The proteasome usually completely degrades its target proteins, but it can also degrade a handful of proteins in a limited and site-specific manner. The molecular mechanism for such limited degradation is unknown. The repressor forms of Gli2 and Gli3 transcription factors are generated from their full-length proteins through limited proteasome-mediated protein degradation. In this study, we have taken advantage of the fact that Gli3 is efficiently processed, whereas Gli2 is not, and identified a region of ~200 residues in their C termini that determines differential processing of the two proteins. This region, named processing determinant domain, functions as a signal for protein processing in the context of not only Gli2 and Gli3 protein sequences but also a heterologous hybrid protein, which would otherwise be completely degraded by the proteasome. Thus, the processing determinant domain constitutes a novel domain that functions independently. Our findings explain, at the molecular level, why Gli2 and Gli3 are differentially processed and, more importantly, may help understand a probably general mechanism by which the proteasome degrades some of its target proteins partially rather than completely.

The ubiquitin-proteasome proteolytic system plays an important role in a wide variety of basic cellular processes. In the majority of these processes, the target proteins are completely degraded. In a few cases, however, the proteasome only partially degrades its target proteins. For instance, the generation of the p50 and p52 subunits of the transcriptional regulator NF-κB from their full-length precursors p105 and p100 is mediated by the proteasome in a site-specific manner (1–6). Although the detailed mechanism of NF-κBp100/p105 processing still needs to be worked out (7, 8), such limited degradation is thought to be due to the presence of a glycine-rich region (GRR) located immediately upstream of the NF-κB precursor cleavage site. The GRR region is proposed to serve as a “stop signal” to block further degradation of p50 or p52 (9, 10). More recently, it has been shown that a tightly folded domain upstream of GRR is also required as a part of the processing signal (34). Nevertheless, it is debatable whether GRR can function as an independent processing signal because fusing GRR with several proteins either closely related or unrelated to NF-κB but known to be the substrates of the ubiquitin-proteasome system does not cause the proteins to be processed (10).

The generation of Gli3/Ci (Cubitus interruptus) transcription repressors from their full-length precursors in the Hedgehog (Hh) signaling pathway may be another instance of a protein being partially degraded by the proteasome. Several lines of evidence support this view. First, Ci/Gli3 processing requires the sequential phosphorylation of numerous serine residues at their C-terminal regions by at least three kinases: protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) (11–14). Second, Ci/Gli3 processing is also dependent on the proteasome (13, 15, 16) and on Slimb/βTrCP of the SCF ubiquitin ligase complex (13, 14, 17). Third, we and others have recently shown that only phosphorylated Ci/Gli3 are able to directly bind Slimb/βTrCP, that Gli3 is polyubiquitinated in the cell, and that mutations of 4 lysine residues, the putative ubiquitination sites in the Gli3 C-terminal region, inhibit Gli3 processing (13, 14, 18, 19). Finally, deletions of a small region including the original cleavage sites in the C termini of both Ci and Gli3 cannot block Ci and Gli3 processing (20, 21). These observations further support the notion that Ci/Gli3 processing is carried out by the proteasome because the deletion of the cleavage site is expected to often disrupt the proteasome-mediated site-specific cleavage.

Gli2 is another member of the Gli/Ci family of transcription factors and shares with Gli3 a 44% sequence identity and conserved PKA, CK1, and GSK3 phosphorylation sites, but the protein is inefficiently processed in vivo (22), and its processing is undetectable in cell culture under conditions that have been used previously (22–24). The molecular basis for such differential processing between Gli2 and Gli3 is not known. In this study, by taking advantage of the fact that Gli2 processing is inefficient, but Gli3 processing is readily detected in cell culture, we identified a specific region in Gli2 and Gli3 C termini that determines the extent to which these proteins are processed.
A Gli2/Gli3 Protein-processing Determinant Domain

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—Expression constructs for mouse Gli2 (mGli2) and human Gli3 were described previously (24). Gli2-3CT contained the Gli3 C-terminal sequence starting from residue 648, whereas the Gli3-2CT chimera contained the Gli2 C terminus from residue 585. For all other chimeric constructs, amino acid residues that were swapped between Gli2 and Gli3 or deleted from Gli3 are indicated in diagrams in Figs. 2–4. All constructs were generated by a combination of PCR and restriction digestions. IkBa cDNA was obtained by reverse transcription-PCR. IkBα, which contained S32A and S36A amino acid substitutions, was engineered by PCR. 3-HA-tagged triple hybrid expression constructs, HA-Tub-Gli2PDD-IkBα, HA-Tub-Gli2PDD-IkBα, and HA-Tub-Gli3PDD-IkBα, were created by inserting either the Gli2 sequence (585–780 residues) or Gli3 sequence (648–844 residues) between α-tubulin at their N termini and IkBa or IkBα at their C termini. Two glycine residues were inserted between α-tubulin and PDD to avoid the possible steric interference. A mouse Ikkβ cDNA fragment encoding 1–680 residues, which is catalytically active, was obtained from Hao Wu at Weill Medical College and cloned into NotI and EcoRV sites of the pRK expression vector. Nucleotide sequences for all constructs created by PCR were verified by sequencing analysis.

