Determinants That Control the Distinct Subcellular Localization of p38α-PRAK and p38β-PRAK Complexes*

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The physical location of a protein is essential for its biological function in eukaryotic cells, in which many proteins traffic continuously, whereas others selectively localize to different subcellular compartments. The best understood system for the transport of macromolecules between the cytoplasm and the nucleus is the nuclear import and export pathway (1–4). In this pathway, nuclear import and export of proteins is mediated through the nuclear pore complex by a superfamily of transport receptors known collectively as karyopherins (1). These proteins recognize specific signal sequences, the nuclear localization signal (NLS)3 and nuclear export signal (NES), in the proteins being transported. The typical NLSs contain a cluster of basic amino acids as is exemplified by the SV40 large T-antigen with the amino acid sequence PKKRRKKV and is known to be bound with importin α (5–7). NESs identified up to now are rich in hydrophobic amino acids, such as leucine or isoleucine. The traffic of some proteins between the cytoplasm and nucleus is mediated by a chaperon (8–10). In these systems, the NLS or NES is not in the proteins being transported, but in the chaperon. The transport of p38α MAP kinase (mitogen-activated protein kinase) is chaperoned by either MK2 (MAP kinase-activated protein kinase-2) or PRAK (p38-regulated/activated protein kinase) that carries conventional NLS and NES that carries conventional NLS and NES (11, 12). However, other methods of p38α translocation may exist, since the homologous protein of p38 in yeast, Hog1, can be translocated into the nucleus in an importin α-independent mechanism without interacting with an NLS-containing chaperon (13).

The p38 group of protein kinases is one of the four subfamilies of the MAP kinase family that plays a role in a variety of biological processes that include cell proliferation, differentiation, senescence, and cell death (14–17). None of the p38 group kinases contains NLS or NES sequences, but their subcellular localization can be regulated, at least in some cases, by their interacting proteins. It needs to be noted that they are not passively regulated by their chaperons. An interesting pair in this group is p38α and p38β, which can in turn regulate the nuclear and cytoplasmic localization of their interacting proteins, such

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Transient Transfection, Immunoprecipitation, and Western Blotting—Transient transfections were carried out using a calcium phosphate precipitation method for HEK 293T cells, and Lipofectamine 2000 for NIH3T3 cells according to the manufacturer’s instruction (Invitrogen). For co-immunoprecipitation, a DNA mixture composed of 2 μg of GFP1C1-PRAK and 2 μg of pcDNA3.0-FLAG-p38α or pcDNA3-FLAG-p38β was co-transfected into HEK 293T cells. After 36 h of transfection, cells were harvested with lysis buffer containing 20 mM Tris-HCl (pH 7.5), 120 mM NaCl, 10% glycerol, 1 mM Na3VO4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. The cell lysates were then sonicated and centrifuged at 18,000 × g for 30 min at 4 °C. FLAG-tagged p38α/p38β was immunoprecipitated using mouse monoclonal anti-FLAG M2 beads (Sigma). The samples were then boiled and separated on 10% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Roche Diagnostics). After blocking with 5% skim milk in PBS with 0.1% Tween 20 for 1 h, the membranes were probed with anti-FLAG M2 antibodies (Sigma) for p38α and p38β and rabbit anti-GFP antibodies (Santa Cruz Biotechnology) for GFP-PRAK.

