INTRODUCTION

The helix-loop-helix (HLH) family of transcription factors comprises >200 members, which have been identified in organisms from yeast to man (reviewed in Littlewood and Evan, 1995; Massari and Murre, 2000). In metazoa, HLH proteins function in the coordinate regulation of gene expression, orchestrating cell cycle control, cell lineage commitment and cell differentiation. An essential role has been established for a number of HLH proteins in the development of haemopoietic, myogenic, pancreatic and neurogenic mammalian cell lineages. Four main groups of HLH protein can be distinguished on the basis of the presence or absence of additional functional domains (Fig. 1). The highly conserved HLH region comprises two amphipathic α helices, each 15-20 residues long, which are separated by a shorter intervening loop that has a more variable length and sequence. The HLH domain primarily mediates homo- or hetero-dimerisation, which is essential for DNA binding and transcriptional regulation. Near all HLH proteins possess a region of highly basic residues adjacent to the HLH domain (Fig. 1), which facilitates binding to DNA containing the canonical ‘E box’ recognition sequence, CANNTG. Some HLH proteins also bind the related ‘N box’ sequence, CACNAG (Massari and Murre, 2000). However, a distinct subfamily of HLH proteins, the ID proteins, lack such a DNA-binding region and instead function solely by dimerisation with other transcriptional regulators, principally those of the bHLH type (Fig. 1). Such ID-bHLH heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins (Benezra et al., 1990; Garrell and Mondolell, 1990; Ellis et al., 1990). Since most bHLH proteins positively regulate sets of genes during cell fate determination and cell differentiation, the term ‘ID’ conveniently alludes to the ability of these proteins to inhibit both DNA binding and differentiation.

SUMMARY

The ubiquitously expressed family of ID helix-loop-helix (HLH) proteins function as dominant negative regulators of basic HLH (bHLH) transcriptional regulators that drive cell lineage commitment and differentiation in metazoa. Recent data from cell line and in vivo studies have implicated the functions of ID proteins in other cellular processes besides negative regulation of cell differentiation. ID proteins play key roles in the regulation of lineage commitment, cell fate decisions and in the timing of differentiation during neurogenesis, lymphopoiesis and neo-vascularisation (angiogenesis). They are essential for embryogenesis and for cell cycle progression, and they function as positive regulators of cell proliferation. ID proteins also possess pro-apoptotic properties in a variety of cell types and function as cooperating or dominant oncoproteins in immortalisation of rodent and human cells and in tumour induction in Id-transgenic mice. In several human tumour types, the expression of ID proteins is deregulated, and loss- and gain-of-function studies implicate ID functions in the regulation of tumour growth, vascularisation, invasiveness and metastasis. More recent biochemical studies have also revealed an emerging ‘molecular promiscuity’ of mammalian ID proteins: they directly interact with and modulate the activities of several other families of transcriptional regulator, besides bHLH proteins.

Key words: Helix-loop-helix protein, Cell growth, Cell differentiation, Cell cycle, Tumorigenesis, Transcription factor

INTRODUCTION

The helix-loop-helix (HLH) family of transcription factors comprises >200 members, which have been identified in organisms from yeast to man (reviewed in Littlewood and Evan, 1995; Massari and Murre, 2000). In metazoa, HLH proteins function in the coordinate regulation of gene expression, orchestrating cell cycle control, cell lineage commitment and cell differentiation. An essential role has been established for a number of HLH proteins in the development of haemopoietic, myogenic, pancreatic and neurogenic mammalian cell lineages. Four main groups of HLH protein can be distinguished on the basis of the presence or absence of additional functional domains (Fig. 1). The highly conserved HLH region comprises two amphipathic α helices, each 15-20 residues long, which are separated by a shorter intervening loop that has a more variable length and sequence. The HLH domain primarily mediates homo- or hetero-dimerisation, which is essential for DNA binding and transcriptional regulation. Nearly all HLH proteins possess a region of highly basic residues adjacent to the HLH domain (Fig. 1), which facilitates binding to DNA containing the canonical ‘E box’ recognition sequence, CANNTG. Some HLH proteins also bind the related ‘N box’ sequence, CACNAG (Massari and Murre, 2000). However, a distinct subfamily of HLH proteins, the ID proteins, lack such a DNA-binding region and instead

function solely by dimerisation with other transcriptional regulators, principally those of the bHLH type (Fig. 1). Such ID-bHLH heterodimers are unable to bind to DNA, and hence ID proteins act as dominant negative regulators of bHLH proteins (Benezra et al., 1990; Garrell and Mondolell, 1990; Ellis et al., 1990). Since most bHLH proteins positively regulate sets of genes during cell fate determination and cell differentiation, the term ‘ID’ conveniently alludes to the ability of these proteins to inhibit both DNA binding and differentiation.

