Active Nuclear Import and Export Pathways Regulate E2F-5 Subcellular Localization*  

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Epidermal keratinocyte differentiation is accompanied by differential regulation of E2F genes, including up-regulation of E2F-5 and its concomitant association with the retinoblastoma family protein p130. This complex appears to play a role in irreversible withdrawal from the cell cycle in differentiating keratinocytes. We now report that keratinocyte differentiation is also accompanied by changes in E2F-5 subcellular localization, from the cytoplasm to the nucleus. To define the molecular determinants of E2F-5 nuclear import, we tested its ability to enter the nucleus in import assays in vitro using digitonin-permeabilized cells. We found that E2F-5 enters the nucleus through mediated transport processes that involve formation of nuclear pore complexes. It has been proposed that E2F-4 and E2F-5, which lack defined nuclear localization signal (NLS) consensus sequences, enter the nucleus in association with NLS-containing DP-2 or pRB family proteins. However, we show that nuclear import of E2F-5 only requires the first N-terminal 56 amino acid residues and is not dependent on interaction with DP or pRB family proteins. Because E2F-5 is predominantly cytoplasmic in undifferentiated keratinocytes and in other intact cells, we also examined whether this protein is subject to active nuclear export. Indeed, E2F-5 is exported from the nucleus through leptomycin B-sensitive, CRM1-mediated transport, through a region corresponding to amino acid residues 130–154. This region excludes the DNA- and the p130-binding domains. Thus, the subcellular distribution of E2F-5 is tightly regulated in intact cells, through multiple functional domains that direct nucleocytoplasmic shuttling of this protein.

The E2F family of transcription factors is involved in regulation of cell cycle progression and developmental processes such as axis formation, differentiation, and apoptosis (reviewed in Refs. 1–3). This multigene family includes the E2F proteins (1 through 6), as well as the DP proteins (1 through 3; reviewed in Refs. 1–3). E2F factors play a central role in controlling the G → S transition, by regulating transcription of DNA replication enzymes (4), and cell cycle regulators such as cyclins E and A (reviewed in Refs. 2 and 5). The transcriptional activity of E2F is modulated, in turn, at various levels. One of the most extensively studied mechanisms of regulation involves inhibition of E2F activity through association with pRB family proteins (pRB, p107, and p130; reviewed in Ref. 6). In particular, formation of E2F-p130 complexes appears to be very important in the permanent cell cycle withdrawal characteristic of terminal differentiation. Recently, it has become apparent that other post-translational mechanisms participate in the control of E2F activity, including phosphorylation (7–9), acetylation (10–13), and regulation of subcellular localization (14–18).

In contrast to E2F-1, -2, and -3, which have a consensus NLS and are constitutively nuclear in most cells examined (15, 19), E2F-4 shuttles between the cytoplasm and the nucleus, and the relative proportion in each cellular compartment is, at least partially, cell cycle-dependent. Specifically, E2F-4 is predominantly nuclear in quiescent fibroblasts, and moves into the cytoplasm shortly after these cells are stimulated with serum, and as they approach the S phase (15). These observations suggested that nuclear E2F-4 mediates transcriptional repression of growth-promoting genes in fibroblasts. In a variety of cell lines, exogenously expressed E2F-4 and E2F-5 are cytoplasmic, but they can be forced to translocate to the nucleus by coexpression of nuclear DP-2 or pRB family proteins. These observations, together with the lack of apparent nuclear localization signals, has led to the proposal that E2F-4 and E2F-5 reach the nucleus mainly by association with nuclear partners such as DP-2 or pRB family proteins (15, 18, 19).

To date, the subcellular localization of E2F factors has been examined largely in the context of cell cycle progression in fibroblasts or tumorigenic cell lines rather than during irreversible cell cycle arrest associated with terminal differentiation. The latter situation is especially relevant to tissues such as the epidermis, which are constituted by both undifferentiated keratinocytes capable of proliferating, and their terminally differentiated progeny (20, 21).