**Cell Culture, Transfection, Protein Analysis, and Cell Staining**—Cell culture conditions, cell staining, methods of transfection, pharmacological treatment, and protein analysis for HEK293 cells and chick limb bud cells were as described (22, 24, 25).

**RESULTS**

**The Efficiency of Gli2 and Gli3 Processing Is Determined by Their C Termini**—We have previously shown that the majority of Gli3 protein is proteolytically processed in vivo and that Gli3 processing can be induced by PKA stimulation in cultured cells (24). We have recently demonstrated that there is a very low level of Gli2 processing in vivo (22), but in cultured cells, it is not detectable (22–24) (Fig. 1A). The undetectability of Gli2 processing in cultured cells could be simply due to the inefficient processing of the protein. To test this possibility, we enriched the Gli2 protein using Sepharose conjugated with Gli-binding oligonucleotides prior to immuno blotting (22). Indeed, a weak Gli2 processing was detected in cultured cells treated with forskolin (Fig. 1C, compare lane 4 to lane 3). Thus, the findings made in cultured cells regarding Gli2/Gli3 protein processing recapitulate those found in vivo. Besides the weak processing, full-length Gli2 protein, Gli2–185, is also readily degraded by proteosome, whereas full-length Gli3 protein, Gli3–190, is more stable than Gli2–185 (22, 26). Biochemical analysis has demonstrated that both Gli2 degradation and Gli3 processing are dependent on ubiquitination and proteosome activity and are inhibited by Hh signaling (13, 14, 22, 26, 27). These findings have led us to hypothesize that the Gli2 polypeptide sequence has evolved to inhibit its processing to fulfill its main role as a transcriptional activator, whereas the Gli3 polypeptide sequence has been evolutionally selected for enhanced processing to accomplish its potent repressing function. Based on this hypothesis, the different level of processing between Gli2 and Gli3 must be controlled by their amino acid sequences. To test this prediction, we swapped the entire C-terminal regions between Gli2 and Gli3 to generate chimeric constructs, Gli2-3CT and Gli3-2CT (Fig. 1B, lower panel). When the chimeras were tested for proteolytic processing in a chicken limb bud primary culture, Gli3-2CT did not show any processed protein band that was detectable, whereas Gli2-3CT was processed as efficiently as Gli3 (Fig. 1B), indicating that the
A Gli2/Gli3 Protein-processing Determinant Domain

**A**

| Construct | ZFs | PKA sites |
|-----------|-----|-----------|
| Gli3      |     |           |
| Gli3-1-1260 |   |           |
| Gli3-1-1048 |   |           |
| Gli3-1-946 |   |           |
| Gli3-1-860 |   |           |
| Gli3-1-740 |   |           |
| GST-Gli3-645-1050 |   |           |

**B**

| PKA*+  | 220kD | 97kD | 66kD |
|--------|-------|------|------|
| GST-Gli3-645-1050 |       |      |      |
| PKA*+ | yes | no | no |
| myc-mTrCP | no | yes | no |

**C**

| Construct | Blot: αGli3 | Blot: αGST |
|-----------|--------------|------------|
| GST-Gli3-645-1050 | yes | yes |
| Processed GST-Gli3-645-1050 | yes | yes |

**FIGURE 2. The first half of the Gli3 C terminus is sufficient for processing.**

A, schematic diagrams of expression constructs used in B and C. The numbers following Gli3 refer to amino acid positions. The zinc finger (ZFs) DNA binding domain and PKA phosphorylation sites are indicated. B and C, various Gli3 constructs were transfected into HEK293 cells along with either a constitutively active PKA (PKA*) or PKA* and myc-mTrCP, as indicated. Expression and processing of the Gli3 proteins were examined by immunoblotting using either an anti-Gli3 or an anti-GST antibody.

sequences responsible for the efficiency of Gli2 and Gli3 protein processing reside in the C terminus of the proteins.