Genes encoding ID proteins have been isolated from several metazoan species, but have been most studied in Drosophila, in which a single Id-like locus, extramacrochaetae (emc), encodes an ID-like protein (Garrell and Mondolell, 1990; Ellis et al., 1990), and in mammals (mouse and man), which possess four Id family members (Id1-Id4) (Benezra et al., 1990; Sun et al., 1991; Christy et al., 1991; Biggs et al., 1992; Riechmann et al., 1994). These proteins range in size from 119 residues (ID3) to 199 residues (EMC). Outside the highly conserved HLH domain, the different ID proteins display extensive sequence divergence. Although ID proteins traditionally have been viewed as negative regulators of cell differentiation, recent work has revealed much wider biological roles for this family of regulatory proteins, which impinge on the fields of developmental biology, cell cycle research and tumour biology (see Table 1). Here, I highlight recent developments in our
understanding of the biological functions and mechanisms of action of ID proteins. Comprehensive reviews of earlier work can be found elsewhere (Norton et al., 1998; Israel et al., 1999).

**ID PROTEINS IN DEVELOPMENTAL BIOLOGY**

**Drosophila**

In *Drosophila*, the *emc* locus is required for many developmental processes; sensory organ development and wing morphogenesis have been most extensively studied (reviewed in Jan and Jan, 1993). Although null mutations in *emc* are embryonic lethal, studies on various partial loss and gain-of-function *emc* mutants have revealed a role for the EMC gene product in regulating cell commitment, differentiation and cell proliferation (Jan and Jan, 1993). The EMC protein heterodimerises with and antagonises the functions of several bHLH proteins, including those encoded by the *achaete-scute* complex genes involved in neurogenesis and sex determination (Garrell and Mondolell, 1990; Ellis et al., 1990; Jan and Jan, 1993). These genes are positively autoregulated by the Achaete and Scute proteins and are negatively regulated by EMC (Martinez et al., 1993). Genetic studies strongly support the notion that a critical balance between the relative levels of EMC protein and its bHLH targets is important in regulating cell fate determination in *Drosophila* (Jan and Jan, 1993).

More recent genetic studies have revealed how EMC is integrated with upstream signalling pathways that regulate cell fate during *Drosophila* wing morphogenesis. Proteins that function in the Ras signalling pathway (Torpedo, Vein, Veinlet and Gap) appear to cooperate with EMC during cell proliferation but antagonise it during differentiation (Baonza and Garcia-Bellido, 1999). Strong genetic interaction has also been found between *emc* and genes that encode different components of the Notch signalling pathway (Baonza et al., 2000). Notch signalling defines an evolutionarily conserved cell-cell interaction mechanism that, throughout development, controls the ability of primitive cells to respond to developmental signals (Artavanis-Tsakonas et al., 1999). Data on genetic complementation between different mutants affecting wing morphogenesis are consistent with regulation of the *emc* gene or protein by Notch and its collaboration with other proteins downstream of Notch to control wing vein differentiation (Baonza et al., 2000). The biochemical mechanisms underlying genetic interaction between *emc* and *Notch* remain to be elucidated. Since both Ras and Notch signalling play pivotal roles in higher organisms, these studies may well provide a paradigm for understanding how ID proteins are integrated with upstream signalling pathways in mammals.

**Mammals**

Embryonic lethality of double-knockout mice

As in flies, *Id* function in mice is essential for development. However, because of the functional redundancy among the four

<table>
<thead>
<tr>
<th>Function</th>
<th>References*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Act as 'global' regulators of lineage commitment and cell fate determination</td>
<td>Ellis et al., 1990; Martinsen and Bronner-Fraser, 1998; Yokota et al., 1999; Lyden et al., 1999; Blom et al., 1999; Jaleco et al., 1999; Rivera et al., 2000; Janatpour et al., 2000</td>
</tr>
<tr>
<td>Required for embryogenesis/organogenesis</td>
<td>Garrell and Mondolell, 1990; Ellis et al., 1990; Yokota et al., 1999; Lyden et al., 1999</td>
</tr>
<tr>
<td>Promote cell growth; arrest cell differentiation</td>
<td>Jen et al., 1992; Iavarone et al., 1994; Sun, 1994; Desprez et al., 1995; Atherton et al., 1996; Kondo and Raff, 2000; Morrow et al., 1999</td>
</tr>
<tr>
<td>Required for cell cycle progression</td>
<td>Barone et al., 1994; Hara et al., 1994; Severali et al., 1994</td>
</tr>
<tr>
<td>Induce apoptosis</td>
<td>Norton and Atherton, 1998; Florio et al., 1998; Kim et al., 1999</td>
</tr>
<tr>
<td>Immortalise primary cells</td>
<td>Norton and Atherton, 1998; Alani et al., 1999</td>
</tr>
<tr>
<td>Function as oncoproteins in vivo</td>
<td>Wice and Gordon, 1998; Kim et al., 1999; Morrow et al., 1999</td>
</tr>
<tr>
<td>Required for angiogenesis in vivo and promote tumour invasiveness</td>
<td>Desprez et al., 1998; Lyden et al., 1999; Lin et al., 2000</td>
</tr>
</tbody>
</table>