Immunofluorescent Staining—NIH3T3 cells were grown on glass cover slips in the cell culture medium, as described above, for 16 h. Expression vectors of FLAG-p38α, FLAG-p38β, and GFP-PRAK or various mutations of them were co-transfected into NIH3T3 cells in different combinations as indicated where necessary. 24 h post-transfection, cells were fixed with 4% formaldehyde in PBS for 10 min, followed by treatment with 0.2% Triton/PBS for 5 min. After rinsing three times with PBS, the coverslips were incubated with 10% normal rabbit blocking serum (Santa Cruz Biotechnology) in PBS for 30 min to suppress nonspecific binding of fluorochrome-conjugated IgG, followed by washing in PBS three times. Coverslips were then incubated with anti-FLAG M2 antibodies (Sigma) for 60 min at 37 °C. After washing three times with PBS for 5 min each wash, cells were stained with rhodamine-conjugated rabbit IgG (diluted in 1.5–3% normal rabbit blocking serum) for 45 min at room temperature, followed by extensive washing with PBS. Cells were then stained with the DNA-binding dye 4’,6-diamidino-2-phenylindole (0.1 μg/ml in PBS) for 10 min. Then the coverslips were subjected to rinsing with PBS, and finally mounted with GelMount (Biomeda Corp., Foster City, CA) and kept at 4 °C. Slides were examined under an inverted fluorescence microscope (Olympus 1×51) for GFP fluorescence, rhodamine, and 4’,6-diamidino-2-phenylindole staining at wavelengths of 488/507, 540/625, and 360/460 nm, respectively. Images were captured through a ×100 objective lens by a DP50 microscope digital camera system (Olympus).

Cell Proliferation Assay—NIH3T3 cells were grown on coverslips in media as described above. When cells reached ~60% confluence, they were transfected with 1 μg of expression plasmids of GFP, GFP-PRAK, GFP-PRAK(QTTG), or GFP-PRAK(R361Q) alone, or in different combinations with 1 μg of p38α, p38α(D145G), p38β, or p38β(G145D), as indicated in Fig. 7, by using Lipofectamine 2000 transfection reagent. Cell proliferation was determined by BrdUrd intake assay, employing a 5-Bromo-2’-deoxyuridine Labeling and Detection Kit 1 (Roche) according to the manufacturer’s instructions. In brief,
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24 h post-transfection, the cell culture medium was changed with fresh medium supplemented with BrdUrd labeling solution (1 × concentration). After incubating the cells at 37 °C for 30 min, the BrdUrd labeling medium was aspirated and coverslips were washed in washing buffer three times. Cells were then fixed with the ethanol fixative (add 50 mM glycine solution to 70 ml of absolute ethanol to get 100 ml of fixative, pH 2.0) for 20 min. at −25 °C, followed by rinsing three times with washing buffer. Fixed cells were incubated with mouse anti-BrdUrd working solution for 1 h at 37 °C and washed three times. Hereafter, all coverslips were stained with rhodamine-conjugated anti-mouse secondary antibodies for 1 h at 37 °C. Slides were examined under a fluorescence microscope (Olympus IX-51) for GFP fluorescence and BrdUrd staining. To obtain the rate of nuclear BrdUrd intake, 600 cells positive for GFP were counted for each coverslip and the percentage of BrdUrd-positive nuclei in GFP-positive cells was calculated.

p53 Reporter Assays—NIH3T3 cells were transiently transfected with different combinations of plasmids expressing HA-PRAK, Ha-RasV12, p38α, p38β, p38α(D145G), and p38β(G145D), as indicated in Fig. 7. D and E, together with 0.5 µg of the p53-dependent reporter plasmid PG-Luc. 24 h after transfection, luciferase activities were determined and normalized to the values of β-galactosidase activity derived from cotransfection with 0.5 µg of the pCMV5-LacZ vector. The results are presented as mean ± S.D. from at least five separate experiments.

RESULTS

Sequences in p38α and p38β That Regulate the Subcellular Location of PRAK—Our previous study showed that interaction with p38α or p38β determines the nuclear or cytosolic localization of PRAK in HeLa and HEK293 cells. It was also demonstrated that GFP-PRAK, a PRAK fusion with the reporter protein GFP, adequately mimicked the subcellular localization of PRAK (12). As controls in this study, ectopically expressed GFP-PRAK alone in NIH3T3 cells was found in the nucleus under a fluorescent microscope (Fig. 1A); FLAG-tagged p38α and p38β are generally diffused in the cells with some preference for FLAG-p38α in the nucleus and FLAG-p38β in the cytosol (Fig. 1A). The interactions of GFP-PRAK with FLAG-p38α and FLAG-p38β can be demonstrated by co-immunoprecipitation (Fig. 1B). The interaction of FLAG-p38β and GFP-PRAK leads to cytosolic localization of these two proteins, whereas the FLAG-p38α and GFP-PRAK interaction localize them to the nucleus (Fig. 1C).