To define a specific region within the Gli3 C terminus that is required for processing, a series of C-terminally truncated Gli3 constructs were engineered and tested for processing (Fig. 2A). Both Gli3-1-1260 and Gli2-1-1048, which contain all six PKA sites, were efficiently processed. Furthermore, Gli3-1-946, which contains the first four PKA sites, was still processed, whereas Gli3-1-860, which retains only the first two PKA sites, was no longer processed (Fig. 2B). These results are consistent with the observations that the PKA sites are required for Gli3 processing (13, 14, 24) and indicate that the region from residue 648 to 915, not to 945 as in Gli3, because this still includes the first four PKA sites (Fig. 3A). As predicted, the Gli2-3CTN was processed as efficiently as the Gli2-3CT chimera (Fig. 3B, lanes 2, 11, and 20), indicating that 3CTN and its Gli2 equivalent region determine how efficiently the proteins are processed.

To define a smaller region within 3CTN that makes Gli2 processed efficiently, we created six more constructs: Gli2-3CTNΔC1, Gli2-3CTNΔC2, Gli2-3CTNΔC3, Gli2-3CTNΔN1, Gli2-3CTNΔN2, and Gli2-3CTNΔN3, which contain various lengths of either N-or C-terminal sequences of 3CTN substituted for the equivalent regions of Gli2 (Fig. 3A). When these chimeric molecules were assayed for proteolytic processing, we found that Gli2-3CTNΔC1 and Gli2-3CTNΔC2, which contain the Gli3 sequences from the beginning of the C terminus to either immediately after the second PKA site or before the first PKA site, exhibited a level of processing similar to that of Gli2-3CTN whereas the processing of Gli2-3CTNΔC3, which contains the first 100 amino acid residues of Gli3-CTN but retains all Gli2 PKA sites, was slightly attenuated (Fig. 3B, left panel). In contrast, Gli2-3CTNΔN1 and Gli2-3CTNΔN2, which contain either PKA sites 3 to 4 or 1 to 4 from Gli3, respectively, failed to produce any detectable processed protein, if any at all (Fig. 3B, middle panel). Interestingly, Gli2-3CTNΔN3, which contained the C-terminal half (100 amino acids) of Gli3-CTN, displayed a very low level of processing, and the size of the processed protein was slightly larger than that of the Gli2-3CTN chimera (compare lane 17 to lane 11). Taken together, these data indicate that the sequence responsible for chimeric protein processing is located in the N-terminal half of 3CTN, i.e. from residue 648 to 748 of the Gli3 protein. The fact that Gli2-3CTNΔN3 produces a weakly processed peptide that is slightly larger than that of Gli2-3CTN suggests that the processing site is not fixed and can shift based on the combination of Gli2 and Gli3 sequences present in the region of the Gli2-3CTNΔN3 chimera.

To define the minimal region required for making Gli2 efficiently processed, more sequence from either the N- or C-terminal end of the 648–748-amino acid region of Gli3 (i.e. 3CTNΔC3) was trimmed to generate chimeric constructs: Gli2-3CTNΔC4, Gli2-3CTNΔC5, Gli2-3CTNΔN4, and Gli2-3CTNΔN5 (Fig. 3A). Both Gli2-3CTNΔC4 and Gli2-3CTNΔN5, which contain the 648–692- and 681–692-amino acid sequences of Gli3, respectively, exhibited a reduced level of processing as compared with that of Gli2-3CTNΔC3, whereas both Gli2-3CTNΔN4 and Gli2-3CTNΔC5, which contained either the 693–748 or 648–680 amino acids of Gli3, failed to produce any detectable level of processing (Fig. 3B, right panel). Taken together, these results indicate that the replacement of forms of the fusion protein. Together, these results indicate that the 3CTN region is necessary to and sufficient for Gli3 processing.

Two Amino Acid Changes Are Sufficient to Process Gli2 Efficiently—We then asked if the 3CTN substitution for the equivalent region of Gli2 would be sufficient to make Gli2 efficiently processed. To this end, a chimeric construct, Gli2-3CTN, was engineered and tested for processing. For the convenience of cloning, 3CTN in the chimeric construct contains the sequence from residue 648 to 915, not to 945 as in Gli3, because this still includes the first four PKA sites (Fig. 3A). As predicted, the Gli2-3CTN was processed as efficiently as the Gli2-3CTN chimera (Fig. 3B, lanes 2, 11, and 20), indicating that 3CTN and its Gli2 equivalent region determine how efficiently the proteins are processed.

