Identification of Flotillin-2, a Major Protein on Lipid Rafts, as a Novel Target of p53 Family Members

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Abstract

p73 and p63 are members of the p53 gene family and have been shown to play an important role in development and homeostasis mainly by regulating the transcription of a variety of genes. A subset of these genes encodes secreted proteins and receptors that may be involved in the communication between adjacent cells. We report here that flotillin-2, a major hydrophobic protein on biomembrane microdomain lipid rafts, is a direct transcriptional target of the p53 family member genes. It has been suggested that such rafts could play an important role in many cellular processes including signal transduction, membrane trafficking, cytoskeletal organization, and pathogen entry. We found that the expression of flotillin-2 was specifically up-regulated by either TAp73 or TAp63γ, but not significantly by p53. In addition, flotillin-2 transcription is activated in response to cisplatin in a manner dependent on endogenous p73. By using small interference RNA designed to target p73, we showed that silencing endogenous p73 abolishes the induction of flotillin-2 transcription following cisplatin treatment. Furthermore, we identified a p73/p63-binding site located upstream of the flotillin-2 gene that is responsive to the p53 family members. This response element is highly conserved between humans and rodents. We also found that ectopic expression of TAp73 as well as TAp63 enhances signal transduction by assessing the interleukin-6–mediated phosphorylation of signal transducers and activators of transcription 3. Thus, in addition to direct transactivation, p53 family member genes enhance a set of cellular processes via lipid rafts. (Mol Cancer Res 2008;6(3):395–406)

Introduction

The p53 family consists of three members, p53, p73, and p63. These proteins share a high degree of amino acid sequence similarity and major functional domains. The p53 gene, the first member of the family to be identified, encodes a sequence-specific transcriptional factor playing an important role in suppressing tumorigenic growth by transactivating target genes that facilitate the survival or death of damaged cells (1-4). The other two members, p73 and p63, can also bind to p53 response elements to transactivate a subset of p53 target genes, such as p21 and MDM2, which suggests that the p53 family members have a potential for functional overlap with p53 itself (5-9).

Despite a certain degree of overlapping functions, p73 and p63 have other activities that are different from p53. Unlike p53 which acts as a classical tumor suppressor, p73 and p63 are rarely mutated in human cancers and their roles in cancer development are complex and controversial (10). The TA isoforms act similarly to p53. In contrast, the ΔN isoforms lack the p53-like transactivation domain, but do in fact retain transactivation activity. The ΔN isoforms are also thought to play a role in blocking the transactivation of target genes of p53 and their respective TA isoforms. In general, therefore, the TA isoforms might be expected to have a role in tumor suppression, whereas increased expression of the ΔN isoforms might be oncogenic. Moreover, studies of knockout mice revealed an unexpected functional diversity among the p53 family genes. p73-deficient mice have neurologic, pheromonal, and inflammatory defects (11). On the other hand, p63-deficient mice have major defects in their limbs and craniofacial development, as well as a striking absence of stratified epithelia (12). In humans, heterozygous germ line mutations in the p63 gene are the cause of ectodactyly, ectodermal dysplasia, and facial cleft syndrome (13), suggesting that p63 is required for limb and epidermal morphogenesis. Additionally, p73α and p63α isoforms have a COOH-terminal region showing similarity with the sterile alpha motif domain, a protein-protein interaction domain found in proteins involved in the regulation of development. Thus, these current data suggest that p73 and p63 are mainly involved in development and differentiation (for review, see refs. 14-16).

This functional diversity among the p53 family suggests the existence of specific factors that are regulated by p73 and p63, but not by p53. To date, many genes have been identified as p53 targets (17), but little is known about the target genes specifically activated by p73 and/or p63 (18-26). Thus, identifying the novel specific targets of p73 and p63 is an important research goal.
important step to better understand the roles of these genes in normal development. It also remains to be determined how different stimuli selectively recruit one or more members of the p53 family to achieve specialized transcriptional responses in specific cellular contexts.

Here, we report the identification of flotillin-2 as a direct transcriptional target of the p53 family members p73 and p63. The human flotillin-2 gene contains a response element specific for p73 and p63, which is highly conserved between humans and rodents. Flotillin-2 is a major hydrophobic protein on microdomain lipid rafts. A large number of reports have suggested that lipid rafts play a role in a wide range of important biological processes, including numerous signal transduction pathways, apoptosis, cell adhesion and migration, synaptic transmission, organization of the cytoskeleton, and protein sorting during both exocytosis and endocytosis (27). We also found that ectopic expression of p53 family members enhances interleukin-6 (IL-6)—mediated signaling of signal transducers and activators of transcription 3 (STAT3). Moreover, flotillin-2 knockdown inhibited p63-mediated STAT3 activation. Taken together, our data reveals a novel link between the p53 family and signal organization of the cytoskeleton, and protein sorting during both exocytosis and endocytosis (27). We also found that ectopic expression of p53 family members enhances interleukin-6 (IL-6)—mediated signaling of signal transducers and activators of transcription 3 (STAT3). Moreover, flotillin-2 knockdown inhibited p63-mediated STAT3 activation. Taken together, our data reveals a novel link between the p53 family and signal organization of the cytoskeleton, and protein sorting during both exocytosis and endocytosis (27).

