatogram showed a single peak (Figure 1A) which contained material with an absorption spectrum consistent with proteins. The protein peak was collected and analyzed by SDS–PAGE (Figure 1B). In addition, the fimbrial oligopeptides from endopeptidase Lys C digests were separated by RP–HPLC and numerous peaks were observed in the chromatogram (Figure 2). Three peptides, represented as peaks marked 14.48, 17.6952 and 26.2672 (Figure 2 and inset) were used for obtaining internal amino acid sequences. The N-terminal and three internal amino acid sequences of fimbrial protein are given in Table I. Residues symbolized by an 'X' are not identifiable by conventional methods. It is also noteworthy that the N-terminal and both internal sequences 2 and 3 contain internal lysine residues, even though the enzyme used to generate the oligopeptides was endoproteinase Lys-C. This enzyme cleaves all lysylpeptide bonds efficiently with the exception of lysylproline (Sakiyama and Masaki, 1994).

The four amino acid sequences were compared using the GCG Wisconsin Sequence Analysis package (Devereux, 1994) to all known protein sequences present in release 31.0 of the Swiss-Prot database. All four showed similarity only to collagen.

In total, 49 residues of the fimbrial protein were determined by Edman degradation. The deglycosylated fimbrial protein has a molecular mass of 47 kDa (Figure 1B and Celerin *et al.*, 1995). Since a 47 kDa protein contains ~392 amino residues (average of 119.78 Da per residue), direct protein sequencing determined ~12.5% of the total amino residues present in the fimbrial aglycone. This indicates that at least 12.5% of the total fimbrial protein is similar to the collagen family of proteins.

Amino acid composition

Table II lists the amino acid composition of cesium chloride-purified fimbriae. Of the total amino residues detected, glycine, proline and hydroxyproline were the most abundant. Glycine comprised 31% of the amino acids, while proline and hydroxyproline comprised 14% and 10%, respectively. These results are consistent with all known collagens, where glycine residues comprise about one-third of the amino acids and proline plus hydroxyproline combined constitute 20% or more of the total residues. Additionally, collagen is composed of Gly-X-Y repeats, where X is often proline, and Y is often hydroxyproline, but can represent any of the amino acids, and is Ala in about one-fourth of the repeats. Thus, in most collagens Ala comprises ~12% of the amino residues; in fungal fimbriae, Ala constitutes >10% of the amino residues. Also, hydroxylysine was detected in fungal fimbriae in quantities similar to those found in type I and III collagens (0.4%). Although only 12.5% of the fimbrial protein has been sequenced directly (described above), the amino acid composition results indicate that the entire fimbrial protein is likely a type of collagen.

In addition, since the aglycone of fimbrial subunit is 47 kDa (based on SDS–PAGE) and since the minimum formula weight of the fimbrial protein is 25 995 Da (based on the amino acid composition), we interpret that there are likely two repeated components per fimbrial subunit.

Collagenase digestions of fimbriae

Bacterial collagenase from *Clostridium histolyticum* degrades the triple helical regions of native collagen, preferentially at the Y-Gly bond in the sequence -Gly-Pro-Y-Gly where Y is a neutral amino acid. The enzyme initiates over 200 cleavages per polypeptide and thus causes extensive degradation of the collagen chain (for a review, see Seifter and Harper, 1971).

Native fimbriae were subjected to digestion using bacterial collagenase. Samples of the digests were collected and analyzed by SDS–PAGE (Figure 3). After 5 h of collagenase digestion, the intensity of the 74 kDa band, the subunit of fimbriae, decreased substantially (Figure 3, lane 4) as compared with samples taken after 15 min (Figure 3, lane 2) and 2 h (Figure 3, lane 3). Conversely, the intensity of the 74 kDa protein remained unchanged when the same conditions were used, but the enzyme was omitted (Figure 3, lane 1). In control experiments, RNase A was unaffected by bacterial collagenase after both 15 min (Figure 3, lane 8) and 5 h (Figure 3, lane 7), indicating that the enzyme did not contain substantial quantities of contaminating proteases. Clearly, fimbriae are substrates for bacterial collagenase, although the rate of cleavage appears slower than that of most collagens. After 5 h, collagens I and III were digested completely with bacterial collagenase (Figure 3, lanes 5 and 6). Interestingly, certain annelid cuticle collagens, which contain small amounts of carbohydrate, are partially resistant. We speculate that the high level of glycosylation detected in fungal fimbriae may be contributing to the slowed digestion by bacterial collagenase.