To define a smaller region within 3CTN that makes Gli2 processed efficiently, we created six more constructs: Gli2-3CTNΔC1, Gli2-3CTNΔC2, Gli2-3CTNΔC3, Gli2-3CTNΔN1, Gli2-3CTNΔN2, and Gli2-3CTNΔN3, which contain various lengths of either N- or C-terminal sequences of 3CTN substituted for the equivalent regions of Gli2 (Fig. 3A). When these chimeric molecules were assayed for proteolytic processing, we found that Gli2-3CTNΔC1 and Gli2-3CTNΔC2, which contain the Gli3 sequences from the beginning of the C terminus to either immediately after the second PKA site or before the first PKA site, exhibited a level of processing similar to that of Gli2-3CTN whereas the processing of Gli2-3CTNΔC3, which contains the first 100 amino acid residues of Gli3-CTN but retains all Gli2 PKA sites, was slightly attenuated (Fig. 3B, left panel). In contrast, Gli2-3CTNΔN1 and Gli2-3CTNΔN2, which contain either PKA sites 3 to 4 or 1 to 4 from Gli3, respectively, failed to produce any detectable processed protein, if any at all (Fig. 3B, middle panel). Interestingly, Gli2-3CTNΔN3, which contained the C-terminal half (100 amino acids) of Gli3-CTN, displayed a very low level of processing, and the size of the processed protein was slightly larger than that of the Gli2-3CTN chimera (compare lane 17 to lane 11). Taken together, these data indicate that the sequence responsible for chimeric protein processing is located in the N-terminal half of 3CTN, i.e. from residue 648 to 748 of the Gli3 protein. The fact that Gli2-3CTNΔN3 produces a weakly processed peptide that is slightly larger than that of Gli2-3CTN suggests that the processing site is not fixed and can shift based on the combination of Gli2 and Gli3 sequences present in the region of the Gli2-3CTNΔN3 chimera.

To define the minimal region required for making Gli2 efficiently processed, more sequence from either the N- or C-terminal end of the 648–748-amino acid region of Gli3 (i.e. 3CTNΔC3) was trimmed to generate chimeric constructs: Gli2-3CTNΔC4, Gli2-3CTNΔC5, Gli2-3CTNΔN4, and Gli2-3CTNΔN5 (Fig. 3A). Both Gli2-3CTNΔC4 and Gli2-3CTNΔN5, which contain the 648–692- and 681–692-amino acid sequences of Gli3, respectively, exhibited a reduced level of processing as compared with that of Gli2-3CTNΔC3, whereas both Gli2-3CTNΔN4 and Gli2-3CTNΔC5, which contained either the 693–748 or 648–680 amino acids of Gli3, failed to produce any detectable level of processing (Fig. 3B, right panel). Taken together, these results indicate that the replacement of forms of the fusion protein. Together, these results indicate that the 3CTN region is necessary to and sufficient for Gli3 processing.
A Gli2/Gli3 Protein-processing Determinant Domain

11 Gli2 amino acid residues (616–626 amino acids) with their Gli3 equivalents (681–692 amino acids) is sufficient to increase Gli2 processing to a detectable level, and they suggest that the differences between the Gli2 and Gli3 12-amino acid sequences play an important role in regulating the efficiency of the processing of the two proteins.

To further elucidate the molecular basis of the differential processing of Gli2 and Gli3 proteins, we compared the 616–626 amino acids of Gli2 with the 681–692 amino acids of Gli3 and found that although both sequences contain a consensus sequence for casein kinase 2 (CK2) phosphorylation ((S/T)XX(E/D)), 620TVED623 for Gli2, and 684SKRE687 for Gli3, the 2 middle residues of each, VE and KR, are significantly different in property. We thus reasoned that the difference in the 2 residues might determine how efficiently the two proteins are processed. To test this hypothesis, we mutated 621VE622 to 621KR622 in Gli2 to generate a Gli2-VE->KR mutant construct (Fig. 3A). We found that the mutant protein did indeed exhibit a level of processing similar to those of both Gli2-3CTNA5 and Gli2-3CTNAC4 mutants (Fig. 3B, right panel), indicating that these 2 residues are required to determine the extent of Gli2 processing.

A Region of the First 196 Residues of the Gli2 C Terminus Is Sufficient to Inhibit Gli3 Processing—The fact that a Gli2 alteration of 621VE622 to 621KR622 makes processing efficient prompted us to test whether the difference in these 2 residues can explain the differential processing of Gli2 and Gli3 proteins. To address this question, we generated a reciprocal construct, Gli3-KR->VE. The mutant protein displayed a level of processing similar to those of wild type Gli3 and Gli2-3CT. Interestingly, the processed protein product was significantly larger than Gli3–83 in size, as measured by its migration (Fig. 4B, compare lane 5 with lane 3), indicating that the two amino acid changes caused the processing site to shift C-terminally. From these results, we conclude that the 685KR686 residues are required for Gli3 to be efficiently processed at the correct position.

