Specific and Potent RNA Interference in Terminally Differentiated Myotubes*

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Double-stranded RNA (dsRNA) interference is a potent mechanism for sequence-specific silencing of gene expression and represents an invaluable approach for investigating gene function in normal and diseased states as well as for drug target validation. Here, we report that skeletal muscle myoblasts and terminally differentiated myotubes are susceptible to RNA interference. We employed an approach in which dsRNA is generated by cellular transcription from plasmids containing long (1 kilobase) inverted DNA repeats of the target gene rather than using dsRNA synthesized in vitro. We show that gene silencing by this method is effective for endogenously expressed genes as well as for exogenous reporter genes. An analysis of the expression of several endogenous genes and exogenous reporters demonstrates that the silencing effect is specific for the target gene containing sequences within the inverted repeat. Our method eliminates the need to chemically synthesize dsRNA and is not accompanied by global repression of gene expression. Furthermore, we show for the first time that sequence-specific dsRNA-mediated gene silencing is possible in differentiated, multinucleated skeletal muscle myotubes. These findings provide an important molecular tool for the examination of protein function in terminally differentiated muscle cells and provide alternative approaches for generating disease models.

The availability of whole genome sequences for humans and model organisms has greatly facilitated the identification of genes responsible for inherited human disease. In the post-genomics era, efforts to understand the molecular basis of disease will be limited primarily by the ability to determine the function of proteins encoded by candidate disease genes and to evaluate novel drug targets. The ability to silence expression of specific genes is a powerful mechanism for analysis of gene function. Although targeted gene disruption via homologous recombination is possible in mammals, currently available strategies are costly and time-consuming. The generation of knock-out animal models may also be confounded by unanticipated splice variants that produce functional proteins, despite the removal of targeted exons (1). Furthermore, when embryonic lethality results from gene deletion (2), it may be impossible to evaluate the role of a target protein in fully differentiated cells and adult tissues.

Sequence-specific gene silencing initiated by the presence of aberrant RNA was first observed in plants containing inverted transgenes and referred to as post-transcriptional gene silencing (3, 4). A similar gene silencing phenomenon was observed in Caenorhabditis elegans where insightful genetic studies demonstrate that silencing was mediated by double-stranded RNA and occurred at the level of mRNA abundance (5, 6). The term double-stranded RNA interference (dsRNAi) was coined to describe this RNA-mediated genetic interference. The use of RNAi to target specific mRNAs for degradation provides an alternative method for targeted gene silencing and has now been shown to function in a variety of organisms including nematodes, planaria, trypanosomes, hydra, zebrafish, and Drosophila (5–14). In these systems, the presence of dsRNA composed of protein coding sequence induces degradation of single-stranded mRNA that is complementary to the dsRNA (5, 6, 15, 16). Genetic screens for RNAi-resistant mutants in C. elegans has led to the identification of some components of the RNAi machinery including an RNA-dependent RNA polymerase, eIF-2C, and RNase D (17–19). Although the precise mechanism of RNAi is still unclear, the process has been heralded as revolutionary because of its specificity, catalytic features, and effectiveness in comparison with traditional sense or antisense knock-out technology (20).

Despite the broad species specificity of RNAi (16), its utility for examining gene function in mammalian cells has remained somewhat dubious because of nonspecific repression of gene expression that is induced by cellular responses to cytoplasmic duplex RNA in many mammalian cell lines (21–25). A major component of this response is presumed to be the dsRNA-dependent protein kinase PKR, which upon activation by dsRNA longer than 30 nucleotides, phosphorylates eIF-2α, leading to global down-regulation of protein translation (21, 22). In an important advance, Elbashir et al. (23) and Caplen et al. (24) recently showed that by limiting the length of the synthetic dsRNA to <30 bp, sequence-specific RNAi can be achieved in several commonly used non-differentiated cultured mammalian cell lines. These studies demonstrate that the machinery responsible for sequence-specific RNAi is present in these cells, although the magnitude of the specific decrease in target gene expression varies consider-