*Only selected references are given; for additional references refer to text and to Norton et al., 1998.*
members of the mammalian Id family and their widespread, overlapping expression patterns, only crosses between mice that lack different Id genes are embryonic lethal. All combinations of double knockouts of Id1-Id3 are non-viable and die before birth (R. Benezra, personal communication). However, only the Id1+/ Id3−/ phenotype has so far been reported (Lyden et al., 1999). Early embryos from these mice display aberrant neurogenesis with premature withdrawal of neuroblasts from the cell cycle and inappropriate expression of neural-specific markers. In addition, embryos from Id1−/ Id3−/ mice exhibit vascular malformations and an absence of branching and sprouting of blood vessels into the neuroectoderm. These defects in angiogenesis manifest from aberrant vascular endothelial cell development (Lyden et al., 1999). The apparent restriction of these lesions to the embryonic brain probably reflects the endothelial expression patterns of Id genes. All three Id genes are expressed in endothelia outside of the brain, whereas only Id1 and Id3 are expressed in embryonic endothelial cells in the brain. However, in adult Id1−/ Id3−/ mice, which are viable owing to the presence of a single Id1 allele, the endothelial cell vasculature of tumour xenografts is also defective, and this leads to failure of tumour growth and metastasis (Lyden et al., 1999) (see below). It seems likely that analysis of other combinations of crosses between mice that lack different Id genes will reveal ID functions in other cell lineages that are essential for development.

The embryonic lethality of Id double-knockout mice contrasts markedly with the phenotype observed in mice that lack a single Id gene. Only the Id2-null mouse displays obvious phenotypic abnormalities of retarded growth and neonatal morbidity (Yokota et al., 1999). Male Id2−/− mice exhibit a profound defect in spermatogenesis (F. Sablitzky, personal communication), which is consistent with the stage-specific expression pattern of Id2 during spermatogenesis (Sablitzky et al., 1998).

Id proteins in lymphopoiesis

Surviving Id2−/− mice also display a cell-intrinsic defect in production of natural killer (NK) cells, which are involved in immune function (Yokota et al., 1999). A role for Id function in NK-cell fate determination is also supported by the observation that overexpression of Id3 in primary foetal thymocyte cultures promotes NK cell generation from CD3+ progenitor cells at the expense of T cell production (Heemskerk et al., 1997). Id2-null mice also lack secondary lymphoid follicles of Peyer’s patches in the intestine, although this defect evidently arises from an absence of lymphotoxin-producing, CD4+ CD3− IL-7R+ cells that are essential for Peyer’s patch development (Yokota et al., 1999).

Fig. 2. Tentative scheme showing how the functions of ID proteins are integrated with mitogenic gene signalling cascades and cell cycle progression. During early G1 phase of the cell cycle, mitogenic signalling pathways activate a cascade of gene expression changes and activate the assembly of functional cyclin-dependent kinase (CDK) complexes (see Sherr and Roberts, 1999, for recent review). This leads to sequential phosphorylation of the tumour suppressor protein pRB (and related ‘pocket proteins’, p107 and p130), resulting in its dissociation from members of the E2F family of transcriptional regulators. Free E2F-DP1 (E2F) transcriptional complexes then activate the expression of genes required for progression through S phase of the cell cycle. The functions of ID proteins are integrated at multiple points in G1 cell cycle control (see text). Only ID2 and ID4 interact with pRB. All ID proteins except for ID1 are substrates for CDK2-dependent phosphorylation. The ID1-interacting protein MIDA1 (not shown in the figure) is thought to be coupled indirectly to the cell proliferative response by modulating the expression of growth factor genes (see text).
No abnormalities or defective cell functions have so far been reported for Id1-deficient mice, and data for Id4-deficient mice are not yet available. However, the Id3-null mouse, although apparently normal, displays subtle abnormalities that again affect lymphopoietic cells involved in immune function. Data from studies employing enforced expression of Id3 in lymphopoietic cells also implicate this Id family member in multiple stages of lymphopoiesis. Mature B cells from Id3−/− mice exhibit a defective in vitro proliferative response induced specifically by signalling through the B cell surface immunoglobulin (Ig) receptor (Pan et al., 1999). This probably accounts at least in part for the defective in vivo immune response observed in these mice. B cells from Id3−/− mice are also enhanced in their Ig-class-switching capacity during in vitro differentiation (Pan et al., 1999). The inference from this that ID3 modulates class-switch recombination of Ig genes is further supported by the observation that bHLH transcription factors such as the E2A-encoded E12 and E47 proteins actively promote class switching, whereas enforced expression of Id3 impairs class switching (Quong et al., 1999). ID3 might also play a role in B cell fate determination at an early stage during lymphopoiesis, since enforced expression of Id3 in primitive lymphomyeloid progenitors from foetal liver selectively inhibits B cell, but not NK or myeloid cell, differentiation (Jaleco et al., 1999).