Because mutations in the 2 residues did not block Gli3 processing, we wanted to determine the minimal Gli3 sequence that, when replaced by Gli2, would prevent the Gli3 protein from being processed in cultured cells. Based on the results in Fig. 3, we created three more Gli3-Gli2 chimeric constructs, designated Gli3-2CTNA5, Gli3-2CTNA3, and Gli3-2CTNA2 (Fig. 4A), which corresponded to Gli2-3CTNA5, Gli2-3CTNA3, and Gli2-3CTNA2 (Fig. 3A). When tested for processing in chick limb bud primary culture, Gli3-2CTNA5, in which 12 residues of the Gli3 C terminus were replaced by the corresponding Gli2 sequence, exhibited a significantly reduced level of processing as compared with the Gli3-KR->VE mutant, but it was processed at the same site as Gli3-KR->VE (Fig. 4B, compare lane 7 to lane 5). The extent of processing for Gli3-2CTNA3, which contained the first 100 residues of the Gli2 C terminus, was much less than that for Gli3-2CTNA5 (Fig. 4B, compare lane 9 to lane 7). When the first 197 N-terminal residues of the Gli3 C terminus were replaced by the corresponding Gli2 sequence, the chimeric molecule Gli2-3CTNA2 was no longer processed at a detectable level (lane 11). We designated this region of 196 residues in Gli2 or 197 residues in Gli3 as a PDD. These results indicate that the PDD determines how efficiently the two proteins are processed.

Subcellular Localization Does Not Determine the Differential Processing of Gli2 and Gli3—The extent of processing of the Gli2-Gli3 chimeric molecules described above may not necessarily be due to changes in their intrinsic nature but rather to changes in their subcellular localizations. To rule out this possibility, we examined the subcellular localization of Gli2, Gli3, and three representative chimeras: Gli2-3CT, Gli3-2CT, and Gli2-3CTN, in transfected chick limb bud primary cells. As shown in Fig. 5, Gli2 was exclusively localized in the nucleus,
A Gli2/Gli3 Protein-processing Determinant Domain

A

Gli3
Gli3-KR>VE
Gli3-2CTN\(\Delta N5\)
Gli3-2CTN\(\Delta C3\)
Gli3-2CTN\(\Delta C2\)

Gli2
Gli2-PK

ZF
PKA sites

Gli3 regions (aa) replaced by Gli2 replacing Gli3 Processing
None None None -
None None None + + +
685-686 620-621 + + + + a
681-692 616-626 + + a
648-748 585-676 + a
648-844 585-780 -

a: The processing site of these proteins shifts C-terminally.

B

FIGURE 4.

Replacement of the first 197 amino acid residues of the Gli3 C terminus with the corresponding Gli2 sequence is sufficient to suppress Gli3 processing. A, schematic diagrams showing Gli2, Gli3, and their chimeric proteins. B, an immunoblot showing the expression and processing of Gli3 and Gli3-Gli2 chimeric mutants. Note that the extent of processing for chimeric proteins decreases as more of the Gli2 sequence substitutes for Gli3 sequence and that the processed form of the chimeric proteins exhibits a slower mobility than Gli3, indicating that the processing site has been shifted C-terminally.

whereas Gli3 was predominantly found in the cytoplasm. Although our observation for Gli2 localization is consistent with a recent report, the predominantly cytoplasmic localization of Gli3 is different (28). We believe that the observed Gli3 localization more closely reflected its subcellular localization in vivo, because the Gli3 construct we used was not fused with any epitope tag. The localization of the three chimeras did not correlate to whether or how extensively they were processed. Most Gli2-3CT-expressing cells showed cytoplasmatic staining (5 nuclear versus 15 cytoplasmic), and almost all Gli3-2CT-transfected cells exhibited nuclear staining (18 nuclear versus 2 cytoplasmic), whereas Gli2-3CTN was found in the nucleus in most of the transfected cells (19 nuclear versus 1 cytoplasmic). These results suggest that the sequences that determine the localization of Gli2 and Gli3 proteins are located within their C-terminal regions and also argue strongly against the notion that subcellular localization determines how efficiently Gli2 and Gli3 proteins are processed. We thus conclude that the differential processing of Gli2 and Gli3 proteins is determined by the PDD of the Gli2 and Gli3 C termini.