Results

*TAp73 and TAp63 Induce the Expression of Flotillin-2 mRNA and Protein*

To express the human p53 family genes, we generated replication-deficient recombinant adenoviral vectors Ad-p53, Ad-p73α, Ad-p73β, Ad-p63γ, and Ad-p63α for the p53, TAp73α, TAp73β, TAp63γ, and TAp63α genes, respectively (28). To determine the relative efficiency of adenovirus-mediated gene transfer, cells were infected with adenovirus containing the bacterial *lacZ* gene (*Ad-lacZ*). We used several human cancer cell lines that showed highly efficient gene transfer, with 90% to 100% of the cells staining for β-galactosidase activity at a multiplicity of infection (MOI) of 25 to 100. After infection with Ad-p53, Ad-p73α, Ad-p73β, Ad-p63γ, and Ad-p63α, expression of exogenous p53, TAp73α, TAp73β, TAp63γ, and TAp63α were observed, respectively (in Saos2 cells, for example; Fig. 1). We used TAp73β and TAp63γ isoforms in this study because we and others have shown that transcription of a p53 response reporter gene was activated more strongly in TAp73β than TAp73α and in TAp63γ than TAp63α, respectively (6, 29, 30). In an effort to identify specific targets regulated by p73 and p63, we did Affymetrix Genechip analyses and compared expression patterns in a human osteosarcoma cell line Saos-2 transfected separately with Ad-p53 and Ad-p63γ (data not shown). Using this approach, we found that the *flotillin-2* gene reproducibly up-regulated at least 4-fold in p63-transfected cells as compared with p53-transfected cells. Using the *flotillin-2* pDNA as a probe, we performed Northern blot analysis using seven human cancer cell lines including Saos-2, SW480 and DLD1 (colorectal cancers), U373 and LN308 (gliomas), and BM314 and MKN28 (stomach cancers). The status of the endogenous *p53* gene in these lines is mutant for SW480, DLD1, U373, LN308, BM314 and MKN28, and p53-null for Saos2. As shown in Fig. 2, *flotillin-2* mRNA was clearly induced by TAp73α, TAp73β, and TAp63γ in all cell lines tested, but not significantly by p53. Although flotillin-2 was still induced in response to TAp63γ in two of the four cell lines tested (U373 and SW480), the degree of induction was less remarkable than that of TAp73 isoforms. In contrast, p53 induced *p21* significantly in all cell lines tested (Fig. 2, middle).

To determine whether the increase in *flotillin-2* mRNA was accompanied by an increase in protein expression, we then examined the level of flotillin-2 protein by immunoblot using an antibody against human flotillin-2. Figure 3A shows increased total cellular flotillin-2 protein in p73α-transfected, p73β-transfected, or p63γ-transfected cells, consistent with the Northern blot analyses. In addition, we did time course experiments to examine the expression levels of flotillin-2 protein. Saos-2 cells were infected with Ad-p53, Ad-p73β, or Ad-p63γ at a MOI of 25, and total protein was collected at the indicated time periods and subjected to immunoblotting with anti–flotillin-2 antibody. As shown in Fig. 3B, the expression of flotillin-2 protein was dramatically increased by infection with Ad-p73β or Ad-p63γ, in a time-dependent manner, but not by infection with Ad-p53. The flotillin-2 protein induction was observed as early as 12 hours after TAp73β or TAp63γ overexpression. Even when Saos-2 cells were infected with Ad-p53 at a MOI of 100, flotillin-2 was not significantly induced by p53 (Fig. 3B, right). Furthermore, these data were confirmed by immunofluorescence staining that showed increased endogenous flotillin-2 protein in p63γ-transfected Saos-2 cells (Fig. 3C). The subcellular localization of the flotillin-2 protein coincided with that reported previously (31). Altogether, the results presented here suggest that the *flotillin-2* gene is a specific target of p73 and p63.

**Induction of Flotillin-2 in Human Cancer Cells in an Endogenous p73-Dependent Manner**

The effect of p53 family members on flotillin-2 induction shown above was based on their overexpression. To address whether this effect could occur under more physiologic conditions, we used cisplatin, a DNA-damaging agent known to activate endogenous p73 through transcriptional activation and protein stabilization (32-35). As seen in Fig. 4A,
endogenous p73α protein increased in U373 and SW480 cells following exposure to 10 μmol/L of cisplatin for 24 hours. We then examined whether flotillin-2 expression could be induced after cisplatin treatment of these two cell lines. Northern blot analysis showed a parallel increase of flotillin-2 mRNA in U373 and SW480 cells following cisplatin treatment (Fig. 4A, FLOT-2). To further validate that flotillin-2 mRNA increase after cisplatin treatment was dependent on p73, we down-regulated endogenous p73 specifically using double-stranded RNA oligonucleotides (small interference RNA; siRNA) corresponding to a part of the p73 cDNA sequence. Four different siRNAs were synthesized: si-p73-1, si-p73-2, si-p73-3, and si-p73-4 (see Materials and Methods for details). These p73 siRNAs are expected to inhibit the gene expressions of both TAp73 and ΔNp73. Transfection of each of p73 siRNAs separately in DLD1 and SW480 cells in vitro strongly inhibited the accumulation of p73 protein following cisplatin treatment without affecting the levels of β-actin [Fig. 4B, CDDP(+)]. Notably, p73 siRNAs, but not untreated [siRNA (-)] or control siRNA, inhibited basal expression as well as the induction of flotillin-2 mRNA after cisplatin treatment (Fig. 4B). We also analyzed the endogenous mRNA expression of TA and ΔN.
isoforms of p73 using semiquantitative reverse transcription-PCR (RT-PCR). mRNA expression of ΔNp73, a dominant-negative isoform, was low in these two cell lines (Fig. 4B, TAp73 and ΔNp73; bottom). These results show that activation of endogenous TAp73 following cisplatin treatment mediates the induction of the flotillin-2 gene.