Unlike bacterial collagenases, which cleave collagen throughout the length of the polypeptide, interstitial collagenase, gelatinase A and gelatinase B cleave collagens at a limited number of sites. Figure 4 shows the results of

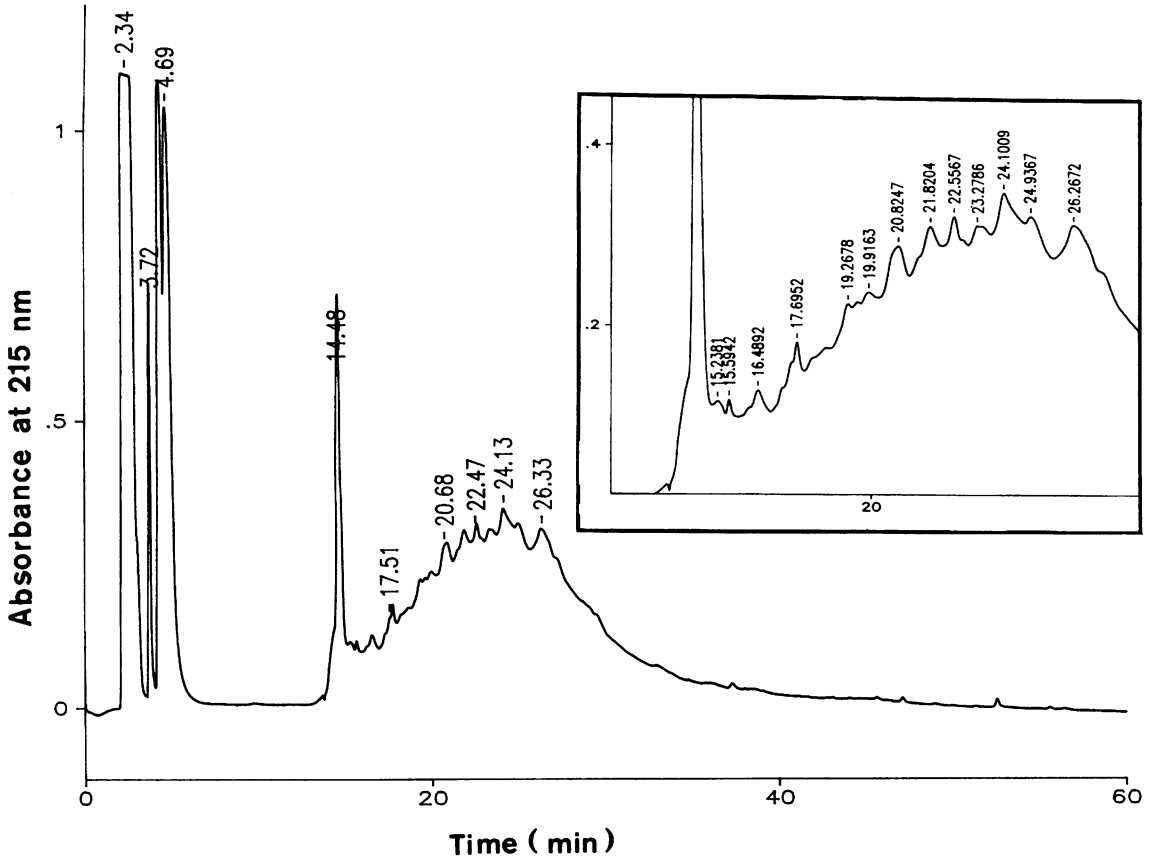

Fig. 2. RP–HPLC analysis of fimbrial oligopeptides. The oligopeptides were generated by digestion of the deglycosylated and irreversibly reduced fimbrial polypeptide with endoproteinase Lys-C. The inset is an enlargement showing more detail of peaks between 12 and 28 min. The individual peptides were separated, collected and three (marked 14.48, 17.6952 and 26.2672 min) were used to obtain internal amino acid sequences (Table I).

the digestion of fimbrial protein using the latter three collagenases. The products were separated by SDS–PAGE, and appreciable quantities of some products are evident.

Fimbrial protein in the native fimbrial conformation was resistant to digestion with gelatinase B and interstitial collagenase as indicated by the seemingly unaffected 74 kDa protein in SDS–PAGEs (Figure 4A and C), even after 20 h of incubation (data not shown). However, fimbrial protein present in native fimbriae was somewhat susceptible to digestion with gelatinase A (Figure 4E). Partial digestion was first detected after 1 h (Figure 4E, lane 4), but the small quantities of product were difficult to visualize. However, the presence of digestion products (61 and 56 kDa) were confirmed using a laser densitometer. A relatively stable product (53 kDa) was also first detected at this time, and eventually it emerged as the only stable product (Figure 4E, lane 1). Nonetheless, the percentage of the protein digested appears to be small, and it may simply represent the amount of partially unfolded protein which is readily susceptible to gelatinase A digestion (Figure 4F).

Partial heat-denatured fimbrial protein, in contrast to native fimbrial protein, was significantly more susceptible to digestion by all three of the collagenases. Figure 4B shows that the digestion of the partially heat-denatured fimbrial protein with gelatinase B began almost immediately following addition of the enzyme. With time, the product from the digestion accumulated, indicating that they are stable.

Table I. Partial amino acid sequences of the fimbrial protein

Sequence location	Amino acid sequence
N-terminus	GFPGLPGPXGE
Internal #1	GEPKPXGA
Internal #2	KVLPGPMGPSGETGP
Internal #3	GFPGLPGXPAEPXGFKGENG

The timed digests of the partially heat-denatured fimbrial protein with interstitial collagenase were also analyzed by SDS–PAGE (Figure 4D). After 4 h of digestion, a considerable quantity of a stable product (62 kDa) was formed.