During the final stages of thymocyte differentiation, the ID3 protein also plays a role in positive selection of T cells in the cell-mediated immune response. Id3−/− mice exhibit defective positive selection of both MHC-class-I- and MHC-class-II-restricted thymocytes (Rivera et al., 2000). Furthermore, enforced expression of Id3 in committed thymocyte progenitors inhibits commitment to and production of the αβ subset but not the γδ subset of T cells (Blom et al., 1999). Finally, enforced expression of Id3 promotes maturation of an immature T cell line (Bain et al., 1999); this finding is consistent with the observation that, in the absence of the bHLH protein E47, mature-T-cell production is increased (Bain et al., 1999). ID3 probably plays a role partially overlapping but complimentary to that of Id1 and Id2 during lymphopoiesis. Enforced expression of Id1 or Id2 in committed thymocytes in transgenic mice leads to a block in further maturation (Kim et al., 1999; Morrow et al., 1999). Similarly, enforced expression of an Id1 transgene in early B cells blocks B lymphocyte maturation at the pro-B-cell stage (Sun, 1994).

**ID PROTEINS IN CELL CYCLE CONTROL**

As might be expected for regulatory molecules that have a major role in negative regulation of cell differentiation, ID proteins also act as positive regulators of cell growth, and indeed their functions are required for cell cycle progression in cell line models. Genetic studies in *Drosophila* and mice (discussed above) now provide direct evidence for a role for ID proteins in cell proliferation and cell cycle control in vivo. Quiescent cells express low/undetectable levels of Id genes. However, following mitogenic stimulation of, for example, fibroblasts, Id expression is rapidly induced (within 1-2 hours) as part of a cascade of ‘delayed’ early response genes. After an initial decline, Id expression is sustained throughout the G1 phase of the cell cycle and is further upregulated as cells enter S phase (Hara et al., 1994; Norton et al., 1998 and references therein). ID proteins function at multiple stages in cell cycle control by modulating the transcription of several known target genes, in some instances by directly interacting with non-bHLH proteins (see Table 2).

Fig. 2 summarises the known pathways through which ID proteins are integrated into mammalian cell cycle control. Following mitogenic signalling, ID proteins downregulate the expression of immediate early genes (e.g. c-fos and egr-1) by antagonising the ETS domain proteins SAP-1 and ELK-1, which are components of the ternary complex factor (TCF) responsible for upregulating immediate-early-gene expression (Yates et al., 1999). Interestingly, the expression of Id1 (and probably also of Id3) is itself positively regulated by EGR-1 (Tournay and Benezra, 1996; Deed et al., 1994) as part of a
Table 2. Non-bHLH proteins whose functions are modulated by direct interaction with ID proteins

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinoblastoma (pRB) tumour suppressor protein and related ‘pocket’ proteins, p107 and p130</td>
<td>Only ID2 and ID4 display this interaction, which antagonises pRB function. Involved in cell cycle and growth control</td>
<td>Iavarone et al., 1994; Lasorella et al., 1996; R. Deed, F. Sablitzky and J. Norton, unpublished observations</td>
</tr>
<tr>
<td>ETS-domain transcription factors</td>
<td>Demonstrated for the ternary complex factors (TCF), SAP-1 and ELK-1. Interaction with ID1-ID3 inhibits DNA binding and inhibits immediate early gene induction coupled to MAP kinase signalling</td>
<td>Yates et al., 1999</td>
</tr>
<tr>
<td>MIDA1, a Z-DNA-binding protein displaying an additional sequence-specific DNA-binding function</td>
<td>Implicated in positive regulation of cell growth. Association with ID1 potentiates sequence-specific DNA binding of MIDA1</td>
<td>Shoji et al., 1995; Inoue et al., 1999; Inoue et al., 2000</td>
</tr>
<tr>
<td>ADD1/SREBP-1c (adipocyte determination and differentiation factor 1/sterol regulatory element binding protein 1c)</td>
<td>bHLH-leucine-zipper transcription factor controlling expression of adipocyte genes. Interaction with ID2/ID3 inhibits ADD1-regulated promoter function implicated in lipogenesis</td>
<td>Moldes et al., 1999</td>
</tr>
</tbody>
</table>