To better understand the structure basis of the different functions of p38α and p38β, a series of plasmids were constructed to express chimeric proteins (M1–M10) of p38α and p38β (Fig. 2A). Each of these chimeric proteins was coexpressed with GFP-PRAK in NIH3T3 cells to determine their interaction with PRAK and their effects on PRAK subcellular localization. All of these chimeric proteins retained the ability to interact with GFP-PRAK (Fig. 2B). Analysis of the subcellular localizations of GFP-PRAK and FLAG-chimeric proteins revealed that the chimeric proteins containing the p38α sequence from amino acids 132 to 170 (M2, M3, M4, M8, and M10) exhibited the same effect as wild type p38α in regulating the distribution of PRAK (Fig. 2C), whereas the chimeric mutants that contain the same sequence as p38β in the region from amino acid 132 to 170 (M1, M5, M6, M7, and M9) were co-localized with GFP-PRAK in the cytoplasm (Fig. 2C). Therefore, it is the sequence between amino acid residues 132 and 170 that determines the different functions of p38α and p38β in regulating the subcellular distribution of p38α-PRAK and p38β-PRAK.

There are four amino acids differences in the region from residues 132 to 170 between the p38α and p38β sequences (Fig. 3A). Eight mutants of p38α and p38β were generated by swapping each of these residues between p38α and p38β. None of these mutations affected the interaction between p38α (or p38β) and PRAK in the co-immunoprecipitation experiments (Fig. 3, B and C). However, Asp145 to Gly (D145G) and Leu156 to Val (L156V) mutations of p38α altered the subcellular localization of its complex with PRAK to the cytosol, much like the behavior of p38β in regulating PRAK subcellular localization.
The Subcellular Location of MK2 Can Be Regulated by p38α and p38β in the Same Way as That of PRAK—Several protein kinases, including MK2 and MNK1 (MAPK-interacting kinase 1), also interact with p38α (11, 21, 22), and it is of interest to determine whether they can be regulated by p38α and p38β to their respective subcellular locations in a manner similar to that of PRAK. Both MK2 and MNK1 can interact with p38α and p38β (Fig. 4A). When expressed individually in NIH3T3 cells, GFP-MK2 and GFP-MNK1 were localized to the nucleus and cytosol, respectively (Fig. 4B). This is consistent with results obtained by other groups (11, 23, 24). Coexpression of either p38α or p38β did not affect the cytosolic localization of MNK1 (Fig. 4C). This is not surprising, as MNK1 and PRAK are quite different in the structural location of their putative NLS. NLS in MNK1 is located at the N terminus but in PRAK it is located at the C terminus. As expected, the subcellular distribution of MK2, with its NLS being aligned well with that in PRAK, can be regulated by p38α and p38β in the same way as that of PRAK (Fig. 4D). Additionally, the mutants of p38α and p38β regulate the subcellular localization of MK2 in the same fashion as that of PRAK (Fig. 4, E and F).