Identification of a Specific Binding Sequence for p73 and p63 in the Flotillin-2 Gene

To determine whether flotillin-2 is a direct target of transcriptional activation by p73, we searched for consensus p53-binding sequences in the genomic locus encoding human flotillin-2 (GenBank accession number, NC000017), as the p73 and p63 proteins could potentially bind to the consensus sequences (5-8). We found four putative p53-binding sites within 10 kb and around exon 1 of the flotillin-2 gene. These four candidate sequences were identified at nucleotide positions −4029, −3637, +526 and +7230, where +1 represents the transcription start site (Fig. 5A). Subsequently, we did chromatin immunoprecipitation (ChIP) assays to determine whether the p73 and p63 proteins could bind to these candidate sites in vivo. For the ChIP assay, we used Saos-2 cells infected with a control adenovirus Ad-LacZ and adenoviruses expressing FLAG-tagged p53 family genes (Fig. 5B, left; Ad-Fp53, Ad-Fp73a, Ad-Fp73β, Ad-Fp63β, and Ad-Fp63α). Cross-linked chromatin complex from these cells was immunoprecipitated with an anti-FLAG monoclonal antibody (mAb). We then measured the abundance of candidate sequences within the immunoprecipitated complexes by PCR amplification. The ChIP assay revealed that one DNA fragment containing the candidate sequence at −4029 was reproducibly present in the immunoprecipitated complex containing TAp73α, TAp73β, or TAp63γ protein (Fig. 5B, right; top row, lanes 11, 13, and 15). We designated this p73/p63-binding sequence RE-FLOT2 (for response element in flotillin-2). The RE-FLOT2 consists of three copies of the 10-bp consensus p53-binding motif separated by 0 and 2 bp (see Fig. 5A). In contrast, p53 protein binding to the RE-FLOT2 site was not significant in Ad-Fp53–infected cells, as assessed by the ChIP assay with an anti-FLAG antibody and subsequent PCR amplification (Fig. 5B, right; top row, lane 9). The other candidate sequences were amplified in the input-positive control for PCR, but not in the immunoprecipitates (for example, +526 in Fig. 5B). As a positive control for the ChIP assay, we analyzed the interaction of p53 family proteins with the MDM2 promoter that contains a p53 response element. As expected, p53, p73, and p63 proteins resided at the MDM2 promoter in vivo (Fig. 5B, bottom; lanes 9, 11, 13, 15, and 17). The results of these ChIP assays indicate that both

**FIGURE 4.** Regulation of flotillin-2 expression by endogenous p73. A. Induction of flotillin-2 mRNA in response to cisplatin (CDDP) treatment. Anti-p73 immunoblots are shown containing whole extracts (20 μg) from U373 and SW480 cells untreated or treated with 10 μmol/L of cisplatin for 24 h. Endogenous p73 protein accumulated in response to cisplatin treatment in both cell lines (TAp73). To verify equal loading, the blots were reprobed with an antibody against control protein β-actin (actin). Northern blot analysis shows an elevation of flotillin-2 mRNA levels in cells exposed to cisplatin for 24 h (FLOT2). Ethidium bromide staining of 28S ribosomal RNA (28S) shows that equal amounts of RNA were loaded in each lane (bottom). B. p73 siRNA antagonizes the activity of cisplatin on flotillin-2 induction. DLD1 and SW480 cells were transfected twice with p73 siRNA (si-p73-1, si-p73-2, si-p73-3, and si-p73-4) or control siRNA (si-cont) using OligofectAMINE or untransfected [siRNA (−)]. Twenty-four hours after the last transfection, cells were exposed to 10 μmol/L of CDDP (+) or left untreated (−). After 24 h, cell lysates were prepared and analyzed for p73 and β-actin by immunoblot (TAp73 and actin). Semiquantitative RT-PCR revealed that p73 siRNA inhibits flotillin-2 mRNA induction by cisplatin (FLOT-2). Transcription of the GAPDH gene was examined as a negative control (GAPDH).
TAp73 and TAp63 proteins could be selectively associated with the RE-FLOT2 site in Saos-2 cells in vivo.

To determine whether the RE-FLOT2 sequence confers p73- or p63-dependent transcriptional activity, we did a heterologous promoter-reporter assay using a luciferase vector pGL3-RE-FLOT2 prepared by cloning the oligonucleotide corresponding to RE-FLOT2 upstream of a minimal promoter (see Materials and Methods). Saos-2, H1299, and HEK293 cells were

**FIGURE 5.** A response element for p73 and p63 in the flotillin-2 gene. A. The position and nucleotide sequence of a p73/p63 response element RE-FLOT2 (open boxes, exons). RE-FLOT2 is located 4029 bp upstream of the first exon and consists of three copies of the consensus 10 bp motif of the p53-binding sequence. Upper case letters, consensus sequences; "-", spacer sequences between the 10 bp motifs; lower case letters, mismatches with the consensus sequence. Alignment of the conserved p73/p63 binding sites in chimpanzee, mouse, and rat sequences from the FLOT2 gene (bottom). Mismatched nucleotides from the human genome are underlined. A mutated sequence corresponding to potentially critical nucleotides of RE-FLOT2 used in the luciferase assay is also indicated (RE-FLOT2-mut). Species and sequence accession numbers are as follows: human, NT010799; chimpanzee, AADA01132584; mouse, NT096135; rat, NT010799; R, purine; Y, pyrimidine; W, adenine or thymine.