The Gli3 PDD Functions Independently as a Signal for Protein Processing—We next wanted to know whether the Gli3 PDD could serve as a functional domain that independently mediates protein processing. To address this question, we fused an HA-tagged \(\alpha\)-tubulin to the N-terminal end and I\(\kappa\)B\(\alpha\) to constructs alone or together with I\(\kappa\)B\(\beta\) to induce the degradation of the fusion proteins. As shown in Fig. 5, only when I\(\kappa\)k\(\beta\) was coexpressed, a significant fraction of the full-length HA-Tub-Gli3PDD-I\(\kappa\)B\(\alpha\) fusion protein was processed to form one dominant and several faint larger protein products (Fig. 5, compare lane 8 to lane 7). The faint protein bands were presumably the intermediate processed products. Comparison of the size of the dominant processed fusion protein with that of HA-tagged \(\alpha\)-tubulin (HA-Tub) alone revealed that the processing occurred at a site where the full-length Gli3 protein is normally processed (Fig. 5, compare lane 8 to lane 2). In contrast, although coexpression of I\(\kappa\)k\(\beta\) also induced a dramatic reduction in the amount of full-length HA-Tub-Gli3PDD-I\(\kappa\)B\(\alpha\) fusion protein, only a small fraction of the fusion protein was processed (Fig. 5, compare lane 4 with lane 3), indicating that the fusion protein mainly underwent a complete degradation. To further verify that the processing and degradation of the fusion proteins were indeed induced by I\(\kappa\)k phosphorylation and mediated by the SCF\(^{\text{IkB}}\)-dependent ubiquitin/proteasome pathway, we repeated the same experiments using two fusion proteins (HA-Tub-Gli2PDD-I\(\kappa\)B\(\alpha\) and HA-Tub-Gli3PDD-I\(\kappa\)B\(\alpha\) that contained Ala substitutions for Ser-32 and Ser-36 of I\(\kappa\)B\(\alpha\). As predicted, the mutant fusion proteins were neither processed nor degraded, even when I\(\kappa\)k\(\beta\) was coexpressed (Fig. 5, lanes 6 and 10). These results indicate that

the C-terminal end of either the Gli2 or Gli3 PDD to create HA-Tub-Gli2PDD-I\(\kappa\)B\(\alpha\) and HA-Tub-Gli3PDD-I\(\kappa\)B\(\alpha\) (Fig. 6A), respectively. I\(\kappa\)B\(\alpha\) was chosen because the mechanism of its degradation has been well understood. I\(\kappa\)B\(\alpha\) is first phosphorylated at Ser-32 and Ser-36 by the I\(\kappa\)B kinase (I\(\kappa\)k), and the phosphorylated I\(\kappa\)B\(\alpha\) is then bound and ubiquitinated by SCF\(^{\text{IkB}}\)-CP ubiquitin ligase and is subsequently completely degraded by the proteasome (29, 30). Thus, if Gli3 PDD functions independently as a processing signal, we predict that, upon phosphorylation by I\(\kappa\)k, the HA-Tub-Gli3PDD-I\(\kappa\)B\(\alpha\) fusion protein would be processed approximately up to the first 50 residues of PDD from the C-terminal end based on the approximate processing site of the full-length Gli3 protein (24). In contrast, the HA-Tub-Gli2PDD-I\(\kappa\)B\(\alpha\) fusion protein would be completely degraded by the proteasome and/or processed at a very low efficiency.

To test the above prediction, HEK293 cells, which are known to contain all necessary components for I\(\kappa\)B\(\alpha\) degradation (29), were transfected with each of the two

constructs alone or together. As predicted, the mutant fusion proteins were neither processed nor degraded, even when I\(\kappa\)k\(\beta\) was coexpressed (Fig. 6B, lanes 6 and 10). These results indicate that...
A Gli2/Gli3 Protein-processing Determinant Domain

FIGURE 5. Subcellular localization of Gli2, Gli3, and their chimeric proteins. Chick limb bud primary cells overexpressing Gli2 (A), Gli3 (B), Gli2-3CT (C and D), Gli3-2CT (E and F), Gli2-3CTN (G and H) (see Figs. 1 and 3A for constructs) were immunostained with an anti-Gli3 (B, E, and F) or anti-Gli2 (A, C, D, G, and H) antibodies. Images were taken by a fluorescent microscope. Images were classified according to their subcellular localization listed in parentheses (C, cytoplasmic; N, nuclear).

Gli3 PDD acts independently as a processing domain that mediates protein processing.