Nuclear Import but Not Export Is Important in Determining the Subcellular Location of p38α-PRAK and p38β-PRAK—As we mentioned earlier, p38α and p38β do not contain NES and NLS sequences, whereas PRAK has both (Fig. 5A). To determine whether NES-mediated nuclear export plays a role in the cytosolic localization of p38β-PRAK, we used a PRAK mutant, GFP-PRAK(SSS), in which the NES motif was mutated (12). Mutation of NES in PRAK did not affect the interaction of PRAK with p38α/β (data not shown). As expected, mutation of NES did not affect nuclear localization of PRAK when it was expressed alone in NIH3T3 cells (Fig. 5B) or coexpressed with p38α (data not shown). Yet, when coexpressed with p38β, GFP-PRAK(SSS) was mainly localized to the cytoplasm (Fig. 5C, bottom), reminiscent of the distribution pattern of wild type PRAK, indicating that NES in PRAK is not required for cytosolic locali-
zation of the p38β-PRAK complex. Treatment with leptomycin B, an inhibitor of nuclear export that binds to the chromosomal region maintenance 1, had no effect on the cytosolic localization of p38β-PRAK (Fig. 5C, middle), consistent with the notion that nuclear export has no role in the cytosolic localization of the p38β-PRAK complex.

Because NLS overlaps with the docking motif in PRAK (Fig. 5A), mutation of the four amino acids RKKK to QTTQ in PRAK not only abolishes its nuclear localization but also eliminates its interaction with p38α/β (12). To separate the docking function from NLS, a mutation of R361Q in PRAK was generated, which did not alter the PRAK interaction with p38α and p38β (Fig. 5D). The R361Q mutant is localized to the cytosol when expressed alone in NIH3T3 cells, similar to the QTTQ mutant (supplemental Fig. 2), indicating that the NLS function was interrupted. As shown in Fig. 5E, wild type PRAK was localized to the nucleus when coexpressed with p38α. In contrast, the R361Q mutant was localized to the cytosol. This data suggests that NLS in PRAK is essential for the nuclear localization of p38α-PRAK.

Asp145 and Leu156 in p38α Are Located on Two Different Surface Patches—The complex structures of the MK2 peptide and its complex with p38α were reported recently (25, 26). Leu156 in p38α is located in the vicinity that interacts with MK2. Asp145 on the other hand is located on the other side of p38α close to the P-loop surface. Due to the high level of homology to p38α, we believe Gly145 and Val156 in p38β are located at the same places as their corresponding Asp145 and Leu156 in p38α. Based on the published structures of the p38α-MK2 complex, models of p38α-PRAK and p38β-PRAK complexes were made (Fig. 6A). Leu156 or Val156 (purple spheres) are located in a β strand that interacts with the docking peptides from PRAK (or other substrates such as MEF2A (red ribbon)). It is known that NLS in PRAK overlaps with its docking site for p38α and p38β. Perhaps Leu156 in p38α and Val156 in p38β affects the conformation of the NLS differently for PRAK, therefore the binding with p38β, but not with p38α, may interfere with the interaction of NLS with importin α for PRAK, preventing the nuclear import.

Asp145 in p38α or Gly145 in p38β (red spheres) are located on the other side of the surface that interacts with PRAK (Fig. 6A). They may have no role in influencing the function of NLS in PRAK, but could alter the interactions with the protein(s) of import/export machinery to affect the traffic of p38α/β-PRAK complexes. Interaction with nucleoporin was shown recently to be important in ERK2 nuclear import (27). Sequence comparison between p38α and p38β revealed that the sequence corresponding to the nucleoporin binding region in ERK2 is conserved between p38α and p38β, suggesting that the different functions of p38α and p38β in regulating PRAK localization is not related to their nucleoporin-binding ability.