B. TAp73α, TAp73δ, and TAp63γ proteins bind to the RE-FLOT2 site in vivo. Expression of FLAG-tagged p53, p73, and p63 in adenovirus-infected Saos-2 cells (left). Cells were infected with adenovirus at a MOI of 25 and were harvested 24 h after infection. Immunoblot analysis was done on lysates (10 μg) of cells infected with Ad-lacZ, Ad-Fp53, Ad-Fp73α, Ad-Fp73δ, Ad-Fp63α, Ad-Fp63γ, and Ad-Fp63δ. In vivo recruitment of p63 and p73 proteins to the RE-FLOT2 site (right). Cross-linked chromatin was extracted from Saos-2 cells after infection with Ad-lacZ, Ad-Fp53, Ad-Fp73α, Ad-Fp73δ, Ad-Fp63α, and Ad-Fp63δ, and then immunoprecipitated with an anti-FLAG antibody. The immunoprecipitated material was amplified using primers specific for RE-FLOT2 (RE-FLOT2) and the MDM2 promoter (2nd panel). "Input chromatin", a portion of the sonicated chromatin prior to immunoprecipitation was used as a positive control for the PCR (lanes 1-6). Immunoprecipitates in the absence of antibody [Ab (-), lanes 8, 10, 12, 14, 16, and 18] served as negative controls. The DNA fragment containing the RE-FLOT2 sequence was amplified in the immunoprecipitated samples using Ad-Fp73α, Ad-Fp73δ, and Ad-Fp63α-infected cells (RE-FLOT2; lanes 11, 13, and 15). The other candidate sequences were amplified in the input control, but not in the samples immunoprecipitated with an anti-FLAG antibody (for example, +526). C. The RE-FLOT2 sequence is responsive to p73 and p63. H1299, Saos-2, or 293 cells were transiently transfected with the pGL3-promoter vector containing the RE-FLOT2 or its mutant (RE-FLOT2-mut) along with pNL-TK by using LipofectAMINE 2000 reagent. Cells were cotransfected with a control pcDNA3.1 vector or a vector that expresses p53, TAp73α, and TAp63γ 24 h prior to performing the luciferase assay. Luciferase activity was measured using the Dual-Luciferase Reporter Assay system with the Renilla luciferase activity as an internal control. All experiments were done in triplicate; columns, mean; SD, bars. D. Regulation of flotillin-2 transcription by endogenous p73. ChIP assay for the presence of p73 protein at RE-FLOT2 was carried out on nontreated (CDDP (-); lanes 4 and 5) and cisplatin-treated (CDDP (+); lanes 6 and 7) Saos-2 and DLD1 cells.
Indeed responsible for p63 (Fig. 6A). Moreover, to test whether the p53 family is specifically reduced in the mRNA levels of p73 for 3 consecutive days. Semiquantitative RT-PCR revealed a siRNA, and cells were transfected with siRNAs every 24 hours sequence homology with any human gene was used as a control were designed to target all isoforms. An siRNA lacking si-mp73-2) and p63 (si-mp63-1 and si-mp63-2). These siRNAs used double-strand siRNAs to knock down p73 (si-mp73-1 and induction of the Flotillin-2 protein was activated STAT3, and then treated with 10 ng/mL of Flotillin-2 is one of the ubiquitous markers of lipid rafts, which act as membrane platforms for regulating signal transduction, including IL-6 receptor signaling (36). The released IL-6 preferentially activates STAT3 protein with the phosphorylation of Tyr705 via the JAK signaling pathway. We then investigated the role of the p53 family members on IL-6–STAT3 signal activation as a marker of biological functions of lipid rafts. Figure 7A shows an up-regulation of the Flotillin-2 protein in the raft-rich membrane components of Saos-2 cells after Ad-p73β or Ad-p63γ infection, but not after infection of Ad-p53; these results are consistent with those of the Northern blot analysis (Fig. 2). Moreover, we tested whether Flotillin-2 induction mediated by p63 could promote IL-6–mediated signaling, as measured by STAT3 activation. Saos-2 cells were transfected separately with Ad-lacZ, Ad-p53, and Ad-p63γ at a MOI of 25. After 16 hours, cells were incubated in serum-free minimal essential medium for 3 hours to reduce basal levels of activated STAT3, and then treated with 10 ng/mL of recombinant human IL-6 in serum-free medium for 30 minutes. In the absence of IL-6, transduction of Ad-p53 or Ad-p63γ did not stimulate STAT3 phosphorylation (Fig. 7B, p-STAT3, lanes 3 and 5). In p63γ-transfected cells, a high level of Stat3 Tyr705 phosphorylation was induced by IL-6 treatment (Fig. 7B, p-STAT3, lane 6). The specificity for the response to IL-6 was then confirmed using a neutralizing anti–IL-6R antibody. When cells were preincubated with this neutralizing antibody prior to IL-6 treatment, IL-6–mediated phosphorylation of STAT3 protein was inhibited (lane 7). Additionally, we treated cells with filipin III, a lipid raft inhibitor that depletes cholesterol and destroys lipid rafts. As expected, filipin III

transiently cotransfected with pGL3-RE-FLOT2 together with a p53-, p73β-, or p63γ-expressing plasmid. Figure 5C shows that luciferase activity from pGL3-RE-FLOT2 is higher in cells cotransfected with either TAp73β or TAp63γ than in those cotransfected with p53. Negative control reporter plasmid pGL3-RE-FLOT2-mut was constructed by altering potentially critical nucleotides of RE-FLOT2 (see Materials and Methods).

In contrast, the mismatches in the RE-FLOT2 (pGL3-RE-FLOT2-mut) significantly abolished the transactivation by p73β or p63γ (Fig. 5C). Finally, using a ChIP assay, we showed that endogenous p73 protein interacted with the chromatin region containing the specific binding site RE-FLOT2 in Saos-2 and DLD1 cells treated with cisplatin (Fig. 5D, lane 6). These results are consistent with the strong induction of endogenous flotillin-2 by p73 and p63 in cancer cells, and support the idea that flotillin-2 is a direct target of the p53 family members p73 and p63.

