Nuclear Localization and Regulation of Id Protein through an E Protein-mediated Chaperone Mechanism

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Richard W. Deed, Suzanne Armitage, and John D. Norton:

From the Cancer Research Campaign Department of
Gene Regulation, Paterson Institute for Cancer
Research, Christie Hospital National Health Service
Trust, Wilmslow Road, Manchester M20 9BX,
United Kingdom

Members of the Id family of helix-loop-helix proteins function as negative regulators of DNA binding, E protein, helix-loop-helix transcription factors in the control of cell growth, differentiation, and development. By using transient transfection analysis of COS cells, we show that in the absence of its E protein target, the Id3 protein is localized exclusively to the cytoplasm/perinuclear region. Co-transfection with E protein (E47) results in nuclear translocation of the Id3 protein, a process requiring both a functional Id helix-loop-helix dimerization domain and an E protein nuclear localization signal. Id3 that is associated with E protein displays an extended half-life, while the E protein itself is more rapidly turned over. These observations demonstrate that E protein, by nuclear chaperoning Id, can regulate the available cellular pool of its own inhibitory partner.

Transcription factors belonging to the family of basic, helix-loop-helix (bHLH) proteins regulate gene expression accompanying the processes of cellular lineage commitment, growth, and differentiation in both vertebrates and invertebrates (1). The bHLH domain itself comprises two amphipathic α helices separated by an intervening loop, bounded by a short, N-terminal, basic DNA-binding region (1,2). DNA binding and transcriptional activation of target genes through recognition of the “E box” consensus sequence (CANNTG) (3) is mediated by a bHLH dimer configuration, typically comprising one of several cell type-specific bHLH proteins, exemplified by the muscle determining MyoD protein together with a member of the ubiquitous “E” protein family (1, 4). The activities of positively acting bHLH proteins are, in turn regulated by a set of dominant negative “Id” (inhibitor of differentiation/DNA binding) HLH proteins that lack a basic, DNA binding domain and function by sequestering their target bHLH proteins by heterodimeriza-

tion, into an inactive state that is unable to bind DNA (1,5). Originally isolated as a class of growth factor-inducible genes (6,7), the Id family, of which there are four members in mammals (Id 1-4) (1), share a similar overall organization consistent with evolution from a common ancestral Id gene (8).

It is becoming increasingly clear that the function of various bHLH proteins in regulating gene expression/cellular function is critically dependent on subtle changes in their cellular activities. This is dramatically demonstrated in the examples of sex determination in Drosophila (9), MyoD-driven myogenesesis (10), and recently in E2A-regulated B lymphopoiesis, where only a 2-fold decrease in bHLH protein activity is translated into an equivalent decrease in probability of cell fate determination (11). An understanding of the mechanisms regulating the activities of the Id bHLH antagonists is therefore of central importance to understanding how the above cellular processes are controlled. In this report we have used human Id3 (12) as a model to explore how the intracellular localization and stability of Id proteins is regulated by the presence of their target bHLH E proteins.

EXPERIMENTAL PROCEDURES

Expression Plasmids—Plasmids encoding Id1 (13), Id2 (8), Id3 (12), E47 (14), and E12r (15) proteins were constructed by subcloning the respective cDNAs into the vector, pcDNA3 (Invitrogen). The plasmid encoding the VP16/Id3 hybrid polypeptide was generated by insertion of the Id3 cDNA coding sequence modified by addition of a BamHI recognition sequence at the ATG start site (12), into the BamHI-EcoRI-cut pVP16 NcoI vector (16). The expression and reporter constructs, pGal4E47 and pGS5ElBac (17), were generously supplied by Dr. R. Baer. The plasmid Id3 HB, encoding the Ser-49 → Pro mutation in the Helix 1 of Id3 was generated by a two-step overlap PCR method (18). The sequences of the mutational primers were: 5′-aacctcgactccg-3′ and 5′-ccgagcggggtgctggtt-3′. Attachment of a sequence encoding the SV40 nuclear localization signal to the C terminus of the Id3 cDNA to generate Id3NLS was carried out essentially as described by Vastrik (19), with the Id3 cDNA as template for PCR mutagenesis using the following primer 5′-ggtagccagttcatttcttttcc-3′, in combination with a T7 RNA polymerase primer. The resultant PCR product, after digestion with EcoRI, was cloned into the vector pcDNA3. All plasmid constructs were sequenced on both strands using a Prism Cycling sequence kit (Applied Biosystems) and an Applied Biosystems 373 DNA sequencer.