Finally, the timed digests of partially denatured fimbrial protein with gelatinase A show the most dramatic effect. The digestion of fimbrial protein began as soon as the activated enzyme was added. However, these initial products (70 and 56 kDa) were short-lived (Figure 4F, lane 1). A more stable, prominent product (53 kDa) was evident after 15 min of digestion (Figure 4F, lane 2), and this became the only significant product after 20 h of digestion (Figure 4F, lane 6).

The difference in the susceptibility to the collagenases of native compared with partially heat-denatured fungal fimbriae suggests that there exists a digestion-resistant conformation which is disrupted with mild heating (50°C, 15 min). The native, three-dimensional structure of fimbrial protein is presumably important in its ability to resist

Table II. Amino acid composition of the fimbrial protein

Amino acid	pmol	Mol%	Amino acid ratio	Amino acid composition
Asp/Asn	3856.0	6.368	18.002	32.548
Thr	1293.4	2.136	6.038	10.917
Ser	1883.5	3.110	8.793	15.898
Glu/Gln	5379.4	8.883	25.114	45.407
Pro	8383.2	13.844	39.137	70.762
Gly	18742.8	30.951	87.501	158.206
Ala	6244.0	10.311	29.150	52.705
Val	1365.2	2.254	6.373	11.524
Met	299.5	0.495	1.398	2.528
Ile	807.7	1.334	3.771	6.818
Leu	1091.4	1.802	5.095	9.212
Tyr	214.2	0.354	1.000	1.808
Phe	608.5	1.005	2.841	5.136
His	243.0	0.401	1.134	2.051
Lys	1755.0	2.898	8.193	14.814
Arg	1796.3	2.966	8.386	15.162
HO-Pro	6338.0	10.466	29.589	53.498
HO-Lys	255.6	0.422	1.193	2.157

HO = hydroxy-; minimum formula weight = 25 995.0 Da.

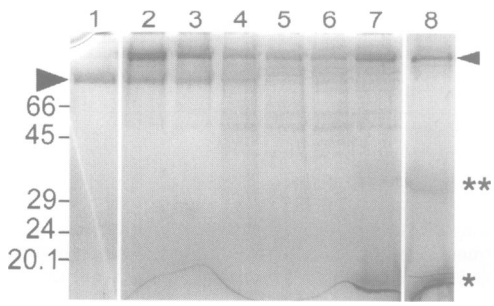

Fig. 3. Analysis by SDS–PAGE of fimbriae digested using bacterial collagenase. Lane 1, fimbriae were incubated for 5 h using digestion conditions, but with bacterial collagenase omitted; lanes 2–4, samples were taken during digestion of fimbriae at 15 min (lane 2), 2 h (lane 3) and 5 h (lane 4); lanes 5 and 6, collagen (type I, lane 5; type III, lane 6) was digested for 5 h with bacterial collagenase; lanes 7 and 8, RNase A was subjected to digestion with bacterial collagenase for 15 min (lane 8) and 5 h (lane 7). Arrowheads indicate location of the 74 kDa fimbrial subunit (large) and the bacterial collagenase (small). RNase A monomer and dimer are indicated by single (*) and double (**) asterisks, respectively.

digestion by the collagenases that are present in the fungus' external environment (Donly and Day, 1984; Iakovleva and Kozel'tsev, 1994). However, it is difficult to discern if the native fimbriae are truly resistant to collagenase digestion or if the digestion is simply retarded and the small quantity of product is not easily detected.

Immunochemical relatedness of fimbriae and collagens

Antibodies, raised against numerous structural proteins, were used in Western blot analyses to ascertain whether fimbrial proteins contain any regions which are epitopically similar. The results of these experiments are summarized in Table III. The only antibodies that detected fimbrial proteins were generated against either fimbriae or collagens. Interestingly, the commercially available antibodies against collagens did not detect fimbrial protein. However, the antibodies provided by H.Kleinman, raised against collagens IV and V, were effective in identifying the