If the flotillin-2 gene has an important role in the downstream effect of p53 family members, flotillin-2 activation would be conserved among species. Thus we did a sequence comparison of the p73/p63 response element, RE-FLOT2, among mammalian flotillin-2 genes. Nucleotide sequence comparison revealed that the RE-FLOT2 sequence is well conserved among chimpanzee, mouse, and rat at a nearly identical position within their flotillin-2 gene (see Fig. 5A). Moreover, expression of flotillin-2 mRNA was increased in mouse embryonic fibroblasts (MEF) 24 hours after infection with Ad-p73β or Ad-p63γ, but was not significantly increased after infection with Ad-p53 or Ad-LacZ; these results are similar to the induction of the Jag2 gene, which is a specific target of p73 and p63 (Fig. 6A). Moreover, to test whether the p53 family is indeed responsible for flotillin-2 expression in MEF cells, we used double-strand siRNAs to knock down p73 (si-mp73-1 and si-mp73-2) and p63 (si-mp63-1 and si-mp63-2). These siRNAs were designed to target all isoforms. An siRNA lacking sequence homology with any human gene was used as a control siRNA, and cells were transfected with siRNAs every 24 hours for 3 consecutive days. Semiquantitative RT-PCR revealed a specific reduction in the mRNA levels of p73 and p63 in cells transfected with p73 and p63 siRNAs, respectively (Fig. 6B, TAp73 and TAp63). We then evaluated the expression of flotillin-2 in primary MEFs after treatment with p73 and p63 siRNAs. Flotillin-2 transcript levels were significantly reduced in the p73- or p63-silenced MEFs when compared with mock or untreated cells (Fig. 6B, FLOT2). These findings indicated that transcription of the flotillin-2 gene is regulated by p73 and p63 in mouse normal cells as well as in human cancer cells.

**p53 Family Members Enhance IL-6–Induced STAT3 Phosphorylation via Lipid Rafts**

Flotillin-2 is one of the ubiquitous markers of lipid rafts, which act as membrane platforms for regulating signal transduction, including IL-6 receptor signaling (36). The released IL-6 preferentially activates STAT3 protein with the phosphorylation of Tyr705 via the JAK signaling pathway. We then investigated the role of the p53 family members on IL-6–STAT3 signal activation as a marker of biological functions of lipid rafts. Figure 7A shows an up-regulation of the flotillin-2 protein in the raft-rich membrane components of Saos-2 cells after Ad-p73β or Ad-p63γ infection, but not after infection of Ad-p53; these results are consistent with those of the Northern blot analysis (Fig. 2). Moreover, we tested whether flotillin-2 induction mediated by p63 could promote IL-6–mediated signaling, as measured by STAT3 activation. Saos-2 cells were transfected separately with Ad-lacZ, Ad-p53, and Ad-p63γ at a MOI of 25. After 16 hours, cells were incubated in serum-free minimal essential medium for 3 hours to reduce basal levels of activated STAT3, and then treated with 10 ng/mL of recombinant human IL-6 in serum-free medium for 30 minutes. In the absence of IL-6, transduction of Ad-p53 or Ad-p63γ did not stimulate STAT3 phosphorylation (Fig. 7B, p-STAT3, lanes 3 and 5). In p63γ-transfected cells, a high level of Stat3 Tyr705 phosphorylation was induced by IL-6 treatment (Fig. 7B, p-STAT3, lane 6). The specificity for the response to IL-6 was then confirmed using a neutralizing anti–IL-6R antibody. When cells were preincubated with this neutralizing antibody prior to IL-6 treatment, IL-6–mediated phosphorylation of STAT3 protein was inhibited (lane 7). Additionally, we treated cells with filipin III, a lipid raft inhibitor that depletes cholesterol and destroys lipid rafts. As expected, filipin III

**FIGURE 6.** Induction of the flotillin-2 mRNA by TAp73 and TAp63 in mouse normal cells. A. Semiquantitative RT-PCR analysis showing Flot2 mRNA induction after adenovirus-mediated transfection of proteins from the human p53 family into mouse cells. MEFs were infected with adenovirus at a MOI of 100. Total RNAs were prepared from the cells 24 h after infection. RT-PCR analysis was done as described in Materials and Methods. We also did semiquantitative RT-PCR analysis of a known p53 target gene, p21, and a known p73/p63 target gene, Jag2. GAPDH mRNA levels were examined as a control for integrity of the cDNA. B. MEF cells were transfected with control siRNA (si-cont), p73 (si-mp73-1 and si-mp73-2) or p63 (si-mp63-1 and si-mp63-2) siRNAs, every 24 h for 3 consecutive days using Oligofect-AMINE. Twenty-four hours after the last transfection, expression of the corresponding mRNA was determined by semiquantitative RT-PCR.
Flotillin-2 as a Target Gene for p53 Family Members

Treatment significantly inhibited the IL-6–induced STAT3 activation in p63γ-transfected cells in a dose-dependent manner (lanes 8 and 9). Because neither IL-6R nor gp130 mRNA were significantly induced by p73, p63, or p53 in Saos-2 cells (Fig. 7C), we concluded that p63 modulates IL-6–triggered phosphorylation of STAT3 via flotillin-2 induction. Furthermore, we investigated the role of p53 family members on IL-6–Stat3 signal activation in normal cells. MEF cells were transfected separately with lacZ, p53, TAp73β, and TAp63γ. After 16 hours, we examined the IL-6–mediated tyrosine phosphorylation of STAT3. Figure 7D shows that a high level of STAT3 phosphorylation was induced by IL-6 treatment in both p73β and p63γ-transfected MEF cells (p-Stat3, lanes 6 and 10). Because the flotillin-2 gene is also up-regulated by TAp73β and TAp63γ in MEF cells (Fig. 6), we concluded that TAp73 as well as TAp63 enhances IL-6–triggered phosphorylation of STAT3 via flotillin-2 induction in normal cells.