Primary epidermal keratinocytes can be isolated and cultured under conditions that maintain an undifferentiated population. These cells differentiate by culture in medium with extracellular calcium levels > 0.05 mM, to mimic the morphological changes, induction of differentiation markers and irre-
versatile entry into quiescence observed in mature epidermal keratinocytes in situ (22, 23). We have shown that E2F factors are differentially regulated in the epidermis, with E2F-2 and E2F-5 mRNA expression localized, respectively, to undifferentiated and differentiated keratinocytes (24, 25). A similar switch in E2F protein expression occurs in primary cultured murine keratinocytes, which express E2F-1, -2, and -3 in the undifferentiated, proliferative state. Induction of differentiation by elevated extracellular Ca$^{2+}$ causes a reduction in these E2F forms, with a concomitant increase in E2F-5 protein, and no substantial change in E2F-4 levels (26). Notably, the up-regulation of E2F-5 in differentiating keratinocytes is accompanied by formation of complexes containing E2F-5, p130, and histone deacetylase 1, which appear to be involved in irreversible cell cycle withdrawal (26). The presence of E2F-5:p130 species in the nucleus of differentiated keratinocytes prompted us to investigate the mechanisms controlling E2F-5 subcellular localization. We now report differential regulation of E2F-4 and E2F-5 subcellular distribution upon induction of keratinocyte maturation. Furthermore, we demonstrate that E2F-5 has distinct domains that determine its subcellular localization. Specifically, E2F-5 has an intrinsic nuclear localization domain, which allows it to translocate into the nucleus independently of interactions with DP or pRB family proteins, and a nuclear export domain, which contributes to cytoplasmic localization mediated by active nuclear export through CRM1.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies—** Anti-E2F-4 (sc-866), anti-E2F-5 (sc-999), and monoclonal anti-GST (sc-138) antibodies were purchased from Santa Cruz Biotechnology. Rabbit Anti-GST (71–7500) and monoclonal anti-FLAG M5 antibodies were, respectively, from Zymed Laboratories Inc. and Eastman Kodak. Restriction enzymes were from Invitrogen or from New England Biolabs. Digitonin was from Calbiochem. Essential minimal Eagle’s medium without Ca$^{2+}$ was purchased from BioWhittaker; all other cell culture materials were from Invitrogen. Cy3- and FITC-conjugated goat anti-mouse or goat anti-rabbit antibodies were purchased from Jackson ImmunoResearch Laboratories.

**Cell Culture and Transient Transfections—** HeLa, MCF-7, and HEK293 cells were purchased from the American Type Culture Collection. IMDM cells are a line of spontaneously immortalized dermal fibroblasts that we isolated from the dermis of a 2-day-old CD-1 mouse. All cell lines were maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum in a humidified atmosphere. Primary mouse keratinocytes were isolated, cultured, and infected with recombinant adenovirus as described (26). Primary keratinocytes were maintained as proliferating, undifferentiated cells in medium containing 50 μM Ca$^{2+}$ or induced to differentiate in medium containing 0.1 mM Ca$^{2+}$. Transient transfections on HeLa, MCF-7, and HEK293, or IMDM cells were conducted by the calcium phosphate method, using 1 μg of DNA/well in a 12-well culture plate. Primary mouse keratinocytes were transfected with LipofectAMINE Plus (Invitrogen). For nuclear export studies, cells were plated on glass coverslips in 12-well culture dishes so that they were 40–50% confluent the next day. At that time, they were transfected with indicated plasmids and, 24–48 h after DNA addition, they were processed for fluorescence microscopy. To inhibit CRM1-mediated nuclear export, transiently transfected cells were cultured with cycloheximide (10 μg/ml, final) and leptomycin B (10 ng/ml, final) for 4 h prior to processing for microscopy.

**Plasmid Constructs—** GST-E2F-1 and GST-E2F-2 plasmids were gifts from Drs. P. Hamel (University of Toronto) and D. C. Heinbrook (Merck Research Laboratories). GST-E2F-3 was obtained by excision of a 2.5-kb BamHI/BglII fragment from CMV-E2F-3 (27) and cloning in-frame into pET-41a (Novagen). GST-E2F-5 was produced by cloning a 2.5-kb SalI/EcoRI fragment from CMV-E2F4 (28) into pET-41b (Novagen). FLAG-tagged E2F-5 was constructed by cloning in-frame an EcoRI fragment form CMV-E2F-5 corresponding to the mouse E2F-5 cDNA (29) into pBK-CMV-LacZ-FLAG (a modified pBK-CMV expression vector (Stratagene) lacking the β-galactosidase CDNA), which contained FLAG-encoding sequences. The resulting plasmid (FLAG-E2F-5) encodes the full-length E2F-5 DNA downstream from the FLAG tag.

GFP-E2F-5 was generated by cloning in-frame a 1-kb BamHI/HindIII fragment encoding the tagged E2F-5 cDNA from FLAG-E2F-5 into pEGFP-C2 (CLONTECH). GFP-E2F-5-(315–332), which lacks the pRB family-binding domain, GFP-E2F-5-(405–435), and mutants containing C-terminal deletions were obtained by PCR. GFP-E2F-5-(Δ84–129) was obtained by EcoRV/BglII excision of a 146-bp fragment, followed by Klenow treatment and ligation. GFP-E2F-5-(1–156) was obtained by excision of a 650-bp BglII/BamHI fragment corresponding to amino acids 131–323 from GFP-E2F-5.

Wild type GST-E2F-5 was obtained by cloning in-frame a 1-kb EcoRI fragment from FLAG-E2F-5 into pGEX-KG (a pGEX2T derivative with a polyclgyine linker at the thrombin cleavage site). GST-E2F-5-(315–332) was constructed by isolating a 1-kb HindIII/EcoRI fragment corresponding to mouse E2F-5-(315–332) and cloning in-frame into pET-41c. GST-E2F-5-(405–435) was constructed by cloning a 670-bp BglII/HindIII fragment encoding mouse E2F-5 amino acids 105–346 from FLAG-mE2F5 in-frame into pET-41c. GST-E2F-5-(1–179) were constructed by removal from GST-E2F-5-(315–332) of fragments corresponding to E2F-5 amino acids 57–346 and 180–332, respectively, followed by ligation. GST-GFP was constructed by cloning a 738-bp NeoI/EcoRI fragment encoding the EGFP cDNA from pEGFP-C2 (CLONTECH) into pET-41a(+). GST-GFP-NLS was constructed by phosphorylation of residues 619–627 (E. coli) of FLAG-E2F-5-(1–156) and GST-GFP-NLS-(619–627) fragment corresponding to the NLS of SV40 large T antigen (PKKKKRV) into GST-GFP. All mutant cDNAs were verified by sequencing using dideoxy methodology.