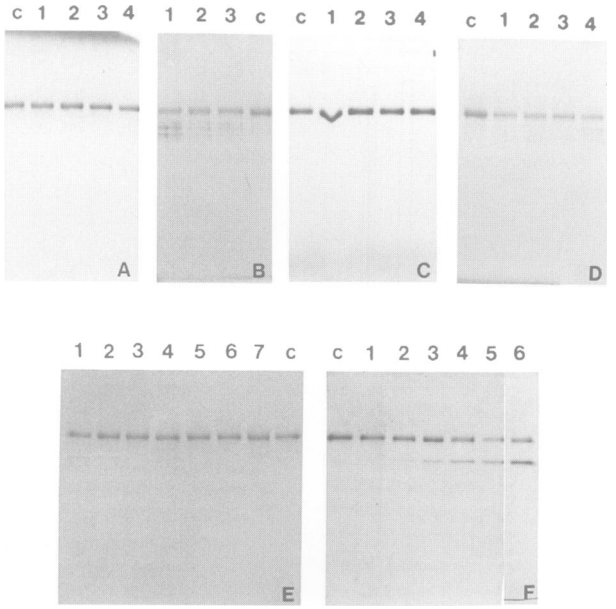

Fig. 4. Analysis by SDS–PAGE of eukaryotic collagenase-digested fimbrial subunits. CsCl-purified fimbriae were subjected to digestion with gelatinase B (**A** and **B**), interstitial collagenase (**C** and **D**), and gelatinase A (**E** and **F**). In (B), (D) and (F), fimbriae were heated briefly before enzymatic digestion. Control lanes (c) contain fimbriae subjected to the same conditions and for maximum period of digestion, but enzyme was omitted in each case. Duration of digestions were as follows: (A): lane 1, 0 h; lane 2, 1 h; lane 3, 2 h; lane 4, 4 h. (B): lane 1, 2 h; lane 2, 1 h; lane 3, 0 h. (C): as (A). (D): as (A). (E): lane 1, 4.5 h; lane 2, 2 h; lane 3, 1.5 h; lane 4, 1 h; lane 5, 0.5 h; lane 6, 0.25 h; lane 7, 0 h. (F): lane 1, 0 h; lane 2, 0.25 h; lane 3, 0.5 h; lane 4, 1 h; lane 5, 1.5 h; lane 6, 4.5 h; lane 7, 20.5 h.

fimbrial protein. The converse experiments, namely using fimbrial-specific antibodies in an attempt to detect various collagens on Western blots, did not show any antigenic relationship.

The antigenic similarity of three collagens (I, III and IV) to fimbriae was analyzed by ELISA using an anti-fimbrial protein antibody (Av-3). The end-point values for the assays were determined to be 1:512, 1:265 and 1:64, respectively. The pre-immune serum was tested at the same time and in all three cases the end-point titer was 1:8. Unlike the results of the Western blot experiments listed in Table III, these ELISAs demonstrate clearly that the fimbrial-specific antibodies are able to detect native collagens. The denatured collagens that were used in the Western blot analyses were not detected with this antibody.

Adhesion assays

Cultured animal cell lines require the presence of specific extracellular matrix (ECM) components for adhesion and proliferation. Adhesion of human melanoma cells to a fibronectin substratum resulted in changes in the cytoarchitecture (Figure 5). These alterations were manifested as spreading cells with numerous peripheral cell attachment sites (Figure 5A). No characteristics of cell adhesion were observed in the absence of fibronectin or fimbriae (Figure 5B). Fimbriae alone were capable of functioning as an ECM for human melanoma cells (Figure 5C–F). Indeed, 100 μg of fimbriae was sufficient to permit the adhesion and spreading of this cell line (Figure 5C). Nevertheless, the interaction was more complex than expected, since

Table III. Immunodetection by Western blot analysis of structural proteins using various antibodies

Antibody	Type	Fimbriae	Actin	Tubulin	Collagen I	Collagen III	Collagen IV	Ovalbumin	BSA
Pv-1[a]	P	–	–	–	–	–	–	–	–
Pv-3[b]	P	–	–	–	–	–	–	–	–
Av-1[a]	P	+	–	–	–	–	–	–	–
Av-3[b]	P	+	–	–	NT	NT	NT	–	–
Anti-actin[c]	P	–	+	–	NT	NT	NT	–	–
Anti-tubulin[c]	M	–	–	+	NT	NT	NT	–	–
Anti-vimentin[d]	M	–	–	NT	NT	NT	NT	–	–
Anti-collagen I,II,III,IV,V[e]	P	–	NT	NT	+	+	+	–	–
Anti-collagen IV[c]	M	–	NT	NT	–	–	+	–	–
Anti-collagen IV[f]	P	+	NT	NT	NT	–	+	–	–
Anti-collagen V[f]	P	+	NT	NT	NT	–	–	–	–

+, detected; –, not detected; NT, not tested; P, polyclonal; M, monoclonal.
[a]Celerin *et al.* (1995).
[b]Present study.
[c]Sigma.
[d]Boehringer Mannheim.
[e]Cedar Lane.
[f]H.Kleinman.

apparently there was an inverse relationship between fimbrial concentration and cell adhesion. Increasing the concentration of fimbriae inhibited cell adhesion. This was evident as successively fewer and fewer cells attached with increasing amounts of fimbriae (Figure 5D–F). This correlation was quantified as the amount of stain taken up by attached cells compared with the amount of fimbriae present as substratum. Figure 6 shows that a non-linear relationship exists between cell adhesion and the quantity of fimbriae used as substratum.

Discussion

Extensive analysis of fungal fimbrial protein has revealed that it exhibits an unexpected similarity to the collagens. This resemblance is evident at the levels of the peptide composition (direct amino acid sequencing, amino acid composition), the epitopic sites present (ELISAs and Western blot analyses), the presence of collagenase recognition motifs (collagenase digests) and the presence of modified residues consistent with hydroxylation (amino acid composition). In combination, these findings demonstrate that fimbriae are members of the collagen family of proteins. Consequently, the protein component of fungal fimbriae will now be referred to as a fungal collagen.