To assess the significance of flotillin-2 on p63-mediated STAT3 activation, we designed siRNAs targeting human flotillin-2 (si-FLOT2-1 and si-FLOT2-2) and a nonspecific siRNA as a control, and introduced them separately into Saos-2 cells. Twenty-four hours after siRNA transfection, flotillin-2 expression was analyzed by RT-PCR. As shown in Fig. 8A, both flotillin-2 siRNA oligonucleotides effectively blocked flotillin-2 mRNA expression whereas control siRNA had no effect on flotillin-2 expression. Although a low level of flotillin-2 mRNA remained in Saos-2 cells exposed to flotillin-2 siRNAs, the expression of GAPDH mRNA was unaffected by the flotillin-2 or control siRNAs (Fig. 8A). Moreover, pretreatment of Saos-2 cells with flotillin-2 siRNAs strongly inhibited the flotillin-2 protein accumulation in a raft-rich component following TAp63γ overexpression (Fig. 8B). We then examined the effect of the siRNAs on the IL-6–mediated tyrosine phosphorylation of STAT3. Importantly, flotillin-2 siRNA inhibited the induction of p63-mediated STAT3 activation in response to IL-6 (Fig. 8C; p-STAT3, lanes 4 and 6). These results strongly suggest that p53 family members regulate IL-6–STAT3 signal activation mainly by transactivation of the flotillin-2 gene.

Discussion

Here, we present a novel target of the p53 family members, flotillin-2, a major protein on lipid rafts. Northern blot and immunoblot analyses revealed that flotillin-2 expression levels increase dramatically and rapidly in response to the over-expression of TAp73 or TAp63 (Figs. 2 and 3). We also showed that cisplatin treatment induced flotillin-2 mRNA in an endogenous p73-dependent manner (Fig. 4).

The ability of p53 to transactivate target genes containing specific binding sites is thought to be central to its role as a tumor suppressor (1-4). p73 and p63 can also bind to the p53 response elements and up-regulate a subset of p53 target genes. Here, we report the identification of a specific binding site for TAp73 and TAp63 proteins, RE-FLOT2, in the human flotillin-2 gene by a ChIP assay (Fig. 5B). A reporter assay showed that RE-FLOT2 is important for p73/p63-dependent transactivation (Fig. 5C). The RE-FLOT2 sequence consists of three copies of the 10 bp p53-binding motif separated by 0 and 2 bp nucleotides, whereas nearly all p53 response elements previously reported...
contain two adjacent copies of the 10-bp motif (1-4, 37, 38). Our laboratory and others have shown that response elements for p73 and p63 are found in Aquaporin-3 (19), the p73 gene itself (39), Jagged1 (21), IL-4Rα (23), PEDF (25), and Sonic hedgehog (26) genes, and consist of three or more copies of the 10-bp motif separated by spacer sequences. Therefore, spacing between at least three copies of the 10-bp motif may be important for determining the binding specificity of the p53 family member proteins. Consistent with our data, p53 and p63-y differentially activate a luciferase reporter gene under the control of response elements with a one-nucleotide spacer between the two 10-bp motifs having three mismatches from the consensus sequence (40). Moreover, a comparison of the human and rodent flotillin-2 genes revealed that this transactivation is mediated by the conserved RE-FLOT2 sequence upstream of the transcription start site (Fig. 5). We concluded that the flotillin-2 gene is a direct and evolutionarily conserved transcriptional target of the p53 family member genes.

Although several studies have tried to better define the features of the p53 consensus sequence, it is still far from being satisfactorily described. Moreover, further studies will be needed to define whether preferential target genes of p73/p63 have a specific response element with particular structural features which differentiate them from response elements in p53 preferential target genes or whether p53 and p73/p63 interact with different proteins to activate different target genes selectively. It is likely that sequence conformation may provide additional specificity over the primary sequence alone and that conformation based on chromatin structure may provide greater specificity for p53 target genes. Recently, it has been shown that the specificity of the consensus is correlated with the propensity of DNA to bend (41, 42). The energy of DNA bending could contribute significantly to the site-specific binding of p53 and its family members with the response elements.

Lipid raft microdomains represent cholesterol- and glycosphingolipid-enriched dynamic patches in the plasma membrane and organize the plasma membrane into functional units. These raft domains act as platforms for conducting a variety of cellular functions, including cytokine signaling (27, 36, 43). Several protein families have been reported to contribute to the organization and structure of lipid rafts, including membrane integral proteins such as caveolins and flotillins, and lipid chain–modified proteins. The flotillin family contains flotillin-1 and flotillin-2 (44); this family of scaffolding proteins interacts with several lipid-modified proteins involved in signal transduction, including heterotrimeric G proteins (45), protein kinase C isozymes (46), endothelial nitric oxide synthase (47), and estrogen receptors (48). Lipid rafts are also implicated in the assembly of diverse signaling pathways such as those mediated by growth factors and antigen receptors on immune cells (49, 50). Recently, abnormalities in microdomains and associated molecules have been found in various diseases, including neurogenerative disorders (51). Furthermore, lipid rafts function as the entry site in host cells for parasites, bacteria, and viruses (52-55). We show further that p73 and p63 stimulated cytokine signaling mediated by transactivating the flotillin-2 gene, as assessed by IL-6–mediated STAT3 phosphorylation in cancer and normal cells (Figs. 7 and 8). Although our results indicate that flotillin-2 induced by the p53 family member genes exerts IL-6–mediated signaling, largely via lipid rafts, further studies should be done to determine whether the p53 family member proteins play additional roles in normal development through modulating other signaling pathways.

Our results suggest that the two p53 family proteins could play a role distinct from p53 in normal development by inducing specific target genes. IL-6 is a multifunctional cytokine that plays important roles in the immune system, hematopoiesis, and inflammatory responses (56). Various cells, including immune cells, are capable of secreting IL-6 upon stimulation of cytokines or endotoxins during inflammation (57, 58). STAT3 is a member of functionally related STAT proteins that can be activated by cytokines or growth factors and plays a key role in biological processes including cell growth, differentiation, apoptosis, transformation, inflammation, and immune responses (59). The present results suggest an association between two p53 relatives and IL-6–STAT3 signaling, which are not shared by p53, and therefore raise
the possibility that the p53 family members play a role in normal development in part by modulating the IL-6 signaling pathway. Because p73-deficient mice have severe inflammatory defects (11), the p73-directed regulation of IL-6–STAT3 signaling provides a potential mechanism by which p73 could participate in the inflammatory response. Moreover, IL-6, a key proinflammatory cytokine, is involved in a spectrum of diseases, including atherosclerosis, osteoporosis, arthritis, diabetes, and certain cancers (56).