**Bacterial Expression and Purification of GST Fusion Proteins—** GST fusion proteins containing E2F-1, -2, or -5 sequences were expressed in E. coli BL21(DE3)-RIL (Stratagene), and those containing E2F-3 or E2F-4 sequences were expressed in E. coli BL21(DE3)-RP. All other GST fusion proteins were expressed in E. coli BL21(DE3). To induce protein production, bacteria were cultured at 37 °C in NYZ medium to an optical density of 1 (600 nm) and then supplemented with 0.5 mM isopropyl-1-thio-galactopyranoside for 2 h. The bacteria were harvested by centrifugation, resuspended in lysis buffer (phosphate-buffered saline, 1% Triton X-100, 1 mM phenylmethylsulfonfly fluoride, 4 μg/ml leupeptin, 2 μg/ml aprotinin), and lysed by passing twice through a pre-chilled French press at 1000–1500 p.s.i. After clearing the lysates by centrifugation (14,000 rpm, 15 min, 4 °C), the supernatants were incubated for 30 min at 22 °C with 100 μl of a glutathione-Sepharose slurry (Amersham Biosciences). The bound proteins were washed thrice with ice-cold lysis buffer, followed by elution with 100 μl of glutathione elution buffer at 4 °C. With the exception of GST-E2F-5, no significant degradation products for each GST fusion protein were detected by SDS-PAGE analysis (not shown). Full-length GST-E2F-5 was purified from partial-length fusion proteins by fast protein liquid chromatography on a SP-Sepharose column. All experiments conducted contained fractions with ≥90% full-length GST-E2F-5.

**In Vitro Import Assays—** Transport assays were performed essentially as described previously (30, 31). Briefly, HeLa cells were plated on glass coverslips 24–48 h prior to use. The cells were rinsed three times with transport buffer (hereafter termed TB; composed of 20 mM HEPES, pH 7.3, 110 mM KOAc, 2 mM Mg(OAc)$_2$, 2 mM DTT, 1 mM EDTA), and permeabilized for 10 min at 4 °C with TB containing 2 mM DTT, 1 mM phenylmethylsulfonfly fluoride, 4 μg/ml leupeptin, 2 μg/ml aprotinin, and 40 μg/ml digitonin. After two washes with TB, the coverslips were immersed in TB supplemented with protease inhibitors and 2 mM DTT, and incubated at room temperature (22–23 °C) for 5 min. Excess buffer was removed, and the permeabilized cells were incubated with transport reaction mixture consisting of complete transport (CT) buffer, HeLa cytosol extract (with protease inhibitors), and the appropriate GST fusion protein for 30 min at the temperature indicated in individual experiments. CT buffer contained TB plus an ATP regeneration system (0.5 mM ATP, 0.5 mM GTP, 5 mM creatine phosphate, and 20 units/ml creatine phosphokinase). Assays in the absence of an energy-regenerating system were conducted with TB, no ATP or GTP, and apyrase (10 units/ml). For WGA treatments, permeabilized cells were incubated in the presence of 2.05 mg/ml WGA in TB for 15 min prior to the transport reaction. HeLa cytosol extract was prepared as described previously (32). Cell permeabilization and absence of nuclear membrane damage were verified by the absence of nuclear diffusion of FITC-labeled dextran (M, 70,000). After the import assay, cells were rinsed with ice-cold transport buffer, and fixated with 3% paraformaldehyde at 4 °C for 40 min. After rinsing, GST fusion proteins were detected by fluorescence microscopy. To this end, the cells were treated with...
were blocked at room temperature in phosphate-buffered saline with 1% bovine serum albumin for 1 h, rinsed, and probed with an anti-GST antibody for 1 h at room temperature. After three rinses, the cells were probed with the appropriate Cy3- or FITC-labeled secondary antibody at room temperature for 1 h. After removal of the secondary antibody, the cells were rinsed twice, incubated with Hoechst 33258 (10 μg/ml) for 5 min at room temperature, rinsed five times, and mounted. All photomicrographs were obtained with a Leica DMIRBE microscope equipped with an Orca charge-coupled device camera, using Openlab software (Improvision). The proportion of cells exhibiting the presence or absence of the tested GST fusion protein in the nucleus was calculated from examination of 500–1400 cells in random microscope fields, and each experiment was repeated four to nine times with similar results.