To understand the role of Asp145 (or Gly145) in regulating PRAK subcellular localization, a series of point mutations were generated in p38α for comparative analysis of their roles in the regulation of p38α for PRAK localization. Amino acid 123 in p38α is Thr and in p38β is Ser. Converting Thr123 in p38α to

![FIGURE 3. Amino acids 145 and 156 are crucial for p38α and p38β to localize PRAK to nuclear or cytosolic locations. A, sequence alignment of p38α and p38β showing the different amino acids (boldfaced) in the region between amino acids 132 and 170. B, four point mutants of p38α, I134L, D145G, L156V, and K165R (lie145, Asp145, Leu156, and Lys165 of PRAK subcellular localization, a series of point mutations were introduced. As shown in Fig. 5E, wild type PRAK was localized to the nucleus when coexpressed with p38α. In contrast, the R361Q mutant was localized to the cytosol. This data suggests that NLS in PRAK is essential for the nuclear localization of p38α-PRAK.](image-url)
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Ala 325 in p38α is Gln and in p38β is Gla. A Q325G mutation in p38α resulted in cytosolic localization of coexpressed PRAK (Fig. 6B). In contrast, PRAK coexpressed with p38α mutants containing single changes of H107T, L122G, L130G, I134G, R149G, V158A, or V319E were localized to the nucleus, just as with wild type p38α (Fig. 6C). A summary of point mutations on the three-dimensional structure of p38α complexed with PRAK is shown in Fig. 6D. All of these mutants retained the ability to interact with GFP-PRAK (Fig. 6E). Because the structures of importin α in complex with NLS peptides are available (5–7), we included importin α into our modeling (Fig. 6D). Based on this modeled structure, Asp145, Thr123, and Gly125 in p38α are in the interacting distance with importin α (<4 Å), whereas His107, Leu122, Leu130, Ile134, Arg149, and Val158 are not able to interact importin α. Although Val139 is in the distance for interaction with importin α, it may not be important for interacting with importin α because its mutation did not affect the localization of p38α-PRAK. Collectively, these data suggest that the interaction with importin α is also required for nuclear import of the p38α-PRAK complex, in which Asp145 in p38α plays a vital role.

PRAK-induced Growth Inhibition of NIH3T3 Cells Depends on Its Nuclear Location—It is of great interest to determine whether different subcellular localization of PRAK affects its function. We have shown previously that PRAK is essential in Ras-induced senescence, and overexpression of PRAK can inhibit the proliferation of NIH3T3 cells (18, 20). We employed the BrdUrd intake assay to examine whether the inhibitory effect of PRAK on proliferation of NIH3T3 cells is dependent on its specific subcellular localization. The data showed that GFP-PRAK robustly inhibited the proliferation of NIH3T3 cells compared with the control GFP (p < 0.001) (Fig. 7A). GFP-PRAK(QTTG) and GFP-PRAK(R361Q), two mutants that failed to be distributed in the nucleus, showed impaired inhibitory effects on the proliferation of NIH3T3 cells (Fig. 7A) compared with GFP-PRAK (p < 0.001), indicating that nuclear localization of PRAK is essential for its inhibitory effect on cell proliferation. Expression of GFP did not influence NIH3T3 cell proliferation (Fig. 7B). Coexpression of p38α and GFP (Fig. 7B, second column) showed some inhibitory effects on cell proliferation in comparison with GFP alone (Fig. 7B, first column) (p < 0.001). In contrast, coexpression of p38β with GFP (Fig. 7B, third column) exhibited little or no inhibitory effects on NIH3T3 cell proliferation (p > 0.05 compared with GFP alone). p38α enhanced the PRAK-mediated cell growth inhibition (Fig. 7B, fifth column versus the fourth column, p < 0.01). In contrast, p38β, which docks PRAK to the cytosol, strongly blocked the inhibitory effect of GFP-PRAK on cell proliferation (Fig. 7B, sixth column), consistent with the idea that nuclear localization of PRAK is essential for its ability to inhibit cell proliferation.
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To confirm that the effect of p38β on PRAK-mediated inhibition of NIH3T3 cell proliferation is due to its docking of PRAK to the cytosol, we used a p38α mutant (D145G) that redistributes PRAK to the cytosol and p38β(G145D) that docks PRAK to the nucleus, to determine their effects on the PRAK-mediated inhibition of cell proliferation. As predicted, p38α(D145G) (Fig. 7C, second column) had no effect on cell proliferation in comparison with wild type p38α (Fig. 7B). On the contrary, p38β(G145D) (Fig. 7C, third column) had an enhanced inhibitory effect on cell proliferation. Coexpression of p38α(D145G), which docks PRAK to the cytosol, eliminated PRAK-mediated cell growth inhibition (Fig. 7C, fifth column), whereas cotransfection of p38β(G145D), which causes PRAK to be localized to the nucleus, showed enhanced effects on PRAK-mediated suppression of cell proliferation (Fig. 7C, sixth column).