In conclusion, we have found that flotillin-2 is a direct downstream target of p53 family member genes. Because both p73- and p63-deficient mice have developmental defects, future experiments will determine whether flotillin-2 plays a role in normal development. The study described here will open a new window into the role of the p53 family members in the regulation of cellular processes via lipid rafts.

Materials and Methods

Cell Culture and Recombinant Adenovirus

The human cancer cell lines used in this study were purchased from American Type Culture Collection or the Japanese Collection of Research Bioresources. MEFs were prepared according to standard methods (18) from day 9.5 embryos and were maintained in DMEM containing 10% FCS. The generation, purification, and infection procedures of embryos and were maintained in DMEM containing 10% FCS. Prepared according to standard methods (18) from day 9.5 Japanese Collection of Research Bioresources. MEFs were purchased from American Type Culture Collection or the

Flotillin-2 as a Target Gene for p53 Family Members

In conclusion, we have found that flotillin-2 is a direct downstream target of p53 family member genes. Because both p73- and p63-deficient mice have developmental defects, future experiments will determine whether flotillin-2 plays a role in normal development. The study described here will open a new window into the role of the p53 family members in the regulation of cellular processes via lipid rafts.

Microarray

Total RNA was isolated from adenovirus-infected Saos-2 human osteosarcoma cells using TRIzol reagent (Invitrogen). U133 plus 2.0 GeneChip, which contains oligos representing 47,000 unique human transcripts, was purchased from Affymetrix. Experimental procedures were done according to the GeneChip Expression Analysis Technical Manual. Washes and staining of the arrays were done with an Affymetrix Fluidics Station 450, and images were obtained using an Affymetrix GeneChip Scanner 3000. Initial analysis and quality assessment of the array data were done using GeneChip Operating Software 1.1.

Northern Blot and Semiquantitative RT-PCR

For Northern blot analysis, total RNA (10 μg) was electrophoretically separated on a 1% agarose gel containing 2.2 mol/L of formaldehyde and blotted onto a nitrocellulose membrane (Schleicher&Suell). RNA was visualized with ethidium bromide to ensure that it was intact and loaded in similar amounts and to confirm proper transfer. Hybridization was done as described previously (21). cDNA probes for flotillin-2 (nucleotides 1616-1715) and p21 (nucleotides 11-429) were amplified by RT-PCR and were sequenced to confirm proper transfer. Hybridization was done as described previously (21). cDNA probes for flotillin-2 (nucleotides 1616-1715) and p21 (nucleotides 11-429) were amplified by RT-PCR and were sequenced to verify their identity. For semiquantitative RT-PCR analysis, cDNAs were synthesized from 5 μg total RNAs with SuperScript Premiapplication System (Invitrogen). The RT-PCR exponential phase was determined within 20 to 30 cycles to allow semiquantitative comparisons among cDNAs from identical reactions. The PCR conditions involved an initial denaturation step at 94°C for 2 min, followed by 30 cycles (for flotillin-2) or 25 cycles (for GAPDH) of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. Oligonucleotide primer sequences were as follows: human Tαb 73p73 sense 5′-CACCCTGAGGGGACATGACTAC-3′, human ΔNp73 sense 5′-ACCATGCTTACGTGGTAC-3′, human p73 antisense 5′-TTGGTGTGGCAGAGGCA-3′, mouse p73 sense 5′-GAGCACCTGTGGGATTCTTCTAGAG-3′, mouse p73 antisense 5′-GGATATTGGAAGGGATGACAGGCG-3′, mouse p63 sense 5′-AAAGAACCAGCGGATGTGACCAGA-3′, mouse p63 antisense 5′-GGCAATGGAGATGGGAAGGAGGAGA-3′, human flotill-2 sense 5′-CATGCCATCTTTCATCACAGGT-3′, human flotillin-2 antisense 5′-TTATTTTACCAAGAAGGACAGGC-3′, mouse flotillin-2 sense 5′-TGTGTTGGCAGAGGCAA-3′, mouse flotillin-2 antisense 5′-GCTCACGCTTAAGTTTCACT-3′, mouse jagged-2 sense 5′-TGGGATCTCCGCTGAGGTA-3′, mouse jagged-2 antisense 5′-ACGGGCACACAGCACTGTT-3′, mouse p21 sense 5′-CCCGGAGACGTTGAACATTT-3′, mouse p21 antisense 5′-CCTCTCTGACCCACAGCGAA-3′, human and mouse GAPDH sense 5′-ACACCAGCTCCATGCAATC-3′, and
human and mouse GAPDH antisense 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR products were visualized by electrophoresis on 1.5% agarose gels.

**Immunofluorescence Microscopy**

Saos-2 cells (5 × 10^5) were incubated with Ad-Fp63y at a MOI of 25 in a four-well slide chamber (Lab-tek chamber slide, Nalge Nunc). After 16 h, cells were fixed in 4% paraformaldehyde for 30 min. They were then incubated for 2 h with mouse anti–flotillin-2 mAb (1:1,000 dilution) together with rabbit anti-FLAG polyclonal antibody (5 µg/mL). The slides were then stained with Alexa 594–conjugated goat anti-rabbit IgG (Invitrogen) and Alexa 488–conjugated mouse anti–flotillin-2 mAb (1:1,000 dilution) together with mouse IgG and Alexa 488–conjugated goat anti-rabbit IgG (Invitrogen) for 1 h, and counterstained with 4',6-diamidino-2-phenylindole. After each incubation, unbound antibodies were removed by rinsing with PBS. The specimens were examined using a laser-scanning confocal microscope (Fluoview; Olympus, Japan).