negative feedback loop in mitogenic signalling cascades (Fig. 2). Two distinct pathways couple ID function to positive regulation of the cell cycle machinery during mid-late G1 phase. The first involves a direct interaction between the retinoblastoma proteins (pRB, and related ‘pocket’ proteins p107 and p130), and ID2 and ID4 (but not ID1 or ID3) (Iavarone et al., 1994; Lasorella et al., 1996; R. Deed, F. Sablitzky and J. Norton, unpublished observations). This is thought to potentiate S phase progression by attenuating pRB suppression of E2F-DP1 transcription factors, which drive expression of genes required for S phase progression (Fig. 2). The second pathway, described for ID1 but probably also used by other ID family members, involves inhibition of E2A bHLH-regulated expression of the gene encoding the cyclin-dependent kinase (CDK) inhibitor p21Cip1/Waf1 (Prabhu et al., 1997). This relieves p21 suppression of cyclin-A/E-CDK2 activity, which in turn leads to phosphorylation of pRB. The phosphorylation of pRB causes its dissociation from E2F-DP1 complexes (Fig. 2), which can then activate genes required for progression into S phase.

Interestingly, ID2, ID3 and ID4 (but not ID1) are themselves substrates for CDK2-dependent phosphorylation during late-G1/early-S phase (Hara et al., 1997; Deed et al., 1997). This alters the bHLH dimerisation specificity of the ID2 and ID3 proteins and is essential for cell cycle progression, since mutants of ID2 and ID3 that lack CDK2 phosphorylation sites elicit S phase arrest and cell death (Deed et al., 1997). The p21-CDK2 pathway provides an elegant feedback mechanism for temporal control of ID phosphorylation during late G1 (Fig. 2). The functions of the phosphorylated forms of ID proteins during S and G2/M phases of the cell cycle are not known.

ID1 also associates with a novel transcription-factor-like protein, MIDA1 (mouse ID-associated protein 1 – see Table 2), which is ubiquitously expressed (T. Inoue and M. Obinata, unpublished observations) and is required for cell growth, at least in erythroleukaemia cells (Shoji et al., 1995). The association with ID1 potentiates the sequence-specific DNA binding of the MIDA1 protein to a 7-bp sequence, GTCAAAGC, present in the 5’ flanking region of several growth-factor/cytokine genes. MIDA1 might therefore couple ID function to the cell cycle control apparatus through indirect mechanisms (Inoue et al., 1999; Inoue et al., 2000).

ID PROTEINS IN TUMOUR BIOLOGY

Oncogenic properties of ID proteins

Early studies showed that enforced expression of Id genes in immortalised fibroblast cell lines causes disruption of cytoskeletal organisation and loss of adhesion (Deed et al., 1993). Subsequently, Id genes have been shown to function as either cooperating oncogenes or as dominant oncogenes in various contexts. For example, Id genes can immortalise primary rodent fibroblasts when co-transfected with the Bcl-2 gene (Norton and Atherton, 1998), and Idl can immortalise primary human keratinocytes (Alani et al., 1999). The extended lifespan of Idl-immortalised keratinocytes is accompanied by activation of telomerase activity and inhibition of pRB function (Alani et al., 1999). Enforced expression of Idl also promotes mammary epithelial cell invasion of basement membranes and is associated with induction of a 120-kDa gelatinase activity (Desprez et al., 1998). Enforced expression of Idl targeted to intestinal epithelial cells in transgenic mice is associated with development of intestinal adenomas (Wice and Gordon, 1998). Similarly Idl and Id2 transgenes expressed in thymocytes induce an aggressive thymic lymphoma in vivo (Kim et al., 1999; Morrow et al., 1999).