Yamada *et al.* (1980) defined the collagen family of proteins by distinctive common structural properties, including: (i) the presence of glycine residues at every third amino acid; (ii) an abundance of prolines and lysines, many of which are hydroxylated; (iii) the characteristic configuration of the molecule, composed of three subunits which interact with each other to form a triple helical structure; and (iv) the presence of many inter- and intramolecular cross-links which result in a higher-order structural organization. Collagens are initially subdivided into two main classes based on their macromolecular organization, namely fibril-forming and non-fibril-forming collagens. The former are visible as bundles of striated fibers (e.g. fibrils), while the latter are discernible only in the electron microscope as individual narrow filaments, the smallest having a diameter of 8 nm. Additional features are then used to delineate 17 types or classes of collagen

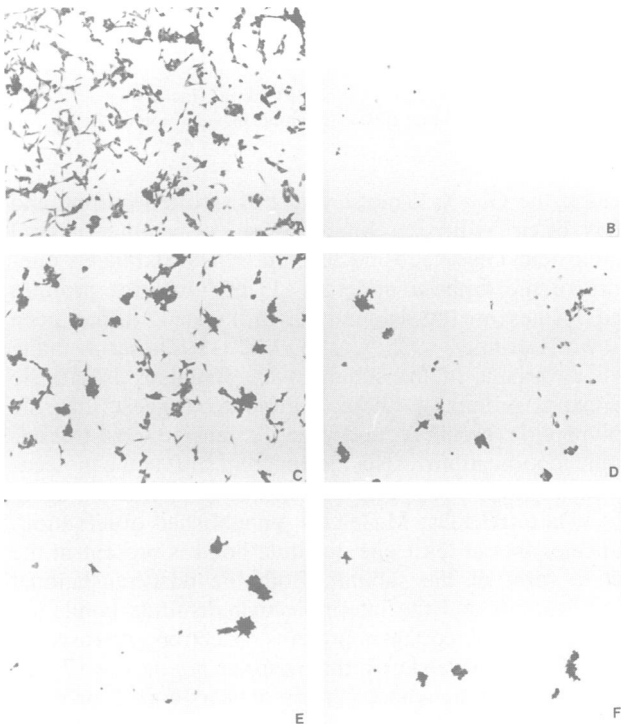

Fig. 5. Adhesion of cultured human melanoma cells to fimbrial collagens. (A–F) Representative photographs of human melanoma cells adhering to either fibronectin or fimbriae of various concentrations. In each plate well assay, 100 µl of the protein suspension was permitted to adhere before the addition of the cultured cells. (**A**) Positive control: fibronectin adhered at 2.0 µg/ml. (**B**) Negative control: no protein. (**C**) Fimbriae adhered at 0.10 µg/ml. (**D**) Fimbriae adhered at 0.25 µg/ml. (**E**) Fimbriae adhered at 0.50 µg/ml. (**F**) Fimbriae adhered at 1.00 µg/ml. (Magnification: ×500.)

(Vuorio and de Crombrugghe, 1990; Li *et al.*, 1993), including sequence homology, subunit molecular weights, length of uninterrupted helical domain, intron–exon structure (Miller and Gay, 1987) and susceptibility to collagenase digestion.

Fungal collagen appears to fulfill the criteria required to be designated a member of the collagen family of proteins. All four of the amino acid sequences obtained

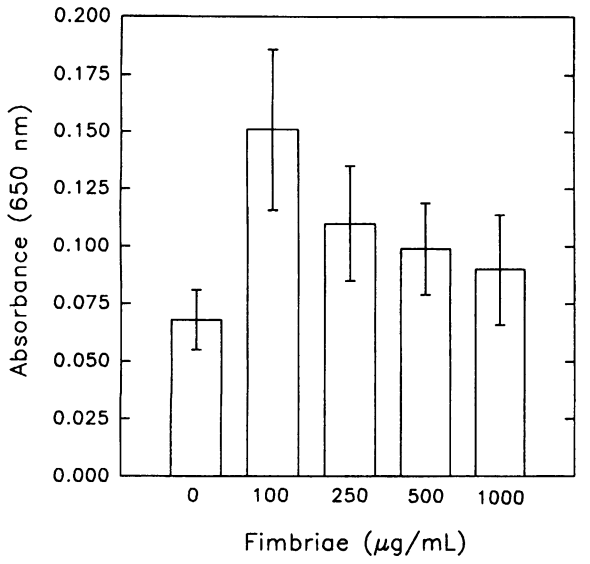

Fig. 6. Quantification of human cell adhesion to fungal fimbriae. Bar graph shows the amount of cultured human melanoma cells adhered as a function of the concentration of fungal fimbriae. Each bar represents the mean of three adhesion trials. The quantity of adhered cells is represented as the relative absorbance at 650 nm. The greatest amount of adhesion is observed at 0.1 μg/ml of fimbriae. Increasing concentrations of fimbriae appear to inhibit the adhesion of the cultured cells.