**RNA Interference**

Four double-stranded RNA oligonucleotides corresponding to human p73, si-p73-1 (5'-AAUAGUCUCUGUACCCUUUGG-GAGGGUG-3'), si-p73-2 (5'-AAAGACCGUCAAUCUGG-GAAUCCGUC-3'), si-p73-3 (5'-UAUCCACAGAAUAGUAGAAGAGGAUGG-3'), and si-p73-4 (5'-CGGAUCAUCCGACGGACGUGUdTdT-3'); two human FLOT2 siRNAs, si-FLOT2-2 (5'-CCUGUGCCUUCCUGGUAAdTdT-3') and si-FLOT2-3 (5'-GGGAUUCUCUAGAAGAAUUdTdT-3'); two mouse p73 siRNAs, si-mp73-1 (5'-UGAGAUCUGUUAAAGAAGUCAA-3') and si-mp73-2 (5'-CACCACUUCUUGAAGAAUGUA-3'); two mouse p63 siRNAs, si-mp63-1 (5'-CUGCUAGAUCUAGAAGAAUA-3') and si-mp63-2 (5'-UAGCAGUAAGAAGAAACCAAA-3'), and nonspecific control siRNA (non-specific control VII; target sequence 5'-NNACTCCTATCAGCCACGTTCGC-3') were purchased from Invitrogen or Qiagen and used according to the manufacturer's protocols. Cells were plated at 1 × 10^5 cells per well in six-well plates with 2 mL of medium in each well. The following day, siRNAs were transfected using OligofectAMINE (Invitrogen) to result in a final RNA concentration of 50 nmol/L per well. Transfection was repeated 24 h later and a DNA-damaging agent was added to the cells 6 h after the last transfection.

**ChiP Assays**

ChIP assay was done as described (21, 60) using the ChIP Assay Kit (Upstate Biotechnology). Cells (2 × 10^6) were cross-linked with a 1% formaldehyde solution for 15 min at 37°C. The cells were then lysed in 200 µL of SDS lysis buffer, and sonicated to generate 300 to 800 bp DNA fragments. After centrifugation, the cleared supernatant was diluted 10-fold with the ChIP dilution buffer and split into two equal portions: one was incubated with anti-FLAG or anti-human p73 antibody (5 µg) at 4°C for 16 h, and the other portion was used as a control (no antibody). A 1:50 volume of total extract was used for PCR amplification as the input control. Immunocomplexes were precipitated, washed, and eluted as recommended. DNA-protein cross-links were reversed by heating at 65°C for 4 h and the DNA fragments were purified and dissolved in 40 µL of 10 mmol/L Tris(pHs) and 1 mmol/L EDTA. Two microliters of each sample was used as a template. PCR amplification of the consensus p53-binding sequence upstream of the flotillin-2 gene (−4029, renamed RE-FLOT2) were done on immunoprecipitated chromatin using the specific primers 5'-GAGGAATTGCAGTTCAGAAG-3' (forward) and 5'-GGTGGCTCAGAGGCTCAGA-3' (reverse). Amplifications were also done on immunoprecipitated chromatin using the specific primers 5'-CGCTGTGGTTTTCTCTGTTTAT-T3' and 5'-AGGCTTGCTAGTTAACG-3' for one of the other candidate sequences (+526); or 5'-GTCACAGGACAGTGACT-3' and 5'-GCTACAAAGCTACTGCTG-3' MDM2 promoter containing a p53-binding sequence. To ensure that PCR amplification was done in the linear range, template DNA was amplified for a maximum of 30 cycles. PCR products generated from the ChIP template were sequenced to verify the identity of the amplified DNA.

**Luciferase Assay**

A 32-bp fragment of the response element for p73 and p63, RE-FLOT2 (5'-GACCTTGGCCAGTCGCCTGGGCTGCTCCT-3') and its mutant form RE-FLOT2-mut (5'-GAAATTTCAGCTGTTGTCGCTGCTCCT-3') were synthesized and inserted upstream of a minimal promoter in the pGL3 promoter vector (Promega) and the resulting constructs were designated pGL3-RE-FLOT2 and pGL3-RE-FLOT2-mut, respectively. Subconfluent cells in 24-well plates were transfected with 2 ng of pRL-TK reporter (Renilla luciferase for internal control) and 100 ng of pGL3 reporter (firefly luciferase, experimental reporter), together with 100 ng of a pcDNA3.1 control vector (Invitrogen) or a vector that expresses p53, TAp73, or TAp63 by using LipofectAMINE 2000 reagent (Invitrogen). Forty-eight hours after transfection, the reporter gene activities were measured by a Dual-Luciferase Reporter Assay (Promega), according to the manufacturer’s instructions. All experiments were done in triplicate and repeated at least thrice.

**IL-6–Mediated STAT3 Activation**

Cells (5 × 10^5) were incubated with purified virus at a MOI of 25 (Saos-2) or 100 (MEF) in medium with 1% FCS. After 16 h, cells were incubated in serum-free minimal essential medium for 3 h to reduce basal levels of activated STAT3, and then treated with recombinant human (for Saos-2) or mouse (for MEF) IL-6 (20 ng/mL) in serum-free medium for 30 min. A proportion of the cells were pretreated with an anti–IL-6R antibody (for Saos-2, 5 µg/mL; Sigma) or an anti–IL-6 antibody (for MEF, 0.25 µg/mL; R&D systems), or filipin III (Sigma), a cholesterol-binding compound that disrupts plasma membrane lipid rafts, 2 h before IL-6 treatment. Cells were lysed as described above and phosphorylation of STAT3 (Tyr705) was examined by 10% SDS-PAGE immunoblotting with a specific anti-phosphorylated STAT3 antibody according to the manufacturer’s recommendations. For comparison, total STAT3 (both phosphorylated and unphosphorylated) were also examined with an anti-STAT3 antibody.
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References


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