RESULTS

Changes in Subcellular E2F-5 Distribution in Differentiating Keratinocytes—Induction of terminal differentiation in mouse epidermal keratinocytes is accompanied by increased E2F-5 protein expression and formation of E2F-5-p130 complexes (26). These complexes are involved in differentiation-induced keratinocyte exit from the cell cycle. To begin to understand the mechanisms regulating the formation of E2F-5-p130 complexes in differentiated epidermal keratinocytes, we examined by fluorescence microscopy the subcellular distribution of E2F-5 in undifferentiated keratinocytes and in cells induced to differentiate with Ca$^{2+}$. Whereas E2F-5 in undifferentiated keratinocytes is scarce and largely cytoplasmic, induction of differentiation is accompanied by up-regulation and concentration of E2F-5 in the nucleus (Fig. 1), consistent with the established formation of the nuclear E2F-5-p130 species.

Interaction with pRB family proteins, such as p130, has been proposed to play a role in regulating E2F-4 and E2F-5 subcellular distribution. Consequently, we next examined whether Ca$^{2+}$-induced retention of E2F-5 in differentiated keratinocytes requires association with p130. It is well established that interactions of E2F with pRB family proteins are disrupted by several viral oncoproteins. The adenovirus E1A protein binds pRB family proteins, thereby releasing E2F (reviewed in Ref. 33). Thus, we examined the subcellular localization of E2F-5 in differentiated keratinocytes in which we induced exogenous expression of E1A by adenovirus-mediated gene transfer and observed maintenance of nuclear distribution of E2F-5 (Fig. 1), indicating that nuclear retention of E2F-5 does not require interactions with pRB family proteins.

E2F-5 shares extensive structural homology and biochemical properties with E2F-4, and these two proteins have frequently been grouped together. Furthermore, nuclear localization of E2F-4 has been associated with cell cycle withdrawal and myoblast differentiation. In contrast to E2F-5, E2F-4 mRNA and protein tend to decrease during differentiation in keratinocytes (25, 26). To investigate if there are also differences between E2F-4 and E2F-5 regulation of subcellular localization in keratinocytes, we conducted immunofluorescence assays. We observed that E2F-4 always distributes throughout the cell, irrespective of the differentiation status of the keratinocytes (Fig. 1). These patterns of distribution differ from the constitutively nuclear localization of E2F-1, -2, and -3 in keratinocytes, which we have determined by analysis of fractionated cell extracts (data not shown), and is similar to their distribution in other cell types (14, 15, 35).

Nuclear Import of E2F Proteins—Given the substantial differences in steady-state subcellular distribution of the various E2F proteins, we first investigated the mechanisms mediating their entry into the nucleus, using GST-E2F fusion proteins in in vitro nuclear import assays on digitonin-permeabilized cells. This system has been extensively used to characterize basic nuclear import mechanisms of a variety of proteins. We observed that GST fusion proteins containing sequences corre-

![Cellular distribution of endogenous E2F-4 and E2F-5 in keratinocytes.](http://www.jbc.org/)

**Fig. 1.** Cellular distribution of endogenous E2F-4 and E2F-5 in keratinocytes. Primary mouse keratinocytes were cultured on glass coverslips and maintained undifferentiated (0.05 mM Ca$^{2+}$) or induced to differentiate by treatment with Ca$^{2+}$ (1 mM, final) for 24 h. Where indicated, the cells were infected with an E1a-encoding adenovirus 24 h following culture in growth medium containing 1 mM Ca$^{2+}$. The cells were then fixed and processed for indirect immunofluorescence microscopy using anti-E2F-4 or anti-E2F-5 antibodies, as indicated. Cell nuclei were visualized with Hoechst 33258 (1 μg/ml, final).

sponding to E2F-1, -2, or -3 localized to the nucleus in ≥97% of permeabilized cells (Fig. 2), consistent with the reported existence of a NLS in these proteins (14, 15, 35). Surprisingly, and in contrast with their predominantly cytoplasmic distribution in many intact cells, E2F-4 and E2F-5 GST fusion proteins were found in the nucleus in about 60% of the cells (Fig. 2). The nuclear distribution of all E2F containing GST fusion proteins also differed from GST alone, which was largely cytoplasmic, suggesting that sequences in all the E2F proteins examined contain nuclear localization domains.

Nuclear Uptake of E2F-5 and E2F-2 Occurs through Nuclear Pore Complexes—We next used in vitro import assays to examine the mechanisms involved in E2F-5 nuclear entry, contrasting them with those of E2F-2 translocation. We chose E2F-2 as a prototype of E2F proteins containing a consensus NLS. We initially verified that nuclear E2F entry was indeed mediated and not due to simple diffusion. To this end, we determined the
with Hoescht 33258 (1 µg/ml). Nuclei were visualized using a rabbit polyclonal anti-GST antibody followed by a FITC-conjugated goat anti-rabbit IgG. Nuclei were visualized with Hoescht 33258 (1 µg/ml).

**Materials and Methods**

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To confirm that GST-E2F family proteins are not essential for nuclear import (Fig. 5). This conclusion was confirmed by the ability of a C terminus deletion mutant containing only amino acids 1–179 to enter the nucleus (~90% nuclear localization, Fig. 5).

We next tested the ability of E2F-5-(105–339) to move into the nucleus. This is a mutant that lacks the N terminus, including the DNA-binding domain, but contains the amino acids required for interaction with both DP and pRB family proteins. This mutant was incapable of nuclear entry (Fig. 5), indicating that sequences in the N terminus of E2F-5 are required for import and that the regions that mediate interactions with DP or pRB family proteins are not sufficient for this process.