Our previous work showed that PRAK-mediated transactivation of p53 plays a role in Ras-induced inhibition of cell growth (20). To determine whether different subcellular distributions of PRAK affect its function in regulating transcriptional activity of p53 upon Ras stimulation, we used a reporter gene assay. As shown in Fig. 7D, PRAK (sixth column) can enhance p53 transcriptional activity by comparison to the control group (first column), and this action can be inhibited by p38β, but not p38α (seventh and eighth columns). As anticipated, p38α(D145G) (Fig. 7D, ninth column) showed inhibitory effects on p53 transactivation, and p38β(G145D) (Fig. 7D, tenth column) acted the same as p38α on p53 transactivation. Overexpression of Ha-RasV12 in NIH3T3 cells induced the expression of the p53 reporter gene (Fig. 7E, sixth column), and this effect was inhibited by p38β, but not p38α (Fig. 7E, eighth and seventh columns, respectively). As expected, these opposing effects of p38α and p38β can be reversed by swapping their amino acid residue 145 (Fig. 7E, ninth and tenth columns). Collectively, we conclude that different subcellular localizations of PRAK, regulated by p38α and p38β, may affect its ability to regulate p53 transactivation.

DISCUSSION

Proteins containing typical NLS and NES mediate their nuclear import and export through interactions of their NLS with a carrier protein complex containing importin α, or their NES with chromosomal region maintenance 1 (1–4). However, protein–protein interactions can also influence the subcellular localization of a protein containing NLS and NES signals (4, 28, 29). PRAK contains both NLS and NES, and it alone is mainly localized to the nucleus (Fig. 1A) (12). Interestingly, interaction with p38α and p38β, both lacking NLS and NES signals and being distributed in both the nucleus and cytoplasm, leads to a solely nuclear distribution of the p38α-PRAK complex and to an exclusively cytosolic localization of the p38β-PRAK complex (Fig. 1). Our mutagenesis analysis has revealed that the differences in the two surface patches on p38α and p38β can influence the subcellular localization of p38α-PRAK and the p38β-PRAK complex (Figs. 2, 3, and 6). Structural modeling analysis suggests that the sequence in p38α and p38β that...
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FIGURE 6. Amino acids 156 and 145 in p38α/β affect the localization of PRAK by different mechanisms. A, models of p38α-PRAK and p38β-PRAK complexes are made based on the published structure of the p38α-MK2 complex. Leu130 in p38α and Val134 in p38β are highlighted in purple spheres. A peptide of MEF2A (red ribbon) is also included. It is noteworthy that the docking motif of PRAK overlaps with its NLS. B and C, NIH3T3 cells were cotransfected with GFP-PRAK and different point mutants of p38α as indicated, followed by determining the localization of p38α or its mutants and PRAK. Of nine point mutants of p38α, only T123A and Q325G function as p38αβ (8), and others behave as p38α in regulating the subcellular localization of PRAK (C). D, structural modeling of possible ternary complex formation of p38α, PRAK, and importin α. Mutations affecting p38α-PRAK subcellular localization are shown as yellow spheres (Thr123 and Gln325). Mutations that do not affect p38α-PRAK subcellular localization are shown as small black spheres (His107, Leu122, Leu130, Ile134, Arg149, Val156, and Val319). E, all of the nine point mutants of p38α show the same affinity as that of wild type p38α in co-immunoprecipitation (IP) assays with PRAK. IB, immunoblot; TCL, total cell lysate.

directly interacts with the NLS of PRAK may affect the function of the NLS differently (Fig. 6A), and some other sequences in p38α may interact with importin α and thus play a role in determining the nuclear localization of the p38α-PRAK complex (Fig. 6D).