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1 The abbreviations used are: dsRNAi, double-stranded RNA interference; dsRNA, double-stranded RNA; eIF, eukaryotic initiation factor; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; SSPN, sarcospan.
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EXPERIMENTAL PROCEDURES

Plasmid Expression Constructs—A human dyxin expression construct was prepared by PCR amplification using primers containing appropriate restriction sites for subcloning into the mammalian expression vector pcDNA3 (Amer sham Biosciences). A human sarcospan open reading frame was prepared by PCR amplification of the sarcospan open reading frame from a human skeletal muscle cDNA library (Clontech, Palo Alto, CA) (31). Both the dyxin (pcDNA.dyxin) and the sarcospan (pcDNA.SSPN) constructs were engineered to encode a Myc tag at the COOH terminus followed by a stop codon. For dsRNA expression constructs, standard cloning methods were used to place oppositely oriented copies of the dyxin open reading frame at either end of the GFPmut3b gene (GenBank accession number AF216709) (29) by using primers containing appropriate restriction sites for subcloning into the pcDNA3 vector. The plasmid was then introduced into C2C12 myoblasts by electroporation.

Transfection of C2C12 Myoblasts—C2C12 myoblasts were transfected by the method of FuGene HD (Roche Molecular Biochemicals) and subjected to Northern blot analysis using probes, representing the sarcospan open reading frame (GenBank(TM) accession number AF216709). Dyxin has two tandem LIM domains in its COOH-terminal region and is highly expressed in skeletal muscle cells. This approach has been dramatically successful in other non-mammalian systems (13) and has recently been shown to be effective for RNAi in embryonic cell lines (28). Using this method, we demonstrate that RNA interference is effective and specific in cultured mammalian muscle cells.

RESULTS

Inherited muscle disease provides an excellent example of a field where the identification of candidate disease genes is outpacing efforts to understand function of the corresponding gene products. We investigated whether RNAi could be used for sequence-specific gene silencing in muscle cells as a tool for analysis of gene function. As a target gene, we chose a novel “LIM and cysteine-rich domain” gene (LMCD1) (29) (GenBank accession number NM014583), also known as dyxin (GenBank(TM) AF216709). Dyxin has two tandem LIM domains in its COOH-terminal region and is highly expressed in skeletal muscle (29). Although the precise function of dyxin is unknown, it is postulated to be involved in DNA-protein interactions during skeletal muscle development (29).

As a model system, we employed C2C12 cells, a well-established murine skeletal muscle cell line (33). C2C12 cells can be maintained in culture as undifferentiated myoblasts or can be induced to fuse and assemble into terminally differentiated, multinucleated myotubes in low mitogen medium (33). Immunoblots of whole cell lysates from C2C12 myoblasts and myotubes were probed with affinity-purified anti-dyixin polyclonal antibodies (Fig. 1). As shown in Fig. 1, dyxin is expressed in mature differentiated myotubes but not in myoblasts. Thus, by employing both myoblasts and myotubes, we are able to assess the utility of RNAi for silencing the expression of exogenous reporter genes (recombinant dyxin in myoblasts) as well as endogenously expressed genes (dyxin in myotubes).

To demonstrate that exogenous dyxin expression is possible in myoblasts, a Myc-tagged human dyxin cDNA plasmid (pcDNA.dyxin) was introduced into C2C12 myoblasts by electroporation. Immunoblots of protein lysates from transfected C2C12 cells were stained with either anti-dyixin antibodies (Fig. 2) or monoclonal antibodies to the Myc tag (data not shown). Dyxin protein was not detected in mock-transfected cells (Fig. 2, lane 1) but is expressed at high levels 30 h post-transfection with pcDNA.dyxin (Fig. 2, lane 2).