exhibit the Gly-X-Y motif where X is often proline and Y is often hydroxyproline, alanine, or another neutral amino acid. Glycine residues comprise approximately one-third of the fimbrial molecule. In addition, the prolines and lysines are occasionally hydroxylated. It has been shown (Gardiner, 1985; Castle *et al.*, 1992) that as many as six variants of the subunit were found by isoelectric focusing, prompting these authors to suggest that the polypeptide probably undergoes extensive post-translational modifications. Also, the apparent shift in the molecular mass of the subunit in the absence of a reducing agent (74 kDa to 63 kDa; M.Celerin, unpublished observation) indicates that at least one disulfide bond is present in the native form of the subunit. Both the post-translational modifications and the intermolecular disulfide bonds are consistent with collagen proteins. Electron microscopic examinations have shown that fimbriae are narrow (7 nm) individual strands whose surface appears coiled, or rope-like (Celerin *et al.*, 1994), similar to the non-fibril class of collagens. In addition, fungal collagens are susceptible to digestion by trypsin (M.Celerin, unpublished observation), which is consistent with the non-fibril-forming collagens. However, mammalian collagens have neither N-linked glycosylation nor RNA associated with them, unlike fungal fimbriae (Celerin *et al.*, 1994, 1995). Thus, it appears that fungal collagens fail to fit readily into one of the established 17 collagen types and apparently constitute a novel, as yet undescribed class of collagen proteins.

Cavalier-Smith (1987) used ultrastructural (flattened, non-discoidal cristae, lack of chloroplasts) and biochemical (chitin biosynthesis, glycogen for carbohydrate storage, mitochondrial codon usage) characteristics to propose that animals and fungi are more closely related mutually than either is to plants. More recently, phylogenies based on 16S-like ribosomal RNA sequences (Wainright *et al.*, 1993), amino acid sequences (Bauldauf and Palmer, 1993; Nikoh *et al.*, 1994), and analyses of conserved oligopeptide insertions (Bauldauf and Palmer, 1993) have all confirmed that animals and fungi are indeed sister groups while plants constitute an independent evolutionary lineage {[(A,F),P], A = Animalia, F = Fungi, P = Plantae}.

Until the present data were obtained, a unique protein, limited in its range to organisms from only the two most closely related kingdoms, had yet to be described. The discovery of fungal collagen fills this gap and thereby supports what appears to be the most accurate phylogenetic relationship between plants, animals and fungi described to date.

All true collagenous proteins currently known are found exclusively in the kingdom Animalia (Garrone, 1978; Garrone *et al.*, 1993; Morris, 1993). They have been used alone (Garrone, 1978) or in combination with other ECM components (Morris, 1993) to argue that animals are a monophyletic group. It has been suggested that the ECM is a primitive feature of multicellularity in animals (Garrone *et al.*, 1993; Morris, 1993; Wainright *et al.*, 1993). However, all of these authors acknowledge that the ECM did not arise *de novo* with the first multicellular animal. Thus, it is implied that components of the ECM evolved before the first Metazoan. This study suggests that one of the major components of the animal ECM, namely collagen, is much more primitive than has been documented previously, and probably evolved prior to the divergence of fungi and animals.

ECM components play numerous roles in animal biology including cell-to-cell interactions, cell motility, cell shape changes, chemotaxis and cell adhesion (Reddi, 1984). The ECM components form multiple interactions with each other, as well as with components on the animal cell surface. In many cases, collagens form the scaffolding onto which the other ECM components are assembled. Strong affinities exist between the collagens and other ECM components, including fibronectin, glycosaminoglycans and proteoglycans (Reddi, 1984).

Cell adhesion is dependent on the presence of either an intact ECM or a solid substratum of certain ECM components such as collagen. Cell adhesion can trigger a multitude of biochemical cascades that can result in cell attachment and growth (Kleinman *et al.*, 1981), differentiation (Hauschka and Konigsberg, 1966; Zuk *et al.*, 1989) and cell polarization (Reddi, 1984). *In vitro* studies have shown that certain ECM proteins alone, or in specific combinations, are sufficient to trigger a cascade of events. Current research indicates that each ECM component is involved in numerous and diverse interactions, although the precise role of each *in vivo* has yet to be determined.

Fimbriae are found throughout the Mycota (Gardiner *et al.*, 1981, 1982; Benhamou and Ouellette, 1987; Castle *et al.*, 1992; Rghei *et al.*, 1992; Celerin *et al.*, 1995). Although the function of fungal fimbriae in general is not yet clear, a role in animal mycopathology can be predicted. The present investigation shows that fimbriae can mimic the roles ordinarily reserved for animal ECM proteins. Numerous studies have described ECM components such as fibronectin (Klotz and Smith, 1991), entactin (Lopez-Ribot and Chaffin, 1994), lamenin (Bouchara *et al.*, 1990) and collagen (Ollert *et al.*, 1993; Tsuchida *et al.*, 1995)

interacting with fungal surface components in order to mediate their adhesion (Klotz, 1990; Tronchin *et al.*, 1991; Vazquez-Juarez *et al.*, 1993). Independently, each of these interactions could be important in the adhesion of the fungus to the host tissues. However, Ollert *et al.* (1993) suggested that there exist multiple molecular mechanisms of fungal attachment to cultured cells. Nonetheless, all of these earlier studies suggested that the fungus has a receptor for ECM components. Conversely, the present study is the first to suggest that a potential host organism may recognize, and thus have a receptor for, a familiar structure on a mycopathogen.