These oncogenic properties of Id genes and their well-documented ability to promote cell proliferation are in accord with the tumour suppressor properties of some bHLH proteins whose activities are antagonised by ID proteins. For example, overexpression of bHLH proteins such as E47 in cell lines typically leads to suppression of growth by inducing cell cycle arrest in G1 (Peverali et al., 1994 and references therein). This probably occurs at least in part through transcriptional activation of the gene encoding the p21 CDK2 inhibitor (Prabhu et al., 1997) (see Fig. 2). Restoration of E12/E47 bHLH protein function to tumour cell lines also leads to growth arrest and in some instances to apoptosis (Park et al., 1999; Engel and Murre, 1999). In addition, mice lacking the E2A gene that encodes the E12 and E47 bHLH proteins develop T cell tumours at a high frequency (Yan et al., 1997). However, crossing of E2A-deficient mice with Idl+/− mice fails to suppress tumour incidence (Yan et al., 1997). Also, crossing of Id3−/− mice with mice lacking the related bHLH protein, HEB, leads to T cell tumorigenesis not seen in individual single-knockout animals (Barndt and Zhuang, 1999). Although the
etiological mechanisms involved are unclear, these observations nonetheless highlight the important role of ID-bHLH interactions in tumorigenesis in vivo.

A feature shared by some positive regulators of mammalian cell cycle progression such as MYC, cyclins D and E and E2F1, is their ability to drive apoptosis when ectopically overexpressed. Like ID proteins, these cell cycle regulatory proteins also display oncogenic properties. Several groups have independently reported on the pro-apoptotic properties of ID proteins in cell line and primary cell models (Norton and Atherton, 1998; Florio et al., 1998; Nakajima et al., 1998; Tanaka et al., 1998; Andres-Barquin et al., 1999). Enforced expression of Id genes in vivo can also induce apoptosis – in transgenic mice expressing an Id1 transgene in thymocytes, the differentiation arrest and T cell lymphomagenesis mentioned above is also accompanied by widespread apoptosis (Kim et al., 1999). Interestingly, ID2 might drive apoptosis through a pathway that is distinct from that utilised by other ID family members. ID2-induced apoptosis is independent of HLH-mediated dimerisation and does not therefore depend on interaction with bHLH or other known ID target proteins (see Table 2). Instead, the ability of ID2 to promote apoptosis resides in the N-terminal region of the protein and is associated with enhanced expression of the pro-apoptotic BAX protein (Florio et al., 1998).

ID proteins in primary tumours

Immortalised cell lines from a number of different tumour types display markedly elevated levels of one or more Id mRNAs (reviewed in Israel et al., 1999). More recent work has extended these studies, analysing protein expression in primary tumours. Table 3 lists the major human tumour types in which ID protein levels have been reported to be deregulated. Note that constitutive Id expression is not simply a generalised feature of tumorigenesis per se – in most leukaemias and lymphomas, for example, the Id expression profile mirrors that of normal haemopoietic progenitor cells (Ishiguro et al., 1996). Given the known ‘oncogenic’ properties of ID proteins and the observation that loss of Id expression in tumour cell lines leads to suppression of cell growth (Hara et al., 1994; Kleef et al., 1998; Lin et al., 2000), it seems likely that ID proteins perform a causal role in tumorigenesis mechanisms. Presumably, the tumour-cell-associated deregulation of Id gene expression arises through perturbations in upstream signalling pathways. A number of oncogenes and tumour suppressor genes encode components of signalling pathways; qualitative and or quantitative changes in these constitute the primary genetic lesion in cancer cells.

Two studies highlight distinct roles for ID proteins in tumorigenesis mechanisms associated with invasiveness and metastasis. In primary breast cancer (Table 3), the expression of Id1 is more frequently associated with infiltrating, more aggressive tumours than with indolent ductal tumours, which suggests a link between Id expression and disease progression (Lin et al., 2000). Overexpression of the Id1 gene, as mentioned above, causes mammary epithelial cells to invade the basement membrane (Desprez et al., 1998). Moreover, constitutive expression of Id1 in a non-aggressive breast cancer cell line confers a more aggressive phenotype, as assessed by growth and invasiveness (Lin et al., 2000). ID1 also regulates steroid-hormone-responsive growth of breast cancer cells.

Table 3. Primary human tumour types in which ID expression is deregulated

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic cancer</td>
<td>Kleef et al., 1998; Maruyama et al., 1999</td>
</tr>
<tr>
<td>Astrocytic tumours</td>
<td>Andres-Barquin et al., 1997</td>
</tr>
<tr>
<td>High-grade neural tumour endothelial cells</td>
<td>Lyden et al., 1999</td>
</tr>
<tr>
<td>Invasive breast carcinoma</td>
<td>Lin et al., 2000</td>
</tr>
<tr>
<td>Seminomas</td>
<td>Sablitzky et al., 1998</td>
</tr>
<tr>
<td>Colorectal adenocarcinoma</td>
<td>J. Wilson and J. Norton, unpublished observations</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>L. Peterson and J. Norton, unpublished observations</td>
</tr>
</tbody>
</table>

These observations implicate loss of appropriate regulation of Id1 expression in the process of tumour progression and suggest that Id1 provides a useful marker for aggressive breast tumours (Lin et al., 2000).