DISCUSSION

Because the proteasome usually degrades its protein substrates completely, it is not clear how Gli2/Gli3 undergo a site-specific degradation by the proteasome and how Gli2/Gli3 are each processed at precise sites and to different extents. In the current study, we have identified the PDD necessary to and sufficient for the efficient processing of Gli3 but inefficient processing of Gli2 in the cell culture. The PDD contains the first 197 amino acid residues of the Gli2/Gli3 C termini and is equivalent to a simple sequence region in Ci, which serves as a part of the processing signal (34). The Ci processing signal consists of the zinc finger DNA binding domain and the subsequent simple sequence region. The zinc finger DNA binding domain is required for Ci processing, because it is thought to form a tightly folded domain. In the case of Gli2/Gli3 processing, the zinc finger DNA binding domain is clearly not required because replacing the Gli2/Gli3 N termini and zinc finger DNA binding domain with GST (Fig. 2) or α-tubulin (Fig. 6) does not prevent the fusion proteins from processing. However, it is possible that the ability of GST or α-tubulin to block the proteasome progression is because of the potential presence of a tightly folded domain in GST and α-tubulin, which would be functionally equivalent to the zinc finger DNA binding domain in terms of preventing the proteasome movement. The PDD is not at all similar to and much larger in size than the GRR found immediately upstream of the processing site of NF-κB. Based on the estimated processing site of Gli3, approximately only the first 50 residues of the Gli3 PDD is mapped upstream of the processing site, whereas the rest is downstream. This is very different from the NF-κB GRR, which is completely located N-terminal to the processing site. The GRR sequence is thought to act as a “stop signal” to inhibit further degradation of NF-κB by the proteasome (9, 10). A database search for proteins that share a sequence similar to that of the Gli3 region did not yield any additional information. This is consistent with a recent finding that the protein processing signal depends on the complexity of the simple sequence rather than on amino acid identity (34). It is also probably in part because only a few proteins are known to be processed by the proteasome.

Mechanistically, as recently proposed for Ci (34), approximately the first 50 residues of Gli3 PDD may have a low affinity for proteasome binding, resulting in the release of the proteasome from the Gli2/Gli3 protein substrates, and the actual domain that inhibits the proteasome movement is located immediately upstream of the 50 residues. Alternatively, the first 50 residues may themselves form a certain secondary structure that prevents the proteasome from unfolding the protein, thus blocking further degradation of the protein, since proteasome can only degrade unfolded proteins. Nevertheless, the structure of the 50 residues may be influenced by the rest of the PDD sequence, because the replacement of the first half of Gli3 PDD
A Gli2/Gli3 Protein-processing Determinant Domain

with the Gli2 equivalent region, which contains 100 residues (Gli3-2CTNΔC3 in Fig. 4), was not sufficient to prevent the proteasome from being processed. The requirement of amino acid sequence downstream of processing site can be explained if Gli3 protein is progressively processed from an internal site rather than from its C-terminal end. It has been shown that proteasome has an endoproteolytic activity that degrades its protein targets from its internal sequence (31–33). The difference between Gli2 and Gli3 PDD sequences should have been favorably selected during evolution so that the function of Gli2 and Gli3 could become more specialized to regulate more complex biological processes in vertebrates. The results from our analysis of the processing of Gli2-Gli3 chimeric molecules are consistent with the above hypothesis. Our observation that a Gli2 to Gli3 change of only 2 amino acids, at residues 620 and 621 of the C terminus (Gli2-VE>KR), is sufficient to make Gli2 efficiently processed suggests that these 2 residues play a critical role in determining how efficiently a protein undergoes processing or degradation. These 2 residues also control the location of the processing site, because the reciprocal amino acid substitutions in Gli3 result in a slight shift of the processing site toward the C-terminal end (Fig. 4B). Nevertheless, the role that these 2 amino acids play in processing must be placed in the context of surrounding sequences because the Gli3 protein with changes in these 2 amino acids is still processed efficiently; processing becomes undetectable only when the first 197 amino acid residues of the Gli3 C terminus are replaced by the equivalent Gli2 sequence. These findings support the notion that the complexity of the PDD sequence rather than amino acid identity determines the extent and position of Gli2/Gli3 processing, as recently suggested for NF-κB and Ci proteins (34), and also argue strongly that Gli3 is processed by the proteasome. The GRR from NF-κB can serve as a processing signal in the context of NF-κB or when inserted between gp10 and GST proteins or between GST and green fluorescent protein (9, 33). These heterologous proteins are not proved proteasome targets. However, when it is fused with several proteins that are either related or unrelated to NF-κB, but all are known to be the proteasome targets, the fusion proteins fail to be processed (9, 10), suggesting that the portability of GRR remains questionable. In contrast, the Gli2/Gli3 PDD identified in this study is able to function independently as a processing domain, because the fusion proteins, in which Gli2 or Gli3 PDD is inserted between α-tubulin and IκBα (a well known proteasome target), undergo either an inefficient or efficient limited degradation, depending on the presence of Gli2PDD or Gli3PDD sequences. To our knowledge, PDD is the first transferable domain identified that can impede complete protein degradation by proteasome, yet whose sequence and size is utterly different from GRR of NF-κB. The cleavage region mapped in Ci (34), which is equivalent to Gli2/Gli3 PDD, may also likely be portable. The identification of PDD in Gli2/Gli3 may help us elucidate a potentially general mechanism by which the proteasome degrades a target protein in a limited and site-specific manner. **Acknowledgments**—We thank Wendy Wang for reading the manuscript and Hao Wu for a Ikkβ cDNA.