It has been demonstrated that several antigenically different fungal fimbriae can occur on a single organism (Xu and Day, 1992). Fimbrial protein from *C.albicans* has been examined extensively (Yu *et al.*, 1994a,b), and the amino acid composition did not show any similarity to collagen. However, this type of fimbriae is not related antigenically to the *M.violaceum* fimbriae described in the present study (Celerin *et al.*, 1995). A second putative fimbrial protein on *C.albicans* has been described that is related antigenically to *M.violaceum* fimbriae (Celerin *et al.*, 1995). Further studies are necessary to characterize other types of fimbriae, in addition to those composed of fungal collagen.

Finally, numerous attempts have been made to isolate the fungal collagen gene (low stringency oligonucleotide hybridizations, PCR, RT–PCR, heterologous collagen probes; data not shown). Although the fungal collagen gene has yet to be isolated, the biochemical evidence for the existence of fungal collagens is substantial and indisputable. The phylogenetic implications of the mere existence of fungal collagens are profound and of major significance to important phylogenetic questions.

Materials and methods

Stock cultures and growth conditions

Stocks of *M.violaceum*, wild-type $\mathbf{a}_1$ and $\mathbf{a}_2$ strains [UWO-1 (a_1) and UWO-1 (a_2), ATCC 22,000 and 22,001; Gardiner *et al.*, 1981] were stored either in liquid nitrogen or in silica at –20°C. Active cultures were maintained on Ustilago complete medium (Day and Jones, 1968), either in liquid culture or on solidified media (1.5% agar) at 22°C.

Fimbrial isolation and purification

Crude fimbriae were isolated from exponential-phase haploid cells grown in well-aerated liquid cultures as previously described (Poon and Day, 1975; Celerin *et al.*, 1994). Fimbriae were purified using CsCl gradients (Celerin *et al.*, 1994). The CsCl gradients generated were transilluminated with visible light and material present in the observed band was collected, dialyzed and examined by electron microscopy.

Electron microscopy of fimbriae

One drop of solution containing dialyzed fimbriae was placed on a formvar-coated copper grid and negatively stained with ammonium molybdate (Poon and Day, 1974). The grids were drained, air-dried and viewed with a Phillips EM 200 electron microscope.

Amino acid sequencing of the fimbrial protein

Purified fimbrial protein was deglycosylated with endoglycosidase F (Celerin *et al.*, 1995), reduced (10 mM dithiothreitol, 50°C, 15 min) and carboxamidomethylated (20 mM iodoacetamide, room temperature, 15 min). The resulting product is referred to as the fimbrial polypeptide. Fimbrial oligopeptides were made by digesting the fimbrial polypeptide (20 mg) with endoproteinase Lys-C (Boehringer Mannheim; 1 mg, 37°C, 15 h) by the method of Stone and Williams (1993). Both the fimbrial polypeptide and oligopeptides were separated by RP–HPLC (Applied Biosystems Microbore), and eluates were monitored at 215 nm. The contents of the peaks were analyzed spectrophotometrically by diode array analysis and those fractions consistent with protein absorption spectra were collected. During both polypeptide and oligopeptide separations, buffer A was 0.1% trifluoroacetic acid (TFA)/0% acetonitrile (AN) in double-distilled water, and buffer B was 0.1% TFA/80% AN in double-distilled water. The runs were 0 to 100% B at flow rates of 1 ml/min. An Aquapore RP-300 (Brownlee) column (60 min run time) was used to purify the polypeptide and a Spheri-5 RP-18 (Brownlee) column (40 min run time) was used to separate the oligopeptides.

Automated amino acid sequencing by Edman degradation was performed on the RP–HPLC-purified polypeptide and three of the fimbrial oligopeptides by J.Lagueux (Service de Séquence des Protéines de l'Est du Québec, Centre de Recherche du CHUL, Ste-Foy, Québec).

Amino acid composition of fimbriae

Fimbriae were purified by CsCl gradient centrifugation (Celerin *et al.*, 1994) and 400 µg of protein were vacuum-dried and hydrolyzed in 6 M HCl with 0.1% methanol at 150°C for 1 h. The vacuum-dried hydrolysate was analyzed using a Beckman 6300 Amino Acid Analyzer by D.McKay (Protein Sequencing Facility, MedBiochem, University of Calgary). Cysteine was not derivatized for the analysis.

Digestion of fimbrial protein with collagenases

Bacterial collagenase from *C.hystolyticum* (chromatographically purified; USB Biochemical, 4000 U) was resuspended in 10 mM $CaCl_2$ and 40 mM Tris–HCl, pH 7.5. 100 µg of two collagens (type I from rat tail, and type III from calf skin; Sigma), RNase A and CsCl-purified fimbriae were each subjected to digestion with bacterial collagenase (10 U in 500 µl of 10 mM $CaCl_2$ and 40 mM Tris–HCl, pH 7.5, 37°C, for up to 5 h). Samples (100 µl) were collected after 15 min, 2 h and 5 h. Samples were solubilized, products were separated on 12% gels using SDS–PAGE and the bands visualized by staining with Coomassie blue G-250 (Neuhoff *et al.*, 1988).