To better delineate the region in E2F-5 necessary for nuclear import, we also tested a mutant containing only the first 56 amino acids from the N terminus of the protein. We found that GST-E2F-5-(1–56) was efficiently imported, showing nuclear localization when fused to GST (Fig. 5). To rule out the possibility that GST-E2F-5-(1–56) permeated the nucleus simply by diffusion due to its small size, we also conducted import assays in the presence of WGA, previously shown to impair nuclear import of wild type E2F-5. Inactivation of transport through the nuclear pore with WGA abolished E2F-5-(1–56) uptake, indicating that the first 56 amino acids in E2F-5 correspond to a bona fide nuclear localization domain, capable to induce nuclear import when fused to GST (Fig. 5). These results also demonstrate that nuclear localization of E2F-5 in permeabilized cells does not depend on its ability to bind DNA, DP, or pRB family proteins. To determine if this 56-amino acid fragment also functioned as a nuclear localization domain in intact cells, we conducted transient transfections of a GFP-E2F-5-(1–56) fusion protein in undifferentiated keratinocytes, HeLa, HEK293, and IMDF cells. GFP is a small protein that can simply diffuse into and out of the nucleus due to its size. In all the cells tested, we found that GFP was exclusively cytoplasmic.
in about 50% of the cells (Table I and data not shown). The presence of the E2F-5 nuclear localization region (amino acids 1–56) yielded a GFP fusion protein that was able to localize to the nucleus in 98% of the cells (Table I). Deletion of any part of this 56-amino acid region resulted in GFP fusion proteins that exhibited subcellular distributions indistinguishable from GFP alone (data not shown). Thus, it appears that the N-terminal 56 amino acids in E2F-5 constitute the smallest fragment capable of inducing nuclear uptake of a heterologous protein.

**E2F-5 Is Subject to Nucleocytoplasmic Shuttling through CRM1-mediated Export in Intact Cells**—The cytoplasmic distribution of E2F-5 in undifferentiated keratinocytes and other cell types contrasts with its inherent ability to enter the nucleus. Hence, we postulated that E2F-5 shuttled between the nucleus and the cytoplasm, and that its steady-state cytoplasmic distribution reflected the dominance of nuclear export mechanisms. To test this hypothesis, we induced exogenous expression of GFP-tagged E2F-5 in a variety of cell types, including undifferentiated keratinocytes, MCF-7, HeLa, HEK293, and IMDF. GFP-E2F-5 was exclusively cytoplasmic in all cell types tested (Fig. 6 and data not shown). To investigate if E2F-5 was actively exported from the nucleus through CRM1-mediated pathways, we examined its distribution in cells treated with the CRM1 inhibitor leptomycin B. Under these conditions, E2F-5 exhibited an exclusively nuclear localization in 97–100% of the cell types tested, thus confirming the

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**Fig. 3. Nuclear import E2F-2 and E2F-5 is mediated through the nuclear pore complex.** *In vitro* import assays of GST-E2F-2 or GST-E2F-5 fusion proteins using digitonin-permeabilized HeLa cells and cytosolic HeLa cell extracts. 22°C, transport with cells and Complete Transport Buffer at 22 °C; 4°C, cells and Complete Transport Buffer at 4 °C; -NTP, cells and transport buffer with apyrase and without ATP and GTP; WGA, pre-treatment of permeabilized cells with WGA to inactivate the NPC prior to import assays in Complete Transport buffer at 22 °C. The indicated GST-E2F fusion proteins were detected by indirect immunofluorescence using rabbit polyclonal anti-GST serum, followed by a FITC-conjugated goat anti-rabbit IgG. Histograms on the right side of the micrographs indicate the percentage of nuclei that were positive (%N) or negative (%C) for uptake of the indicated E2F fusion protein. The results were calculated from four to nine experiments conducted on triplicate samples and by evaluating E2F subcellular localization in 400–1000 cells/sample.
Fig. 4. Schematic of E2F-5 deletion mutants used for in vitro import assays. Fragments of the mouse E2F-5 cDNA were ligated in-frame with GST. The numbers indicate the positions of the E2F-5 amino acid residues included in each mutant. Δ315–332 is an internal deletion mutant lacking amino acid residues 315–332, which inactivates the binding to p130.

operation of active nuclear export mechanisms for E2F-5 (Fig. 6 and Table I). Similar results were obtained with an E2F-5 construct containing a FLAG tag in place of GFP (data not shown).