Previous efforts to use RNAi for sequence-specific gene silencing in cultured mammalian cells have primarily employed dsRNA molecules that were produced and annealed in vitro and then introduced into the cytoplasm of cultured cells by injection or liposome-mediated transfection (23, 24, 26). When
the length of the synthetic dsRNA exceeds 30 bp, the target cells are found to exhibit a generalized suppression of gene expression (23, 24). This nonspecific inhibition of gene expression is presumed to result from interferon-response pathways that are activated by the presence of dsRNA injected into the cytoplasm, a process that may mimic the response of the cell to viral dsRNA (23, 24, 36). In this study, we employed an alternative strategy in which the cell’s own transcription machinery transcribes inverted DNA repeats, producing dsRNA within the cell. For these experiments, we generated a dyxin dsRNA expression vector (pcDNA.dyxin.dsRNA) containing inverted copies of a 1-kilobase portion of the dyxin open reading frame (Fig. 1). The specificity of this gene silencing was tested using a key feature of RNA interference is that it exerts its effect at the post-transcriptional level by destruction of targeted mRNA (for review see Ref. 16). To test whether the loss of dyxin protein occurred at the level of mRNA abundance, we analyzed dyxin mRNA levels by Northern blotting. Total RNA was prepared from the samples analyzed previously for dyxin protein (Fig. 4, lane 1). Expression of sarcospan was not affected by co-expression of dyxin dsRNA (Fig. 4, lane 2).

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Further demonstrate that silencing is specific for dyxin.

The ability to silence specific genes in mammalian cells holds great promise for uncovering the function of proteins that are known to be defective in certain inherited diseases. However, many disease genes are expressed only in terminally differentiated cells and tissues. For example, components of the dystrophin-glycoprotein complex are required for normal muscle function and prevention of Duchenne muscular dystrophy (38) but are expressed only upon differentiation of myoblasts into myotubes (39). To determine whether RNAi can be used for sequence-specific gene silencing in differentiated mammalian cells, we induced the differentiation of C2C12 myoblasts into skeletal muscle myotubes in culture. Myotubes possess fully functional sarcomeres, exhibit contractile properties in culture, and are important models for mammalian skeletal muscle physiology (33). We introduced the dyxin dsRNA expression vector (pcDNA.dyxin.dsRNA) into myotubes by liposome-mediated transfection. Whole myotube protein lysates were analyzed at 0, 12, and 24 h post-transfection by Western blotting with affinity-purified anti-dyxin antibodies (Fig. 6). Endogenous dyxin expression in myotubes was completely abolished.
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12 and 24 h after transfection with the dyxin dsRNA expression vector (Fig. 6). Dyxin levels were constant for all mock-treated samples (Fig. 3) (data not shown). As is the case for myoblasts, dyxin dsRNA-mediated gene silencing is specific to dyxin because β-tubulin, vinculin, and E1P-2a expression remained constant in all samples (Fig. 6) (data not shown).

DISCUSSION

Skeletal muscle fibers are specialized multinucleated cells with well-organized contractile filaments. Investigating the function of muscle-specific gene products is complicated by the limitations of traditional cell lines, which lack the contractile apparatus and therefore lack many muscle-specific proteins. Although cultured myotubes provide an excellent model for studying skeletal muscle physiology, the lack of a method to easily eliminate gene expression in these cells has restricted in vitro analysis of protein function. siRNAs offer a number of advantages over gene disruption, because it is rapid, potent, and can be achieved with a fraction of the effort required for generating knock-out animal models. Recent groundbreaking experiments have demonstrated that sequence-specific RNAi is feasible in certain types of undifferentiated cultured mammalian cells using chemically synthesized or in vitro generated duplex RNAs (23, 26, 27, 40). To extend these studies to terminally differentiated myofibers, we tested a method whereby plasmid DNA containing large inverted repeats of a target mRNA is introduced into myoblasts and myotubes. In this way, dsRNA is generated intracellularly by transcription from the expression vector. We demonstrate that gene silencing induced by this method results in a loss of target gene expression without perturbing overall gene expression. Importantly, we show that the target mRNA can be an endogenous transcript or an exogenous reporter gene and that the loss of the target mRNA is correlated with concomitant loss of target protein. Only the target gene is affected, and inverted repeats containing non-relevant sequences have no effect on target gene expression. In addition, we demonstrate that gene silencing by this approach is effective in mature differentiated skeletal muscle myotubes.