Three human collagenases, gelatinase A (type IV collagenase, 72K GL), gelatinase B (type IV collagenase, 92K GL) and interstitial collagenase (FIB CL) were activated using organomercurials as described previously (Stetler-Stevenson *et al.*, 1989). Zymograms (Novex) were performed as per the manufacturer's instructions and used to confirm that the enzymes were active. In addition, native collagens (1 mg) were digested with activated collagenases (30 ng) and the products analyzed by SDS–PAGE.

Purified fimbriae (4.5 mg) were either pre-treated by heating (55°C, 15 min) and quick-cooling on ice, or not pre-treated. Both pre-treated and non-pre-treated fimbrial proteins were digested with 57 ng of each of the three collagenases for a maximum of 20 h. Aliquots of each digest were taken at various time intervals. Control experiments contained all of the components of the digests, and were subjected to the same conditions, except that the enzymes were omitted. Products were separated by SDS–PAGE (4–20%, Novex) and the bands were visualized by staining with Coomassie blue G-250 (Neuhoff *et al.*, 1988). Gels were analyzed using an LBK Ultrascan XL laser densitometer.

Production of antibodies against fimbrial protein epitopes

A New Zealand White rabbit was used to generate antiserum to fimbrial protein epitopes. Pre-immune serum (Pv-3) was collected and stored as previously described (Celerin *et al.*, 1995). Antibody production was based on the method of Harlow and Lane (1988). Briefly, purified fimbriae were solubilized at 95°C for 10 min in sample buffer (10% SDS, 60 mM Tris–HCl, pH 8, 20% glycerol and 5% β-mercaptoethanol). Fimbrial components were separated by electrophoresis on 10% polyacrylamide gels (Laemmli, 1970). Vertical strips, cut from both sides of the gel, were stained with 0.1% Coomassie blue R-250 (in 40% methanol–10% acetic acid and destained in the same solvent) and re-aligned with the gel. A horizontal strip containing the 74 kDa glycoprotein was cut from the gel, frozen to –80°C and lyophilized (Multi-Dry, FTS Systems Inc.) for 12 h. The desiccated gel strips were ground to a fine powder and a slurry was made by mixing the powder with 2.5 ml milliQ water. The slurry, containing 100 mg of fimbrial protein (based on the Bradford assay, BSA as the standard), was homogenized by repeated passage through an 18-gauge needle attached to a 3 ml syringe. A 1:1 (v/v) emulsification in Freund's incomplete adjuvant (BRL) was made and 0.8 ml of the emulsified antigen was injected subcutaneously (s.c.) into the rabbit. Boosting injections (0.8 ml, s.c.) were given after 35, 57 and 78 days. Exsanguination was performed 106 days after the initial antigen introduction. Serum was separated from cells, labelled Av-3 and stored at –20°C. ELISA end-point titers on both Av-3 and Pv-3 were determined to be 1:16 384 and 1:8 respectively.

Immunochemistry of structural proteins

Fimbrial protein and other structural proteins (listed in Table III) were solubilized, separated by SDS–PAGE and transferred onto nitrocellulose membranes as previously described (Celerin *et al.*, 1995). Membranes were stained with Ponceau S to ensure that proteins had transferred. Western blots were blocked [5% bovine serum albumin with Tween-20 in Tris-buffered saline (B-TTBS)], and probed with various antibodies (Table III) in 1% B-TTBS. Antigenic components were detected by first incubating the primary antibody-labelled blots with secondary antibodies (goat anti-rabbit IgG conjugated to horseradish peroxidase, Sigma; or goat anti-mouse IgG conjugated to horseradish peroxidase, Bio-Rad) followed by detection using chemiluminescence (ECL; Amersham).

In addition, collagen types I (rat tail, Sigma), III (calf skin, Sigma) and IV (mouse, H.Kleinman) were analyzed for the presence of epitopes recognized by fimbrial protein-specific antibodies (Av-3) and compared with that of pre-immune serum (Pv-3). Fungal fimbriae and the three types of collagen were adhered to microtiter plates and ELISAs were performed as described above.

Adhesion assays

Purified fimbriae (0.1–5 μg in 100 μl ddH_2O) or fibronectin (Collaborative Research Inc., 0.2 μg in 100 μl ddH_2O) were permitted to adhere to microtiter wells (Nunc, room temperature, 2 h). Non-specific interactions were blocked (1% BSA, 30 min, room temperature), and excess liquid was removed. Human melanoma cells (A2058; Todaro *et al.*, 1980) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Subconfluent monolayers of cells were treated with trypsin, resuspended in DMEM, counted, and allowed to recover for 1 h. Cells were pelleted and resuspended in DMEM to a concentration of 3×10^5 cells/ml. Resuspended cells (100 μl) were added to each well and incubated at 37°C in a 5% CO_2, humidified chamber. After 5.5 h, the wells were rinsed with phosphate-buffered saline (PBS), and adhered cells were observed microscopically for changes in morphology (from globose to spreading). Cells were fixed and stained with Giemsa (Diff-Quik, Baxter), washed with PBS and photographed. The amount of adhesion was quantified by re-extracting stain from adhered, dried cells (10% methanol, 5 min, room temperature) and read at 650 nm on an ELISA Plate Reader (Bio-Rad). Three replicates per experiment were performed and the results were averaged.

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