DNA Transfection and Radiolabeling of COS-7 Cells—Approximately 3 × 10⁶ cells were seeded into 10-cm dishes and cultured overnight in growth medium (DMEM plus 10% fetal calf serum). The adherent cells were fed with fresh medium and transected 3 h later, using the calcium phosphate procedure essentially as described previously (12). After incubation with the precipitate overnight, the cells were washed twice with phosphate-buffered saline and incubated for another 48 h before labeling and harvesting. For analysis in the two-hybrid assay, cells were harvested 48 h post-transfection and lysed in luciferase buffer (25 μl KH2PO4, 8 μM MgCl2, 1 μM dithiotoilet, 1% Triton X-100, 1% bovine serum albumin, 15% glycerol) and luciferase activities were measured as described elsewhere (17). For metabolic labeling, 48 h post-transfection, the cells were starved for 1 h in cyto-
teine/methionine-free DMEM medium (ICN-Flow) containing 5% dialyzed fetal calf serum, and incubated for an additional 2 h in fresh medium supplemented with 100 μCi mL⁻¹ Promix (Amersham; specific activity 1000 Ci mmol⁻¹). For half-life studies, labeled cells were “cold-chased” with methionine and standard DMEM plus 10% fetal calf serum for the specified times prior to immunoprecipitation. Labeled cells were washed once with phosphate-buffered saline and lysed in 1 mL of either radioimmunoprecipitation assay buffer (50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) for direct immunoprecipitations, or HB buffer (0.3% sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40)
Id Protein Localization

**RESULTS AND DISCUSSION**

Functional Association of Id3 with the E Protein, E47, in Vivo—In common with other Id proteins, Id3 heterodimerizes with several target bHLH proteins in vitro and is capable of preventing their association with target E box DNA sequences in vitro, in band shift analysis (1, 5, 12, 21, 22). Fig. 1A shows that Id3 functionally interacts directly with the target E2A E protein, E47 (14), following transient transfection of COS cells in a two-hybrid assay system.

We tested several Id3/E47-specific antisera for their ability

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For co-immunoprecipitations, both containing a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 100 μg/ml each of leupeptin, bestatin, aprotinin, and 2 mM levamisole).

Subcellular Fractionation—To generate cytoplasmic and nuclear fractions for immunoprecipitation, transfected COS cells were lysed as described by Waters et al. (20). The lysate was then centrifuged briefly at 4°C and the supernatant taken as the cytoplasmic fraction. The nuclear pellet was resuspended in TBS plus 1% SDS and heated to 90°C for 5 min. Both fractions were adjusted to give a salt concentration approximately that of radioimmunoprecipitation assay buffer prior to immunoprecipitation.

Immunoprecipitation—Prior to immunoprecipitation, the lysate was sheared by passage through a 19-gauge needle and particulate matter was collected by centrifugation at 2500 rpm for 5 min in a refrigerated microcentrifuge. The cell lysate was precleared by incubation with 10 μl of normal rabbit serum and 50 μl of protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C with agitation. Supernatants were recovered by centrifugation and used in subsequent immunoprecipitations. For each immunoprecipitation reaction, 500 μl of cleared lysate was incubated with 5 μl of Id3-specific antiserum (“RD-1” raised against an 18 amino acid C-terminal epitope) or 5 μl of anti-E47- or anti-E12-specific antiserum (Santa Cruz Biotechnology) and rocked for 1 h at 4°C, after which 30 μl of protein A/G-Sepharose beads was added and the incubation continued for an additional 1 h. For immunoprecipitation experiments with Id3NLS, a different antibody, RD6,2 raised against full-length bacterially expressed Id3 protein was used. The immunocomplexed antigens were pelleted by centrifugation, washed four times with the respective lysis buffer, and finally resuspended and boiled for 5 min in SDS sample buffer. The precipitated antigens were fractionated on 12% SDS-polyacrylamide gels, fluorographed, dried, and autoradiographed overnight essentially as described previously (12). Gel data were quantitated by phosphorimager analysis analysis (Molecular Dynamics) and, unless otherwise stated, were taken from experiments performed at least in duplicate.

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2 R. Deed, unpublished results.