Characterization of the Nuclear Export Signal in E2F-5—Examination of the E2F-5 amino acid sequence revealed the presence of a potential NES at positions 123–132 (LKAIEIDELEL), just at the beginning of the dimerization domain, which conforms to the rev-type consensus NES LX3/U Lx3/LXL (reviewed in Ref. 36). To investigate the functional significance of this region, we tested a deletion mutant lacking residues 84–128 (E2F-5-(Δ84–128)) and that no longer has the putative NES. This mutant exhibited cytoplasmic distribution in untreated cells and was concentrated in the nucleus in cells treated with leptomycin B, indicating that the nuclear export domain in E2F-5 does not reside in that region (Table I). Consequently, to define the sequences in E2F-5 responsible for nuclear export, we analyzed the subcellular distribution of a panel of E2F-5 C terminus deletion mutants expressed in cells incubated in the presence or absence of leptomycin B (Fig. 6 and Table I). These mutants are schematically depicted in Fig. 7. E2F-5-(1–56) and E2F-5-(1–129) were constitutively nuclear in ≥ 98% of cells in the absence of leptomycin B (Fig. 6 and Table I). These mutants lack the dimerization domain, indicating that interaction with DP proteins is not required for E2F-5 nuclear import, and confirming our earlier results using in vitro import assays on permeabilized cells. In contrast, E2F-5-(1–155) was exclusively cytoplasmic in about 90% of the cells, but concentrated in the nucleus in cells treated with leptomycin B (Fig. 6, Table I), indicating that there is a nuclear export domain in E2F-5 in the 130–154 region. Smaller C terminus deletions yielded mutant proteins that exhibited cytoplasmic or nuclear localization, respectively, in the absence or presence of leptomycin B (Table I). These experiments map a functional nuclear export domain in E2F-5 in the region corresponding to amino acid residues 130–154 and confirm that association of E2F-5 with DP proteins is not a major determinant for nucleocytoplasmic movements of E2F-5 in intact cells.

To specifically investigate the role that binding to pRB family proteins may play in E2F-5 subcellular localization in live cells, we first examined the distribution of a mutant lacking amino acids 315–332, which is incapable of binding to p130. This mutant behaved in a manner indistinguishable from the wild type protein, and exhibited leptomycin B-sensitive export (Fig. 6 and Table I). In a complementary approach, we observed that the N terminus deletion mutant E2F-5-(105–339), which lacks the nuclear localization domain but contains the NES region and p130-binding sequences, was cytoplasmic, irrespective of whether leptomycin B was present or not. The lack of effect of leptomycin B indicates that E2F-5-(105–339) is incapable of entering the nucleus. Together, these studies demonstrate that neither nuclear import nor export of E2F-5 in intact cells has an absolute requirement for interactions with pRB family proteins.

**DISCUSSION**

Differential Regulation of E2F-4 and E2F-5 during Keratinocyte Differentiation—Multiple mechanisms regulate E2F-5 during keratinocyte differentiation, including expression levels, protein-protein interactions (25, 26) and, as shown in this report, subcellular localization. E2F-4 and E2F-5 share extensive sequence homology, and have been proposed to fulfill similar functions in various cell types. Consistent with the concept that different E2F proteins may play distinct cell type-specific roles, E2F-4 and E2F-5 appear to be very differently regulated in keratinocytes. For example, whereas cell cycle withdrawal in serum-starved fibroblasts is associated with nuclear concentration of E2F-4 (15), quiescence in terminally differentiated keratinocytes is not (Fig. 1). Rather, keratinocyte differentiation is associated with up-regulation and nuclear concentration of E2F-5 (Ref. 26 and this report). Distinct changes in E2F subcellular localization have been reported during differentiation of L6 myoblasts (17), in which E2F-1, -3, and -5 are predominantly cytoplasmic, whereas E2F-2 is nuclear and E2F-4 is distributed throughout the cells.

E2F-5 Nuclear Import Does Not Require pRB Family Proteins—In eukaryotes, multiple cellular functions are regulated by the bidirectional transport of macromolecules between the cytoplasm and the nucleus, mediated by nuclear pore complexes. Although molecules smaller than 40 kDa can passively diffuse through the NPC, most macromolecules are transported by active, energy-dependent mechanisms that involve specific interactions of transport factors with either NLS or NES in the substrate proteins (reviewed in Refs. 37 and 38).

In keratinocytes, differentiation induces the nuclear localization of E2F-5 in conjunction with formation of E2F-5-p130 complexes (26). Several mechanisms of E2F-5 nuclear translocation are possible in principle, including direct import of E2F-5 through NPCs, or prior formation of E2F-5-p130 complexes, which then use the NLS present in p130 to translocate through the nuclear pore. Because E2F-4 and E2F-5 lack a consensus NLS, it has been proposed that nuclear localization of these two E2Fs requires interaction with a partner that supplies the NLS in trans (14, 35). However, our studies demonstrate an intrinsic ability of E2F-5 to translocate into the nucleus. Our results also show that interaction with either DP or pRB family proteins is not obligatory for nuclear translocation of E2F-5. We have not ruled out that these proteins may contribute to E2F-5 nuclear localization, because our experiments using an E2F-5 mutant incapable of interacting with pRB family proteins showed a slightly reduced nuclear localization relative to the wild type protein in import assays in vitro. However, our results indicate that E2F-5 nuclear import through interaction with p130 plays a minor role at best.

p130 has recently been shown to shuttle from the nucleus to the cytoplasm following serum stimulation of glioblastoma cells...
Our analyses of fractionated lysates from undifferentiated and differentiated keratinocytes have failed to show p130 in the cytoplasmic fraction, suggesting that the steady-state distribution of p130 in keratinocytes is nuclear. Because E1A expression did not alter the nuclear localization of E2F-5 in differentiated keratinocytes, we conclude that interaction of E2F-5 with p130 is not essential for either nuclear entry or permanence in differentiated keratinocytes. Similarly, overexpression of E1A in U2OS cells did not induce nuclear export of exogenously expressed E2F-5.