Although the mechanism of dsRNA-mediated gene silencing observed here in mammalian muscle cells remains to be determined, several lines of evidence suggest that this is occurring through a phenomenon mechanistically related to RNA interference pathways that have been characterized in C. elegans, Dro sophila, and other systems (16). First, the loss of target gene expression is not accompanied by global down-regulation of protein synthesis. Second, target gene silencing is sequence-specific, because non-homologous dsRNA does not interfere with target gene expression. Third, silencing of the target gene occurs at the level of mRNA abundance. Importantly, the ability to silence endogenously expressed genes demonstrates the utility of this approach for examining the function of proteins that are only expressed in differentiated muscle cells.

Recent work from several laboratories indicates that the effectiveness of long dsRNA for inducing sequence-specific RNAi in cultured mammalian cells varies from cell type to cell type (23, 26, 27, 40). During preparation of this paper, Paddison and colleagues (28) report using a 500-nucleotide synthetic luciferase dsRNA to knockdown expression of an exogenous reporter gene (firefly luciferase) in murine myoblasts. Our results compliment and extend this work by demonstrating potent and sequence-specific silencing of endogenous gene expression in both myoblasts and myotubes. Together, these results suggest that murine myoblasts and myotubes may be more susceptible to sequence-specific RNAi than other mammalian cell types thus far tested as has been previously been reported for embryonic cell lines (28).

We have shown that double-stranded RNA-induced gene silencing is possible in terminally differentiated muscle cell lines. This represents the first example of dsRNA-induced gene silencing in terminally differentiated mammalian cells and demonstrates that the target gene can be an endogenous transcript that is expressed only upon cellular differentiation. These findings provide an alternative to traditional gene “knock-out” studies for generating models of inherited muscle disease. It has recently been reported that RNAi induced by short synthetic interfering RNA duplexes (siRNA) can be used to knockdown expression of several endogenous genes in mammalian cell lines (23, 24, 41). Our studies extend these findings by demonstrating that sequence-specific dsRNA-mediated gene silencing can be used for functional analysis of muscle gene products within the context of a biologically relevant cell culture model for mammalian muscle. With advances in gene-silencing techniques, diseases that are caused by genetic mutations leading to loss of protein expression (i.e. recessive muscular dystrophies) can be investigated with greater ease.