**REFERENCES**

1. Fan, C. M., and Maniatis, T. (1991) *Nature* **354**, 395–398
2. Orian, A., Whiteside, S., Israel, A., Stancovski, I., Schwartz, A. L., and Ciechanover, A. (1995) *J. Biol. Chem.* **270**, 21707–21714
3. Coux, O., and Goldberg, A. L. (1998) *J. Biol. Chem.* **273**, 8820–8828
4. Heusch, M., Lin, L., Gelezunis, R., and Greene, W. C. (1999) *Oncogene* **18**, 6201–6208
5. Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) *Cell* **78**, 773–785
6. Lin, L., DeMartino, G. N., and Greene, W. C. (1998) *Cell* **92**, 819–828
7. Orian, A., Gonen, H., Bercovich, B., Fajerman, L., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Ciechanover, A. (2000) *EMBO J.* **19**, 2580–2591
8. Cohen, S., Aebert-Weiner, H., and Ciechanover, A. (2004) *Mol. Cell. Biol.* **24**, 475–486
9. Lin, L., and Ghosh, S. (1996) *Mol. Cell. Biol.* **16**, 2248–2254
10. Orian, A., Schwartz, A. L., Israel, A., Whiteside, S., Kahana, C., and Ciechanover, A. (1999) *Mol. Cell. Biol.* **19**, 3664–3673
11. Jia, I., Amanai, K., Wang, G., Tang, J., Wang, B., and Jiang, J. (2002) *Nature* **416**, 823–835
12. Price, M. A., and Kalderon, D. (2002) *Cell* **108**, 823–835
13. Wang, B., and Li, Y. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 33–38
14. Tempe, D., Casas, M., Karaz, S., Blanchet-Tournier, M. F., and Conordet, J. P. (2006) *Mol. Cell. Biol.* **26**, 4316–4326
15. Chen, C. H., von Kessler, D. P., Park, W., Wang, B., Ma, Y., and Beachy, P. A. (1999) *Cell* **98**, 305–316
16. Wang, Q. T., and Holmgren, R. A. (1999) *Development* (Camb.) **126**, 5097–5106
17. Jiang, I., and Struhl, G. (1998) *Nature* **391**, 493–496
18. Jia, I., Zhang, L., Zhang, Q., Tong, C., Wang, B., Hou, F., Amanai, K., and Jiang, I. (2005) *Dev. Cell* **9**, 819–830
19. Smelkinson, M. G., and Calderon, D. (2006) *Curr. Biol.* **16**, 110–116
20. Methot, N., and Basler, K. (1999) *Cell* **96**, 819–831
21. Wang, C., Pan, Y., and Wang, B. (2007) *Dev. Dyn.* **236**, 769–776
22. Pan, Y., Bai, C. B., Joyner, A. L., and Wang, B. (2006) *Mol. Cell. Biol.* **26**, 3365–3377
23. Ruiz i Altaba, A. (1999) *Development* (Camb.) **126**, 3205–3216
24. Wang, B., Fallon, J. F., and Beachy, P. A. (2000) *Cell* **100**, 423–434
25. Wang, B., Golemis, E. A., and Kruh, G. D. (1997) *J. Biol. Chem.* **272**, 17542–17550
26. Huntzicker, E. G., Estay, I. S., Zhen, H., Lokteva, L. A., Jackson, P. K., and Oro, A. E. (2006) *Genes Dev.* **20**, 276–281
27. Bhatia, N., Thiagarajan, S., Echeva, I., Saleem, M., Dlugosz, A., Mukhtar, H., and Spiegelman, V. S. (2006) *J. Biol. Chem.* **281**, 19320–19326
28. Varjosalo, M., Li, S. P., and Taijule, J. (2006) *Dev. Cell* **10**, 177–186
29. Spencer, E., Jiang, J., and Chen, Z. J. (1999) *Genes Dev.* **13**, 284–294
30. Winston, J. T., Strack, P., Beer-Romero, P., Chu, C. Y., Elledge, S. J., and Harper, J. W. (1999) *Genes Dev.* **13**, 270–283
31. Liu, C. W., Corboy, M. J., DeMartino, G. N., and Thomas, P. J. (2003) *Science* **299**, 408–411
32. Piwko, W., and Jentsch, S. (2006) *Nat. Struct. Mol. Biol.* **13**, 691–697
33. Moorthy, A. K., Savinova, O. V., Ho, J. Q., Wang, V. Y., Vu, D., and Ghosh, G. (2006) *EMBO J.* **25**, 1945–1956
34. Tian, L., Holmgren, R. A., and Matouschek, A. (2005) *Nat. Struct. Mol. Biol.* **12**, 1045–1053