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 TAp63, 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 ectrodactyly, 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 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 transduction via lipid rafts, which may play an important role in normal development and in the pathogenesis of various human diseases, such as immune disease.

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-p63α, and Ad-p63x for the p53, TAp73α, TAp73β, TAp63γ, and TAp63x 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-p63α, and Ad-p63x, expression of exogenous p53, TAp73α, TAp73β, TAp63γ, and TAp63x 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-p63α (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 cDNA 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 TAp63x 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 p73 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 h-actin [Fig. 4B, CDDP(+)]. Notably, p73 siRNAs, but not untreated [siRNA(−)/C0] 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

FIGURE 2. Induction of the flotillin-2 mRNA by TAp73 and TAp63 in human cancer cell lines. Northern blot analysis shows flotillin-2 induction following adenovirus-mediated transfer of genes of the p53 family into human cancer cell lines. Human cancer cell lines were infected with adenoviruses at a MOI of 25 to 100, and the cells were harvested at 24 h following infection. Total RNA (10 μg) was loaded in each lane, and the same filter was rehybridized with human flotillin-2 and p21 cDNAs. Ethidium bromide staining of ribosomal RNA (bottom) shows that equal amounts of RNA were loaded in each lane.

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 h-actin [Fig. 4B, CDDP(+)]. Notably, p73 siRNAs, but not untreated [siRNA(−)/C0] 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

FIGURE 3. Induction of the flotillin-2 protein by TAp73 and TAp63 in human cancer cell lines. A. Immunoblot analysis was done on total lysates (20 μg) of human cancer cells 24 h after infection with Ad-p53, Ad-p73α, Ad-p73β, Ad-p63γ, and Ad-p63α. Cell extracts were separated by electrophoresis on a 10% SDS/acrylamide gel and analyzed by immunoblotting using the mouse anti-human flotillin-2 mAb. B. Time course analysis of flotillin-2 induction following ectopic expression of p53 family in Saos-2 cells. Cells were infected with adenoviruses (Ad-p73β, Ad-p63γ, and Ad-p53) at a MOI of 25, and the cells were harvested at the indicated times following infection. Total protein was extracted and subjected to immunoblotting. Flotillin-2 expression was not induced 24 h after infection with Ad-p53 at a MOI of 50 or 100. C. Immunofluorescence analysis of flotillin-2 protein expression in Saos-2 cells 24 h after infection with Ad-Fp63γ. Cells were fixed and stained with antibodies to the FLAG epitope (transfected p63γ, green) and to flotillin-2 (red), followed by appropriate secondary antibodies. Nuclei were detected by staining with 4',6-diamidino-2-phenylindole (DAPI, blue).
isoforms of p73 using semiquantitative reverse transcription-PCR (RT-PCR). mRNA expression of ANp73, a dominant-negative isoform, was low in these two cell lines (Fig. 4B, TAp73 and ANp73; 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-Fp73α, 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, NW047336. R, purine; Y, pyrimidine; W, adenine or thymine. B. TAp73α, TAp73β, 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-Fp73β, Ad-Fp63β, and Ad-Fp63γ. 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-Fp73β, and Ad-Fp63γ, 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-Fp73β, and Ad-Fp63γ-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 pRL-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.
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 conserved 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 conserved among mammalian flotillin-2 genes.

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, lane 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](image)
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-6Rα 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 h, 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 overexpression 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 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 10 bp p53-binding motif separated by 0 and 2 bp nucleotides, whereas nearly all p53 response elements previously reported
Although several studies have tried to better define the association between two p53 relatives and IL-6–STAT3 signaling, which are not shared by p53, and therefore raise 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 isoforms (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, hematoipoiesis, 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 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.

FIGURE 8. Flotillin-2 siRNA antagonizes STAT3 phosphorylation activated by TAp63γ. A, Inhibition of flotillin-2 mRNA expression by flotillin-2 siRNAs. Saos-2 cells were transfected with flotillin-2 siRNAs (si-FLOT2-1 and si-FLOT2-2) or control siRNA (si-cont) every 24 h for 2 consecutive days using OligofectAMINE. At 24 h after the last transfection, suppression of flotillin-2 mRNA was determined by semiquantitative RT-PCR. B, Inhibition of flotillin-2 protein expression in the raft-rich component by the flotillin-2 siRNAs. Saos-2 cells were transfected with the flotillin-2 siRNAs or control siRNA every 24 h for 2 consecutive days. Eight hours after the last transfection of siRNA, cells were transfected with Ad-p63γ or Ad-lacZ at a MOI of 25. After 24 h, raft-rich membrane components were prepared as described above. Twenty micrograms of protein were resolved by SDS-PAGE and analyzed by immunoblotting with flotillin-2 and flotillin-1 antibodies. C, Saos-2 cells were transfected with the flotillin-2 siRNAs or control siRNA every 24 h for 2 consecutive days. Eight hours after the last transfection of siRNA, cells were transfected with Ad-p63γ or Ad-lacZ at a MOI of 25. Eighteen hours after transfection with Ad-p63γ or Ad-lacZ, 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 IL-6 (10 ng/mL) in serum-free medium for 30 min (lanes 2, 4, 6, and 8). Cells were harvested and subjected to immunoblotting.
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 injection procedures of replicative-deficient recombinant adenoviruses containing p53 (Ad-p53), TAp73α (Ad-p73α), TAp73β (Ad-p73β), TAp63γ (Ad-p63γ), and TAp63α (Ad-p63α) genes, or the bacterial lacZ gene (Ad-lacZ) have been previously described (25, 28, 29). We also constructed recombinant adenoviruses encoding the p53 family proteins with an amino-terminal FLAG epitope (Ad-Fp53, Ad-Fp73α, Ad-Fp73β, Ad-Fp63γ, and Ad-Fp63α; ref. 25). We carried out the titering of adenovirus for the duplicate samples in order to confirm the reproducibility of the experiments. The relative efficiency of adenovirus infection was determined by X-gal staining of cells infected with a control Ad-lacZ. At a MOI of 25 to 100, 90% to 100% of the cells could be infected (data not shown).

