A Plant Homolog of Animal Chloride Intracellular Channels (CLICs) Generates an Ion Conductance in Heterologous Systems*

Received for publication, July 31, 2006, and in revised form, January 31, 2007 Published, JBC Papers in Press, January 31, 2007 DOI 10.1074/jbc.M607241200

Astrid Elter, Andreas Hartel, Christian Sieben, Brigitte Hertel, Elke Fischer-Schliebs, Ulrich Lüttege, Anna Moroni, and Gerhard Thiel

From the Institute of Botany, Darmstadt University of Technology, D-64287 Darmstadt, Germany, the Dipartimento di Biologia and IBF-CNR, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy, and the INFN-Unità di Milano Università, Via Celoria 16, 20133 Milano, Italy

The genome of Arabidopsis thaliana contains unusual members of the glutathione S-transferase (GST) superfamily with a cysteine in place of a serine at the active site. Four of these genes (at-dhar 1–4) have an appreciable homology to intracellular Cl− channels (CLICs) from vertebrates and invertebrates. Transient expression of AtDHAR1 as wild type protein or as a chimera with GFP in mammalian HEK293 or Chinese hamster ovary cells generated a distinct inward rectifying conductance with a characteristic biphasic kinetics but no apparent ion selectivity. Analysis of the subcellular localization of AtDHAR1:GFP showed that the bulk of the protein was located as soluble form in the cytoplasm; however, an appreciable fraction of it could also be found in association with the non-soluble microsomal fraction. These data suggest that plant members of the GST superfamily have similar to those from animals multiple functions. The increase of ion conductance by AtDHAR1 is better explained by a CLIC-like channel activity than by a modification of endogenous channel proteins.

Anion channels are present in the plasma membrane and endomembranes of all eukaryotes. While the functional properties of these channels in plants were well characterized over the last decade with electrophysiological methods (reviewed in Ref. 1) the molecular characterization of the unerlying channel proteins is far behind. The genome of Arabidopsis thaliana contains several genes with a high homology to animal anion channels of the chloride channel (CLC) family (2); however, proof of their function as anion channels, which have been reported to be present in both a transmembrane and a soluble form (4, 5). It has therefore long been questioned whether these proteins are able to function as ion channels. However, recent data strongly favor the view that they have channel function. Evidence for channel activity relies on the functional reconstitution of the protein in lipid bilayers (5, 7, 9, 10) and the observation that features of the channel in the bilayer share similarities with native channel currents in cells (7). Altogether the available data suggest a model according to which CLIC channels are inserted into the membrane upon a stimulus from the cytoplasm (11). Thus the channels seem to behave in a similar way to other channel-forming proteins such as bacterial toxins, the intracellular Bcl-Xl protein, and annexins (12). Moreover these proteins are able to undergo a reversible transition from a soluble cytosolic into an ion conducting, membrane inserted form (4).

The structure of CLICs suggests that they are part of a protein super family of glutathione S-transferases (GSTs) (4). Among other common features CLICs (from vertebrates), like GSTs, contain the same highly conserved domains for glutathione-dependent dehydroascorbate reduction activity. For two of them a cytosolic localization is predicted; because of a specific targeting sequence the remaining two proteins are thought to be most likely present in the chloroplasts (3).

Animal and plant GSTs are well characterized by their role in detoxification: they are able to conjugate different toxins with glutathione. In addition to this well known function, GSTs from plants and animals also seem to perform other functions. These
include, for example, a peroxidase and isomerase activity (4, 13). With such a broad spectrum of activities it is not surprising that some of them, namely the CLICs, also function as ion channels. This hypothesis of multiple functions is also relevant in view of the fact that the genome of Arabidopsis contains more than 25 genes, which code for GSTs (13, 14). This abundance may also suggest that plant GSTs have multiple functions. In the present work we are examining the possibility that a GST from Arabidopsis, which shares similarities with the animal CLICs, has ion channel activity. By heterologous expression of the plant homolog of animal CLICs in mammalian HEK293 cells, we show with patch clamp recordings that transfected cells reveal an elevated inward-rectifying conductance suggesting that this protein can produce an ion conductance.

**EXPERIMENTAL PROCEDURES**

**PCR Amplification, Cloning, and Expression of AtDHAR1**—Photosynthetic tissue of 3-week-old A. thaliana (Columbia) plants was used as source RNA. RNA was transcribed in cDNA using the RevertAid™ first strand NA Synthesis kit (Fermentas, St. Leon-Rot, Germany).

AtDHAR1 sequence was amplified from cDNA via reverse transcriptase-PCR using TaqDNA polymerase and 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 74 °C for 4 min, with the combinations of the following primers: for AtDHAR1, 5′-ggatcctatgtgcctctggaaatctgtgtg-3′; revAtDHAR1 with stop codon, 5′-cctagaattctcaagggttaactttgggagc-3′; and revAtDHAR1 without stop codon, 5′-cctagaattcagggttaactttgggagccca-3′.