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REFERENCES

1. Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H., and Fishman, M. C. (1993) Cell 75, 1273–1286
2. Williamson, R. A., Henry, M. D., Daniels, K. J., Hrstka, R. F., Lee, J. C., Sunada, Y., Bhringhov-Beskrovnaya, O., and Campbell, K. P. (1997) Hum. Mol. Genet. 6, 831–841
3. Que, C., and Jorgensen, R. A. (1998) Dev. Genet. 22, 100–109
4. Jorgensen, R. A., Cluster, P. D., English, J., Que, C., and Napoli, C. A. (1996) Plant Mol. Biol. 31, 957–973
5. Montgomery, M. K., Xu, S., and Fire, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15502–15507
6. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Nature 391, 806–811
7. Kennerdell, J. R., and Cartewh, R. W. (1998) Cell 95, 1017–1026
8. Timmons, L., and Fire, A. (1998) Nature 395, 854
9. Wargielius, A., Ellingsen, S., and Fjose, A. (1999) Biochem. Biophys. Res. Commun. 263, 156–161
10. Bahramian, M. B., and Zarbl, H. (1999) Mol. Cell. Biol. 19, 274–283
11. Lehmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) Science 285, 110–113
12. Misquitta, L., and Paterson, B. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1431–1456
13. Ngs, H., Tsuchdi, C., Gall, K., and Ullu, E. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14687–14692
14. Li, W., Boswell, R., and Wood, W. B. (2000) Dev. Biol. 218, 172–182
15. Bosher, J. M., Dufourcq, P., Sookhareea, S., and Labouesse, M. (1999) Genetics 153, 1245–1256
16. Hammond, S. M., Caudy, A. A., and Hanlon, G. J. (2001) Nat. Rev. Genet. 2, 110–119
17. Ketting, R. F., Haverkamp, T. H., van Luenen, H. G., and Plasterk, R. H. (1999) Cell 99, 133–141
18. Tabara, H., Sarkissian, M., Kelly, W. G., Fleener, J., Grishok, A., Timmons, L., Fire, A., and Mello, C. C. (1999) Cell 99, 123–132
19. Smardon, A., Spierke, J. M., Stacey, S. C., Klein, M. E., Mackin, N., and Maine, E. M. (2000) Curr. Biol. 10, 169–178
20. Bosher, J. M., and Labouesse, M. (2000) Nat. Cell Biol. 2, E31–E36
21. Clemence, M. J. (1997) Int. J. Biochem. Cell Biol. 29, 945–949
22. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, B. H., and Schreiber, R. D. (1998) Annu. Rev. Biochem. 67, 227–264
23. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 244–248
24. Caplen, N. J., Parrish, S., Imani, F., Fire, A., and Morgan, R. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9742–9747
25. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J., and Conklin, D. S. (2002) Genes Dev. 16, 194–205
26. Wissmann, F., and Zernicka-Goetz, M. (2000) Nat. Cell Biol. 2, 70–75
27. Caplen, N. J., Fleener, J., Fire, A., and Morgan, R. A. (2000) Gene (Amst.) 252, 95–105
28. Paddison, P. J., Caudy, A. A., and Hanlon, G. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 1443–1448
29. Bessolvea, I. N., and Burmeister, M. (2000) Genomics 63, 69–74
30. Chu, D. T., and Klymkowsky, M. W. (1989) Amst. J. Genet. 157, 9742–9747
31. Broccoli, R. H., Heighway, J., Venzke, D. P., Lee, J. C., and Campbell, K. P. (1997) J. Biol. Chem. 272, 32121–32124
32. Crookshak, B. P., Vaile, R. H., and Fulkow, S. (1996) Gene (Amst.) 173, 33–38
33. Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C., and Webster, C. (1985) Science 230, 758–766
34. Yaffe, D., and Saxel, O. (1977) Nature 270, 725–727

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35. Crosbie, R. H., Lebakken, C. S., Holt, K. H., Venzke, D. P., Straub, V., Lee, J. C., Grady, R. M., Chamberlain, J. S., Sanes, J. R., and Campbell, K. P. (1999) J. Cell Biol. 145, 153–165
36. Clemens, M. J., and Elia, A. (1997) J. Interferon Cytokine Res. 17, 503–524
37. Crosbie, R. H., Lim, L. E., Moore, S. A., Hirano, M., Hays, A. P., Maybaum, S. W., Collin, H., Devico, S. A., Stolle, C. A., Fardeau, M., Tome, F. M., and Campbell, K. P. (2000) Hum. Mol. Genet. 9, 2019–2027
38. Allamand, V., and Campbell, K. P. (2000) Hum. Mol. Genet. 9, 2459–2467
39. Belkin, A. M., and Burridge, K. (1995) J. Biol. Chem. 270, 6328–6337
40. Ui-Tei, K., Zenn, S., Miyata, Y., and Saigo, K. (2000) FEBS Lett. 479, 79–82
41. Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T., and Weber, K. (2001) J. Cell Sci. 114, 4557–4565
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