As mentioned previously, the Id1+/− Id3−/− knockout mouse, although viable, has a severely impaired ability to support the growth and metastasis of tumour xenografts, owing to a defect in tumour cell vascularisation (Lyden et al., 1999). Significant regression is observed for some tumour types even in mice lacking just one Id allele (Id1+/− Id3+/+), which implies that there is a gene-dosage effect on ability to support tumour xenografts. The ability to support tumour growth in Id1-Id3-deficient mice is also paralleled by impairment in the ability to support metastasis. This defect in angiogenesis can be readily explained by the loss of αvβ3 integrin and its associated MMP2 metalloproteinase on tumour-infiltrating vascular endothelial cells (Lyden et al., 1999). More recent studies have revealed that tumours of diverse histological origin display a similar defect in growth/metastasis in Id-deficient mice and concomitant haemorrhage, necrosis and tumour regression (R. Benezra, personal communication). Significantly, in at least some high-grade human tumours, for example of neural origin, Id1 and Id3 are expressed in cells of the infiltrating vascular endothelium (Table 3). This contrasts with normal adult vascular endothelial cells, in which ID protein levels are very low (Lyden et al., 1999).

Since growth and angiogenesis of tumour cells appear to be strictly dependent on Id function and can be modulated by small changes in intracellular levels of Id protein, it has been suggested that small-molecule-based inhibitors of Id proteins might provide useful drugs in the treatment of human cancers (Lyden et al., 1999; Lin et al., 2000). Whether therapeutically useful drugs can be developed with sufficient specificity for ID proteins remains to be determined.

MECHANISMS OF ACTION OF ID PROTEINS

ID-bHLH protein interactions

Compelling biochemical and genetic data from studies in both Drosophila and mammals support the view that the primary mechanisms through which ID proteins function is through antagonism of bHLH transcriptional regulators. Moreover, several studies illustrate how subtle changes in the equilibrium of heterodimeric interactions between bHLH and ID proteins...
cause dramatic changes in cell fate determination (see Barndt and Zhuang, 1999; Jan and Jan, 1993). The mammalian ID proteins preferentially target the ubiquitously expressed ‘E proteins’, which belong to the Class A bHLH proteins (E12, E47, E2-2, HEB) (Benezra et al., 1990; Sun et al., 1991; reviewed in Norton et al., 1998). These typically function as heterodimeric partners for the larger family of tissue-specific Class B bHLH proteins (Norton et al., 1998; Littlewood and Evan, 1995; Massari and Murre, 2000). Although all ID proteins interact avidly with each of the four E proteins, biochemical data indicate that individual ID proteins have distinct preferences for their E protein targets (Langlands et al., 1997; Deed et al., 1998). More recent data have also revealed differences in the interaction between E proteins and different IDs at a genetic level. The postnatal lethality associated with HEB-deficient mice can be partially rescued by crossing them with Id3−/− but not with Id1−/− mouse strains (Barndt and Zhuang, 1999).

Although ID proteins, because of their lack of a basic, DNA-binding region, are not known to bind DNA, either in vitro or in vivo, under certain circumstances they can act as transcriptional activators. All four mammalian ID proteins, when fused to a heterologous Gal4-DNA binding domain, can activate Gal4-dependent transcription (Bounpheng et al., 1999b). Since this is apparently mediated through sequestration of bHLH protein(s) (which contribute the transactivation function), and ID-bHLH heterodimers do not bind DNA, this mechanism is unlikely to be physiological. However, ID proteins might provide such an adapter function as constituents of DNA-bound, multicomponent transcriptional complexes.