PCR products were purified and ligated into BglII/EcoRI-digested pEGFP-N2 or pIRES-EGFP (both from Clontech, Heidelberg, Germany) for expression in human embryo kidney (HEK293) cells or Chinese hamster ovary (CHO) cells. Cells were transiently transfected using the liposomal transfection reagent (HEK293) or Chinese hamster ovary (CHO) cells. Cells were digested pEGFP-N2 or pIRES-EGFP (both from Clontech, Heidelberg, Germany). This hypothesis of multiple functions is also relevant in view of the fact that the genome of Arabidopsis contains more than 25 genes, which code for GSTs (13, 14). This abundance may also suggest that plant GSTs have multiple functions. In the present work we are examining the possibility that a GST from Arabidopsis, which shares similarities with the animal CLICs, has ion channel activity. By heterologous expression of the plant homolog of animal CLICs in mammalian HEK293 cells, we show with patch clamp recordings that transfected cells reveal an elevated inward-rectifying conductance suggesting that this protein can produce an ion conductance.

**MARCH 23, 2007•VOLUME 282•NUMBER 12 JOURNAL OF BIOLOGICAL CHEMISTRY 8787**

**RESULTS**

AtDHAR1 Has Structural Similarities to CLICs—Search with the human HsCLIC-1 identified four genes in A. thaliana (known as at-dhar1–4 (3)) with an appreciable similarity to the human intracellular Cl⁻ channel. Indeed, the Arabidopsis protein also has a proline-rich stretch. The functionally important amino acids Gly-171 and Pro-66 in HsCLIC1 have their equivalents in AtDHAR1. Notably, however, the loop region with negative amino acids in HsCLIC-1 is absent in AtDHAR1.

**Arabidopsis CLIC-like Channel**

Mapping of CLIC like channels from C. elegans has uncovered the importance of the N-terminal domain for targeting and insertion of the channel in the right membrane of action (18). The respective domain comprises a central α-helix flanked by two β-strands for proper targeting and possible membrane insertion. An alignment of the N-terminal part of AtDHAR1 with several members of the CLICs is shown in Fig. 1B. It reveals that At-DHRA-1 shares essential structural features with the other CLICs. Also in the case of the plant protein the predicted secondary structure is made up from a central α-helix and two flanking β-strands including a number of charges amino acids.

Altogether a comparison between AtDHAR1 and other GSTs from Arabidopsis reveals that the former protein is more similar to most CLIC channels (25% identity to Hs_p64) than to many other Arabidopsis GSTs (11% identity to AtGSTF1). Hence, on the basis of simple sequence comparison, it may be anticipated that AtDHAR1 also functions as a channel.

**AtDHAR1 Is Not Only in the Soluble Phase**—To examine the subcellular localization of AtDHAR1 we constructed a chimeric protein (AtDHAR1-GFP) in which GFP was N-terminally linked to AtDHAR1. The chimera was expressed in HEK293 cells and the distribution of AtDHAR1-GFP examined microscopically. The images revealed GFP fluorescence throughout the cell. Against this huge background it was not possible to iden-
identify a specific association of fluorescence with the plasma membrane (data not shown). We therefore also examined protein localization by Western blotting. For this purpose cells were fractionated in soluble proteins and proteins associated with the microsomal fraction. The blot in Fig. 2 shows that a GFP antibody recognizes, in cells transfected with GFP alone, one single band and only in the supernatant; the molecular mass of the band corresponds to that expected for this soluble protein (27 kDa). In cells transfected with the chimera the antibody labels two proteins: one (27 kDa) corresponds in size to GFP alone, a second larger protein corresponds well with the expected mass (50.5 kDa) of the chimera. Labeling of a protein of the size of GFP alone is unexpected in this preparation because GFP was expressed as fusion protein with AtDHAR1. The reason for this band cannot be fully explained but it is clear that it is not specific for the AtDHAR1 protein. Also expression of a K⁺ channel in the same vector as a fusion protein with GFP in HEK293 cells resulted in the labeling of the chimera and of free GFP (15). A reasonable explanation is that the presence of free GFP is due to protein degradation; a scrutiny of the short linker between the channel protein and the downstream GFP with the analysis software PeptideCutter shows that it contains potential cutting sizes for proteases.