In contrast to previous reports that suggest E2F-4 and E2F-5 do not contain intrinsic NLS (19, 40), we find that both proteins can be efficiently imported into the nucleus in import assays using permeabilized cells. We found that a region encompassing the first 56 amino acids in the N terminus of E2F-5 is necessary and sufficient for nuclear targeting. Furthermore, this sequence constitutes a bona fide nuclear localization domain both in vitro and in living cells, because it is capable of directing nuclear import of heterologous proteins, including GST and GFP. Typical NLS appear to have groups of positively charged amino acids, although there is no stringent consensus sequence. The nuclear localization domain in E2F-5 contains a stretch of arginines not conserved with other E2F family members.

The sensitivity of E2F-2 and E2F-5 nuclear import to WGA treatment indicates the involvement of nucleoporins and, consequently, that of the NPC. E2F-2 nuclear import is mediated by a single NLS with a P(A/V)KR(R/K)L(D/E)L motif, which is also present in E2F-1 and E2F-3, but is absent in E2F-5 (14). We have observed that treatment of permeabilized cells and cell extracts with a blocking anti-importin-[H9225] antibody greatly reduces nuclear uptake of E2F-2, but not that of E2F-5. This suggests that the mode of interaction of E2F-2 with the nuclear import machinery may be different from that of E2F-5. Consistent

### Table I

**Subcellular distribution of GFP-tagged E2F-5 mutants in the presence of active or inactive CRM1-mediated nuclear export**

Percentages of cells in which the indicated GFP-tagged E2F-5 mutant proteins were included (N, nuclear) or excluded (C, cytoplasmic) from the nucleus of transfected cells. The data represent the average of four experiments conducted on triplicate samples, in which 1000 cells/sample were evaluated from random microscope fields. Transfected cells were treated with vehicle only (−LMB) or with leptomycin B and cycloheximide (+LMB) for 4 h prior to fixation and analysis by direct fluorescence from the GFP moiety.

| E2F-5 mutant | −LMB | +LMB |
|--------------|------|------|
|              | %N   | %C   | %N   | %C   |
| WT (1–339)   | 3    | 97   | 98   | 2    |
| 1–56         | 98   | 2    | 96   | 4    |
| 1–129        | 98   | 2    | 97   | 3    |
| 1–155        | 12   | 88   | 96   | 4    |
| 1–176        | 14   | 86   | 97   | 3    |
| 1–198        | 5    | 95   | 97   | 3    |
| 1–225        | 4    | 96   | ND   | ND   |
| 1–252        | 2    | 98   | 98   | 2    |
| 1–279        | 2    | 98   | 98   | 2    |
| 1–305        | 3    | 97   | 95   | 5    |
| 105–339      | 2    | 98   | 3    | 97   |
| Δ84–128      | 4    | 96   | 97   | 3    |
| Δ315–332     | 7    | 93   | 97   | 3    |
| GFP          | 50   | 50   | 47   | 53   |

* WT, wild type E2F-5.

**Fig. 5. In vitro nuclear import of E2F-5 mutants.** Nuclear import assays were conducted with the indicated wild type or mutant GST-E2F-5 fusion proteins on digitonin-permeabilized HeLa cells and Complete Transport Buffer (see “Experimental Procedures”) at 22 °C. The GST-E2F-5 mutant proteins were detected by indirect immunofluorescence using a rabbit polyclonal anti-GST antibody followed by a FITC-conjugated goat anti-rabbit IgG. Nuclei were visualized with Hoescht 33258 (1 μg/ml). Histograms represent data from four experiments conducted on triplicate samples and indicate the percentage of cells positive (%N) or negative (%C) for nuclear uptake of the GST fusion proteins. WT indicates wild type E2F-5; WGA indicates import assays conducted with cells and extracts pre-treated with WGA to inactivate transport through the nuclear pore.

2 L. Dagnino, unpublished.

2 M. Apostolova and L. Dagnino, unpublished observations.
with this concept, it is known that some proteins enter the nucleus directly bound to importin-α, whereas others bind importin-β, which then serves as an adaptor with importin-β (37, 41). Further experiments are necessary to define the exact interactions of different E2F factors with components of the nuclear import machinery.

Mechanism of Regulated E2F-5 Export—Multiple functional domains contribute to determine the subcellular distribution of E2F-5, which depends, in part, on the balance between import and export. We find that in many different cell types (HeLa, MCF-7, HEK293, dermal fibroblasts, and undifferentiated keratinocytes) E2F-5 export is mediated by a leptomycin B-sensitive, CRM1-dependent pathway, which is also present in U2-OS osteosarcoma cells (18). We have identified a nuclear export domain located in the region corresponding to amino acid residues 130–154 in mouse E2F-5. Sequences in this region are poorly conserved with E2F-4, do not conform to consensus, Rev-type leucine-rich NES (Lx3[L/I]x2L), but have a number of hydrophobic residues (LKERE-LQDQQKLWLQDSI). E2F-4 is also exported by CRM1-dependent mechanisms, through two domains further upstream, in the vicinity of the DNA-binding region (18). Intriguingly, although the reported nuclear export signal sequences of E2F-4 are highly conserved and located within the N-terminal 129-amino acid residues in E2F-5, they are devoid of nuclear export properties in the context of the latter protein (Table I). This emphasizes the idea that, despite substantial sequence similarities, E2F-4 and E2F-5 may be subjected to distinct regulatory processes and likely fulfill distinct biological functions in many cell types.