Immunoblot Analysis

The primary antibodies used for immunoblotting in this study are as follows: mouse anti-human p53 mAb (DO-1; Santa Cruz Biotechnology), mouse anti-human p73 mAb (ER-15; Oncogene Research), mouse anti-human p63 mAb (AA4, Oncogene Research), mouse anti-human p21 mAb (EA10; Oncogene Research), mouse anti-human flotillin-2 mAb (BD Transduction Laboratory), mouse anti-human flotillin-1 mAb (BD Transduction Laboratory), mouse anti-human actin mAb (Chemicon), mouse anti-FLAG mAb (M2; Sigma), rabbit anti-FLAG polyclonal antibody (Sigma), rabbit anti–phospho-STAT3 (Tyr705; Cell Signaling Technology), and rabbit polyclonal antibody (Tyr205; Cell Signaling Technology), and rabbit anti-STAT3 polyclonal antibody (Cell Signaling Technology). Whole cell lysates were prepared by scraping cell membranes (Millipore).

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 and blotted onto a nitrocellulose membrane (Schleicher & Schuell). 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 verify their identity. For semiquantitative RT-PCR analysis, cDNAs were synthesized from 5 μg total RNAs with SuperScript Preamplification 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 TA-p73 sense 5′-CACCTGGAGGGGATGACTC-3′, human ΔNp73 sense 5′-ACCATTGCTTACGTCGGTAC-3′, human p73 antisense 5′-TTGGCCTTCCCCAGCGGATGAG-3′, mouse p73 sense 5′-GAGGACACCTGTGGGTTCGTCTAGAG-3′, mouse p73 antisense 5′-GGATTTGGAAGGAGTACAGGC-3′, mouse p63 sense 5′-AAAGAACGCGGATGTCAGA-3′, mouse p63 antisense 5′-GGCATGAGGTGGGAGGAGAGA-3′, human flotillin-2 sense 5′-CAGTGCCACTTTCATCACAGGT-3′, human flotillin-2 antisense 5′-TATTTTATGACAGAGGAGGACAGC-3′, mouse flotillin-2 sense 5′-TTGGTGTGCGAGAGCGAC-3′, mouse flotillin-2 antisense 5′-GCTCTACGCCACCTTAC-3′, mouse p21 antisense 5′-CCTGGTGGGATGAGGAGAGA-3′, mouse p21 sense 5′-GACCGAGGAGGAGGAGGAGA-3′, human and mouse GAPDH sense 5′-ACCACAGTCATGCCATC-3′, and...
human and mouse GAPDH antisense 5'-TCCACCACCCCTGTGCTGTA-3'. The PCR products were visualized by electrophoresis on 1.5% agarose gels.

**Immunofluorescence Microscopy**

Saos-2 cells (5 x 10^5) were incubated with Ad-Fp63γ at a MOI of 25 in a four-well slide chamber (Lab-trek 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 washed with Alexa 594–conjugated goat anti-rabbit IgG (Invitrogen) and Alexa 488–conjugated goat anti-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'-AAUUCUCAUGUACUUCCUGUGGAGGUG-3'), si-p73-2 (5'-AAAGAGCUCCAUCGUGGAAUCCGAUC-3'), si-p73-3 (5'-UAUACACAGGAUGUGACAGGAUGAAAG-3'), and si-p73-4 (5'-CGGAUUCCAGCAUGGACGUdTdT-3'); two human FLOT2 siRNAs, si-FLOT2-1 (5'-GCGAUAUCAACGCUUGGAAUCCGU-3') and si-FLOT2-2 (5'-GCUUGCUUCUUCUGGUAAdTdT-3') and si-FLOT2-3 (5'-GGAUUCUCCAUAGAAAUdTdT-3'); two mouse p73 siRNAs, si-mp73-1 (5'-UGAAGAUCUGAUAAAGUCUA-3') and si-mp73-2 (5'-UCAACACUUGAUGGAUUGAAUA-3'); two mouse p63 siRNAs, si-mp63-1 (5'-CUUCUGUAUGUAUGAAUAUA-3') and si-mp63-2 (5'-UAGGCAUUAAGGAAGCACA-3'); and nonspecific control siRNA (non-specific control VII; target sequence 5'-CUGCUAGA-3' and 5'-GCTACAAGCAAGTCGGTGCT-3'). Subconfluent cells in 24-well plates were transfected with 2 ng of pRL-TK reporter (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, TAp73β, or TAp63β 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 x 10^6) 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 MEF) or an anti–IL-6 antibody (for Saos-2, 5 μg/mL; Sigma) or an anti–IL-6R 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.


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

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