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Fig. 1. In vivo interactions between Id3 and its target E protein, E47. A, COS-7 cells were transfected with 5 μg of the pGal4 reporter construct, pG5E1bLuc, together with 5 μg of each of the VP16 expression constructs indicated. Luciferase activity was determined 48 h post-transfection. Results from two independent experiments are shown. B, COS-7 cells were co-transfected with 5 μg of pcDNA3 expression vectors encoding E47 and Id3 proteins. 48 h post-transfection, cells were [35S]methionine-labeled for 2 h and then extracted under mild lysis conditions. The first immunoprecipitation was performed with either control serum (C) or with anti-Id3 or anti-E47-specific antiserum as indicated. Immunoprecipitates were analyzed directly, while supernatants (sup) were subjected to a second round of immunoprecipitation with the reciprocal antibody and precipitates were analyzed in parallel.

Fig. 2. Intracellular localization of Id3 and E47/E12 bHLH proteins. COS-7 cells were transfected with each of the indicated pcDNA3 expression constructs, and, approximately 72 h later, cells were fixed and stained with different antibodies. DAPI staining (shown in representative cases) was used as a nuclear marker. The photographs (taken at 100-fold magnification) are representative fields of view from a number of independent experiments in which over 100 separate transfected cells were evaluated. Panels C and D are identical fields of view for cells double-stained with FITC (E47) and with TRITC (Id3 protein)
to preserve the relatively weak HLH dimer bonding in order to detect co-immunoprecipitates of Id3-E47 heterodimers in these cotransfection experiments. As shown in Fig. 1B, an antibody raised against a C-terminal 18-amino acid epitope of Id3 co-immunoprecipitated approximately 50% (range 40–60% in five experiments) of the E47 protein that was immunoprecipitable from the same cells with E47 antisera. Similarly, the same E47 antibody raised against an epitope corresponding to amino acids 632–649 of E47 (14) co-immunoprecipitated approximately 35% (range 26–45% in five experiments), of the total Id3 protein as detected following immunoprecipitation with anti-Id3 antisera (Fig. 1B). The variability between values obtained in different experiments appeared to be independent of several experimental parameters such as the time post-transfection when cells were harvested. However, we did observe that prolonged incubation or addition of excessive antibody led to diminished recovery of co-immunoprecipitates (data not shown), implying that our antibody reagents do not completely preserve the integrity of HLH dimers. While the above values should therefore be taken as minimal estimates, they do clearly indicate that a substantial fraction of the cell’s complement of Id3 and E47 proteins exist in a heterodimer configuration in these experiments. Comparable results were also obtained for the Id1 and Id2 proteins (data not shown).

**E47 Acts as a Nuclear Chaperone for Id3—**E proteins such as E47 possess a classical nuclear localization signal (1) and are found exclusively in the cell nucleus (for example, see Fig. 2B). By contrast, Id proteins lack such a nuclear localization signal and have been reported to be distributed in both the nucleus and cytoplasm under physiological conditions in exponentially growing cells (23, 24). Since Ids act as E protein functional antagonists by sequestering them into an inactive (non-DNA-binding) state, we considered the possibility that in the presence of Id, E protein may be preferentially retained in the cytoplasm. Alternatively, the Id-E protein heterodimer might, by virtue of the E protein nuclear localization signal, be translocated to the cell nucleus. To distinguish between these possibilities, we examined the intracellular distribution of Id3 and E47 proteins after transient transfection of COS cells with expression vectors encoding these two HLH proteins either alone or in combination. In this approach, the high levels of exogenous protein generated should obviate interference from their endogenous counterparts, and transiently transfected COS cells have previously been used as a reliable model of protein trafficking in numerous published studies. As shown by the representative immunofluorescence photograph in Fig. 2A, in the absence of E protein, Id3 showed exclusive localization to the cytoplasm/perinuclear region. In further studies using confocal microscopy, no significant intranuclear immunofluorescence above background was observed in several experiments with cells grown under a variety of conditions. Similar results were obtained for the Id1 protein (data not shown). However, when Id3 and E47 vectors were co-transfected together into COS cells, essentially all of the Id3 protein was translocated to the nucleus, while none of the E protein was sequestered into the cytoplasm (Fig. 2, C and D). As shown in Fig. 3, biochemical fractionation of transfected COS cells revealed a similar intracellular partitioning of Id3 and E47 proteins. Also, since essentially all of the Id3 protein is translocated to the nucleus in cotransfected cells, we can infer that E protein is likely to be in functional excess in these experiments.