We show that overexpressed p38α is distributed more in the nucleus than in the cytoplasm, and coexpression of p38α with PRAK leads to both of them being exclusively localized to the nucleus, indicating that the p38α-PRAK complex has a unique property in subcellular localization in comparison with PRAK and p38α alone (Fig. 1, A and C). This notion is supported by coexpression of PRAK and p38β, which shows that p38β-PRAK is exclusively localized to the cytosol, significantly different from the localization of PRAK and p38β when expressed alone (Fig. 1, A and C). It is possible that endogenous p38α and p38β are partially in free form and some are docked with their partners, and therefore both nuclear and cytosolic localization of p38α and p38β have been observed. It is also possible that mammalian p38αβ can be transported, as its yeast counterpart Hog1, in a NLS-independent mechanism (13), and thus both nuclear and cytosolic distribution can be observed. Because endogenous PRAK is primarily cytosolically localized, it most likely docks with protein partners such as p38β. Because nuclear-cytosolic shuttling of PRAK was observed (12), the regulation of PRAK docking interaction could be important for the function of PRAK. Indeed, we show that PRAK-induced cell growth inhibition requires its nuclear localization (Fig. 7).

Our results show that the distinct locations of p38α-PRAK and p38β-PRAK are related to the function of the NLS of PRAK, but not its NES (Fig. 5), indicating that the interaction with import machinery was affected by complexing PRAK with p38α or p38β. This idea was supported by the data that the mutations affect p38α-PRAK importing are on the amino acids that can directly interact with importin α in our modeling analysis and the mutations that did not influence p38α-PRAK nuclear localization cannot reach importin α. These data suggest the cytosolic localization of p38α(D145G)-PRAK and p38α(L156V)-PRAK is due to either D145G or L156V mutations impairing nuclear import of the complex. However, it is still unclear why a single mutation of p38β on either residues 145 or 156 is sufficient to lead to nuclear localization of the p38β-PRAK complex (Fig. 3E). Perhaps some sequences of p38β have some affinity for proteins of the import machinery, which allows nuclear import of p38β-PRAK after a single mutation on either residue 145 or 156. Although NES appears to have no role in determining the subcellular location of the p38α/
ERK2 and p38α/β showed that the region in p38α and p38β that corresponds to the nucleoporin binding region in ERK2 is conserved, suggesting that the difference between p38α and p38β is unlikely to be due to nucleoporin binding. Although the difference between p38α and p38β may not be related to nucleoporin binding, interaction with nucleoporin is still likely to be involved in the p38α-PRAK complex nuclear import. Based on our data and previous published data by us and others (11, 12), we propose that localization of p38α/β and their downstream kinases PRAK and MK2 is determined by multiple factors, including NLS and NES, docking interactions, and their affinity for importin α and other proteins of the importing machinery.

It is known that p38α and p38β have many different functions (16). Although the structural basis for the differences between these two proteins is not fully understood, different subcellular localizations are likely to play a role in the different functions of these two closely related proteins. We have recently found that PRAK plays a role in transactivation of p53 (20). It appears that nuclear localization of PRAK is required for PRAK to activate p53, as overexpression of p38β inhibits PRAK-mediated p53 reporter gene expression (Fig. 7D). p38α is apparently responsible for the activation of PRAK in Ras-induced p53 activation, as p38β overexpression had an inhibitory effect on Ras-induced p53-dependent gene expression, whereas p38α overexpression had a slight enhancing effect (Fig. 7E). The different subcellular localizations of PRAK, resulting from its interaction with p38α and p38β, are also likely to be responsible for the distinct effects of p38α and p38β on PRAK-mediated inhibition of NIH3T3 cells (Fig. 7, A–C). In light of the data in this article and in previous publications, we propose that different subcellular localizations of PRAK, regulated by p38α and p38β, affect its ability to regulate p53 transactivation and thus cell proliferation.