**Interactions with non-bHLH proteins**

In addition to ID proteins in the context of cell cycle and growth control (see above and Table 2), two additional non-bHLH proteins also interact with and are functionally antagonised by ID proteins (Table 2). Members of the paired homeobox family of transcription factors (PAX-2, PAX-5, PAX-8) associate in vitro and in vivo with ID1-ID3 (A. Sharrocks and J. Norton, unpublished observations). As shown for the B-cell-specific, mb-1 promoter, ID proteins disrupt PAX-5 DNA binding and transcriptional activation of PAX-responsive genes. The adipocyte determination and differentiation factor-1 (ADD1), a member of the bHLH-leucine-zipper family of transcription factors (Fig. 1) that controls expression of genes during lipogenesis also interacts with ID2 and ID3 (Moldes et al., 1999). Again, the association with ID protein abrogates binding to and transcriptional activation of ADD1-regulated promoters. These and the other known examples listed in Table 2 illustrate the emerging molecular promiscuity of the ID family. Evidently, ID proteins can form intermolecular associations with diverse structural motifs on different partner proteins. However, as with the interaction between bHLH and ID proteins, in every reported example listed in Table 2, protein interaction is mediated by the ID HLH domain. Also, with the exception of MIDA1 (discussed above; see Table 2), the association with ID protein antagonises the function of the DNA-binding protein, typically by inhibiting DNA binding – as occurs with bHLH proteins. It is important to emphasise, however, that all of the reported functional interactions between ID and non-bHLH proteins (Table 2) have so far only been documented in biochemical and cell line ‘in vivo’ studies. The relevance of these various interactions in mediating biological functions of ID proteins in a truly physiological context in vivo has yet to be established.

**Regulation of ID function**

In mammalian cell lineages, Id expression responds to a multitude of cell surface, ligand-receptor interactions – as might be expected for early response genes that are pivotal in cell fate determination (Norton et al., 1998). Although some data on the mechanisms of regulation of Id expression at the promoter level are available (see Norton et al., 1998), very little is known about the upstream signalling pathways that couple Id expression to extracellular signals. The ID proteins themselves are regulated at the post-translational level. In addition to cell-cycle-linked phosphorylation (see above), intracellular levels of ID proteins are regulated through the ubiquitin-proteasome degradation pathway (Bounpheng et al., 1999a). In common with most other proteins encoded by early response genes, ID proteins rapidly turn over in the cell, having a reported half-life of 20-60 min, depending on the cell type (Deed et al., 1996; Bounpheng et al., 1999a). ID4 is apparently much less sensitive to inhibitors of the 26S proteasome pathway, and, although its degradation is dependent on ubiquitin-activating enzyme activity, this ID family member might also be degraded through an alternative pathway (Bounpheng et al., 1999a).

Heterodimerisation with bHLH proteins extends the half-life of ID3 (and also enhances the degradation of its heterodimeric bHLH protein partner); ID proteins might therefore be less susceptible to degradation by the 26S proteasome pathway in the heterodimer state (Deed et al., 1996; Bounpheng et al., 1999a). In common with several other positive regulators of cell cycle progression, ID proteins are also regulated by subcellular localisation. ID proteins do not possess nuclear localisation signals, whereas their bHLH protein partners possess an efficient nuclear localisation signal. Co-transfection of cells with constructs expressing ID and bHLH proteins leads to sequestration of the ID protein into the nucleus, which suggests that heterodimerisation regulates the subcellular distribution of IDs (Deed et al., 1996). The physiological relevance of such a mechanism has yet to be established.

**CONCLUSIONS**

In the ten year period since ID proteins were first described in flies and mammals, interest in this family of HLH proteins has increased exponentially. Inhibition of cell differentiation through antagonism of bHLH proteins, as originally described ten years ago, still remains the single most important facet of ‘ideology’. However, the diversity of other biological processes in which the functions of ID proteins have been implicated and the apparent promiscuity of molecular interactions that might mediate ID functions now presents a rather bewildering picture. One of the problems associated with the field is the questionable generality of many of the observations made. For example, most of the known molecular mechanisms through which ID functions are integrated in cell cycle control have been modelled in fibroblasts. Given that
cells from different lineages and developmental stages express distinct repertoires of ID proteins and different ID proteins have different properties, it seems highly likely that the mechanisms through which ID proteins regulate the cell cycle machinery will turn out to be cell type specific. Also, the role of ID proteins in lymphopoiesis has been particularly well studied, primarily because of the long-standing interest of developmental immunologists in the bHLH E proteins, whose functions are pivotal for development and function of the lymphopoietic system. However, it is far from clear whether the mode of action of ID proteins in lymphopoiesis, at both the molecular and biological levels, can be extrapolated to other mammalian cell lineages in which ID proteins undoubtedly play a role.

At a mechanistic level, it will clearly be important to establish the biological significance of the interactions between ID proteins and their various non-bHLH protein partners in a physiological context. Some of these ID-interacting proteins, such as pRB, ETS and PAX transcription factors, are well known for their roles in regulating various cellular processes, particularly cell fate determination in diverse cell lineages, and indeed have a functional status to match that of bHLH proteins. Particularly cell fate determination in diverse cell lineages, and ID proteins and their various non-bHLH protein partners in a mammalian cell lineages in which ID proteins undoubtedly play a role.

Work in the author’s laboratory is supported by the UK Cancer Research Campaign and the Leukaemia Research Fund.

REFERENCES