To determine the contribution of Id3-E47 heterodimerization to the ability of E47 to translocate Id3 to the nucleus, we examined the intracellular partitioning of a mutant Id3 protein, generated by site-directed mutagenesis, which incorporates a substitution of a single proline residue at position 49 in place of serine in the helix 1 region of the HLH domain (15). This “helix breaking” mutant of Id3 (Id3HB), was designed from molecular modelling studies (15) and has a drastically reduced ability to form stable heterodimers with E proteins. As shown in Fig. 4, Id3HB does not co-immunoprecipitate with E47 in co-transfected COS cells. The pattern of intracellular...
Id3HB staining in cells cotransfected with E47 (Fig. 2E) was indistinguishable from that seen in the absence of E protein, with very little translocation of Id3HB to the nucleus. Finally, when the Id3 vector was co-transfected with a construct expressing a variant E2A E protein, E12r, which is still able to efficiently dimerize with Id3 (for example see Fig. 5, F and G) but lacks a nuclear localization signal (15), both the Id3 and E12r proteins were localized exclusively to the cytoplasm (Fig. 2, F–H). Thus, we conclude that E47, by virtue of its nuclear localization signal, acts as a nuclear chaperone for its own antagonist Id protein. Such a mechanism would allow the E protein to tightly regulate the available nuclear pool of its inhibitory partner, while still maintaining a cytoplasmic reservoir of Id antagonist.

Stability of Id3 Protein Associated with E47—In common with most early response genes proteins studied to date, Id3 is rapidly turned over in the cell with a half-life of approximately 1 h, as shown by the pulse labeling experiment in Fig. 5A. By contrast, its target bHLH protein, E47, is turned over very slowly (half-life, approximately 24 h; see Fig. 5B). Since the ability of Id3 to antagonize E47 function would be strongly influenced by protein stability of each dimer partner in the heterodimer state, we examined their respective half-lives following co-transfection of COS cells (Fig. 5, C and D). In pulse-labeled cell extracts, the half-life of E47 protein co-immunoprecipitated with Id3 was significantly reduced to about 16 h (Fig. 5C). By contrast, the Id3 half-life was extended to about 3 h when examined following immunoprecipitation with anti-E47 antibody (Fig. 5D). Since only around 35–50% of the Id3/E47 proteins are co-immunoprecipitable from cotransfected cells (see Fig. 1B), we also examined the half-life of the fraction of Id3 protein that is not co-immunoprecipitated with anti-Id3 antibody (Fig. 5D, lower panel). This fraction also displayed an extended half-life indistinguishable from that of co-immunoprecipitated Id3 protein, (Fig. 5D, upper panel) and of total Id3 protein (data not shown). These observations are consistent with E protein being in functional excess (see Figs. 2 and 3) and with most of the Id3 protein being associated with E protein in these co-transfection experiments.

To determine the contribution of nuclear localization to these effects on protein stability, we examined the consequences of co-transfection of Id3 with the truncated E2A variant, E12r, which, although able to efficiently heterodimerize with Id3, does not localize to the cell nucleus (see Fig. 2F). As shown in Fig. 5 (E and F), the half-life of co-transfected E12r detected by co-immunoprecipitation with anti Id3 antibody, like its E47 counterpart, was significantly reduced from 18 to 12 h, while the half-life of co-transfected Id3 protein was extended to about 4 h (Fig. 5G). We conclude that HHLH heterodimerization rather than nuclear localization is the principle functional requirement for these effects on protein stability. Consistent with this, an Id3 variant engineered with a C-terminal NLS, derived from the SV40 T antigen, displayed a half-life indistinguishable from wild type Id3 protein when transfected in isolation, despite being localized exclusively to the nucleus (Fig. 6).

Acting in concert with their nuclear chaperoning function, members of the E protein family, exemplified by E47 and perhaps other bHLH transcriptional activators, are thus able to attenuate the rapid elimination of cellular pools of Id proteins while themselves being more rapidly eliminated in the presence of the inhibitor protein. Precisely how these mechanisms operate in the context of signaling pathways regulating cell growth, differentiation, and development in which Id functions have been implicated (11, 12, 22–27) remains to be determined.

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