β-PRAK complex, NES is required for PRAK shuttling between the nucleus and cytosol (12). It was recently shown that interaction with nucleoporin is important for the nuclear import of ERK2 (27). Sequence alignment between

FIGURE 7. PRAK-induced growth inhibition depends on its nuclear localization. A, QTG and R361Q, two mutants of PRAK impaired for nuclear import, show diminished inhibitory effects on cell growth compared with wild type PRAK. NIH3T3 cells were separately transfected with GFP, GFP-PRAK, GFP-PRAK(QTG), and GFP-PRAK(R361Q). After 24 h of transfection, cell proliferation was determined by a BrdUrd intake assay as described under “Experimental Procedures.” Results are represented as mean ± S.D. of five independent experiments. The statistical analyses were performed by employing unpaired Student’s t test, and the significance is indicated as a p value. *, p < 0.001 compared with control GFP (first column); #, p < 0.001 compared with control GFP. Δ, groups cotransfected with GFP-PRAK, fourth to sixth columns, p < 0.001 compared with the corresponding groups transfected with GFP (first to third columns). ∇, p < 0.01 compared with the group transfected with GFP-PRAK alone (fourth column). *, p < 0.001 compared with the group transfected with GFP-PRAK alone. C, the function of p38α and p38β in regulating the growth inhibition mediated by PRAK can be swapped by interchanging amino acid residue 145. Expression vectors of GFP, GFP-PRAK, p38α(D145G), and p38β(G145D) were transfected into NIH3T3 cells in different combinations as indicated. The data are shown as the mean ± S.D. of five independent experiments. ∇, p < 0.05 compared with control GFP. Δ, groups cotransfected with GFP-PRAK, first column, p > 0.05 compared with control GFP. Δ, represents p < 0.001 when groups transfected with GFP-PRAK were compared with the corresponding groups transfected with GFP (first to third columns), individually. ∇, p < 0.001 compared with the group transfected with GFP-PRAK alone (fourth column). C, the function of p38α and p38β in regulating the growth inhibition mediated by PRAK can be swapped by interchanging amino acid residue 145. Expression vectors of GFP, GFP-PRAK, p38α(D145G), and p38β(G145D) were transfected into NIH3T3 cells in different combinations as indicated. The data are shown as the mean ± S.D. of five independent experiments. ∇, p < 0.05 compared with the group transfected with GFP-PRAK alone. D, different effects of p38α and p38β on PRAK-stimulated p53 transactivation. NIH3T3 cells were transfected with different combinations of expression vectors, p38α, p38β, p38α(D145G), and p38β(G145D), together with p53 reporter PG-Luc and pCMV-lacZ. Luciferase activities were determined 24 h after transfection and normalized to the values of β-galactosidase activity. The data shown are the mean ± S.D. of five independent experiments. ∇, p < 0.01 compared with the control (first column); #, p > 0.05 compared with control GFP. ∇, represents p < 0.001 when groups transfected with GFP-PRAK were compared with the corresponding groups transfected with GFP (first to third columns). ∇, p < 0.05 compared with the group transfected with GFP-PRAK alone (fourth column). F, Ras-induced p53 activation is affected differently by p38α and p38β. Expression vectors of Ha-RasV12, p38α, p38β, p38α(D145G), and p38β(G145D) were transfected into NIH3T3 cells in different combinations as indicated. Luciferase activity was determined as described in D. *, p < 0.001 compared with the control (first column); #, p > 0.05 compared with the group transfected with PRAK alone (sixth column). ∇, p < 0.001 compared with the group transfected with Ha-RasV12 alone (sixth column).
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