The biological role of E2F-5 nucleocytoplasmic shuttling in keratinocytes remains to be definitely established. Ectopic expression of E2F-5 in keratinocytes leads to inhibition of DNA synthesis, suggesting a proliferation inhibitory role for this protein in differentiating keratinocytes (26). A model can be proposed, in which E2F-5 is maintained out of the nuclear compartment in proliferating cells via CRM1-mediated export, but is efficiently imported and maintained in the nucleus of differentiating keratinocytes, to participate in the induction of irreversible cell cycle withdrawal upon terminal differentiation. Whether keratinocyte differentiation activates mechanisms that allow increased nuclear import or decreased nuclear export remains to be determined.
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REFERENCES

1. Muller, H., and Helin, K. (2000) Biochim. Biophys. Acta 1470, M1–M12
2. Nevins, J. R. (2001) Hum. Mol. Genet. 10, 699–703
3. Harbour, J. W., and Dean, D. C. (2000) J. Biol. Chem. 275, 10887–10892
4. Martine-Balbas, M. A., Bauer, U. M., Nielsen, S. J., Brehm, A., and Kouzarides, T. (2000) EMBO J. 19, 662–671
5. Martinez-Balbas, M. A., Bauer, U. M., Nielsen, S. J., Brehm, A., and Kouzarides, T. (2000) EMBO J. 19, 662–671
6. Verona, R., Moberg, K., Estes, S., Starr, M., Vernon, J. P., and Lees, J. A. (1997) Mol. Cell. Biol. 17, 7288–7282
7. Lindeman, G. J., Gautha, S., Livingston, D. M., and Ginsberg, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5993–5998
8. Muller, H., Moroni, M. C., Vigo, E., Petersen, B. O., Bartek, J., and Helin, K. (1997) Mol. Cell. Biol. 17, 5508–5520
9. Gill, R. M., and Hamel, P. A. (2000) J. Cell Biol. 148, 1187–1201
10. Gautha, S., Lees, J. A., Lindeman, G. J., and Livingston, D. M. (2001) Mol. Cell. Biol. 21, 1384–1392
11. Mancia, J., Wu, C. L., Illenye, S., Harlow, E., and Heintz, N. H. (1996) J. Cell Biol. 139, 1717–1726
12. Zinkel, S., and Fuchs, E. (1994) Semin. Cancer Biol. 5, 77–90
13. Fuchs, E. (1990) Cell. Biol. Int. Commun. 14, 1028–1035
14. D’Souza, J. J., Pajak, A., Balazsi, K., and Dagnino, L. (2001) J. Biol. Chem. 276, 23531–23538
15. Krek, W., Ewen, M. E., Shibasaki, S., Arany, Z., Kaelin, W. G. J., and Livingstone, D. M. (1994) Cell 78, 161–172
16. Muller, H., Moroni, M. C., Vigo, E., Petersen, B. O., Bartek, J., and Helin, K. (1995) Oncogene 10, 39–48
17. Krek, W., Xu, G., and Livingston, D. M. (1995) Oncogene 11, 31–38
18. Martin, J. R., Nevins, J. R., and Neel, J. L. (1992) Mol. Cell. Biol. 12, 1079–1091
19. Adam, S. A., Marr, R. S., and Gerace, L. (1990) J. Biol. Chem. 265, 10919–10924
20. Fuchs, E. (1990) Curr. Opin. Cell Biol. 2, 10892–11070
21. Nakielny, S., and Dreyfuss, G. (1999) Cell 99, 1077–1090
22. Gorlich, D., and Kutay, W. (1999) Annu. Rev. Cell Dev. Biol. 15, 607–660
23. Mattaj, I. W., and Englmeier, L. (1998) Annu. Rev. Biochem. 67, 265–306
24. Chestukhin, A., Litovchick, L., Rudich, K., and DeCaprio, J. A. (2002) Mol. Cell. Biol. 22, 453–468
25. Dagnino, L., Fry, C. J., Bartley, S. M., Farnham, P., Gallie, B. L., and Phillips, R. A. (1997) Cell Growth Differ. 8, 553–563
26. Classon, M., and Dyson, N. (2001) EMBO J. 14, 307–313
27. Advani, S. J., Weichselbaum, R., and Roop, D. R. (1988) Ann. N. Y. Acad. Sci. 548, 191–196
28. Advani, S. J., Weichselbaum, R. R., and Roizman, B. (2000) J. Biol. Chem. 275, 3373–3379
29. Advani, S. J., Weichselbaum, R. R., and Roizman, B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7773–7778
Active Nuclear Import and Export Pathways Regulate E2F-5 Subcellular Localization

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