The large protein runs at a molecular mass of ~50 kDa and hence corresponds well with the expected mass (50.5 kDa) of the chimera. This putative AtDHAR1:GFP chimera is most pronounced in the soluble extract but is also visible in the pellet. This result can be interpreted as evidence for the dual localization of AtDHAR1: other than the cytosolic GFP the chimera is...
Expression of AtDHAR1 in HEK293 Cells Causes an Enhanced Plasma Membrane Conductance—To examine the possibility that AtDHAR1 functions as an ion channel we expressed AtDHAR1:GFP in HEK293 cells. Alternatively the protein was also expressed using a bi-cistronic vector with GFP. Channel currents across the plasma membrane of HEK293 cells were measured in medium sized cell-attached patches (~4 MΩ) on green fluorescent cells transfected with AtDHAR1:GFP. For control currents were recorded in mock-transfected HEK293 cells expressing GFP only. The cell-attached configuration was chosen to maintain the cytoplasmic integrity and maintain the redox environment of cells. The patch-pipette contained tetraethylammonium TEA-Cl as the main electrolyte to bias the redox environment of cells. The patch-pipette contained tetraethylammonium TEA-Cl as the main electrolyte to bias the redox environment of cells. The patch-pipette contained tetraethylammonium TEA-Cl as the main electrolyte to bias the redox environment of cells.

Expression of AtDHAR1 in HEK293 Cells—Heterologous expression of ion channels generally bears the hazard that an increase in conductance in transfected cells is not due to the expressed protein but the consequence of an up-regulation of endogenous channels. To test whether the elevated conductance in AtDHAR1:GFP expressing HEK293 cells is an expression artefact, we performed the same experiments in CHO cells. Fig. 4A shows representative currents recorded from CHO cells transfected with AtDHAR1:GFP. Unlike mock-transfected cells, which have a very low, nearly linear back-
ground conductance (Fig. 4B), the AtDHAR1:GFP transfected cells show a high conductance (Fig. 4, A and B). The currents recorded in CHO cells share similar rectification and kinetics with those of HEK293 cells. The fact that two different cell lines transfected with AtDHAR1 show an elevated conductance with similar features supports the view that this protein itself functions as ion channel.

Altogether these data show that the plant protein generates, very much like HsCLIC-1, a conductance in the plasma membrane of mammalian cells. But unlike HsCLIC-1, which is outward rectifying (8, 19), the plant homolog generates an inward rectifying conductance. The different kinetic properties of HsCLIC-1 and AtDHAR1 in the same expression system (e.g. HEK293 cells) strongly supports the notion that the two different conductances are indeed related to the individual proteins and not to an expression artifact.

The AtDHAR1-generated Conductance Can Also be Measured in the Whole Cell Configuration—In further experiments we also tested the possibility of recording the AtDHAR1-generated conductance in the whole cell configuration. Control HEK293 cells or AtDHAR1-expressing cells were therefore examined in a bath solution (14 mM TEA-Cl) in the whole cell configuration with 14 mM TEA-Cl in the pipette solution (see “Experimental Procedures”). The whole cell currents of the control cells revealed the low conductance and quasi-linear I/V relation observed in cell-attached recordings. An exemplary current response of a control cell to a step voltage protocol (holding voltage 0 mV, test voltages in −10 mV steps from +20 or +30 mV to −80 mV) is shown in Fig. 5A. Fig. 5E summarizes the data from 17 recordings in control HEK293 cells in the form of a mean I/V relation.

Recordings obtained from GFP-positive cells transfected with AtDHAR1:GFP exhibited again in ~65% of the cells tested currents, which were appreciably different from the control. Fig. 5B shows a representative example of current responses to a standard step voltage protocol in a transfected HEK293 cell. The current traces as well as the I/V relations from whole cell recordings are qualitatively similar with respect to rectification and kinetics to those obtained in the cell-attached configuration (Fig. 5, B and E, compare Fig. 3).

To obtain information on the selectivity of the AtDHAR1-induced conductance, the same cell of Fig. 5B was also bathed in a solution with either 140 mM TEA-Cl or 14 mM TEA acetate. The resulting currents are shown in Fig. 5, C and D. Comparison of the currents reveals that the overall conductance increases in response to a 10-fold rise in the concentration of extracellular TEA-Cl; an equimolar exchange of Cl− for acetate on the other hand results in a reduction of the overall conductance. To assess the selectivity of the conductance I was plotted as function of voltage; the plot in Fig. 5F shows that the currents reverse at about 0 mV irrespective of the bath composition. The same results were obtained from other experiments in which cells were recorded under the three conditions; the mean reversal voltages were as follows: +4 ± 4 mV (n = 8) with 14 mM TEA-Cl, +2 ± 3 mV (n = 8) for 140 mM TEA-Cl, and −1 ± 3 mV (n = 3) for 14 mM TEA acetate in the bath solution.

To obtain a better resolution of the reversal voltage of the AtDHAR1-induced conductance, we also determined this volt-

**FIGURE 5. Whole cell recordings of AtDHAR1 generated conductance in HEK293 cells.** A–D, typical current responses of HEK293 cell transfected with GFP (A) or with AtDHAR1:GFP (B–D) to standard voltage protocol (holding voltage: 0 mV, test voltages between 20 and −100 mV). Currents were recorded in whole cell configuration with 140 mM TEA-Cl (open symbols) and 14 mM TEA-Cl (closed symbols) as pipette solution and 140 mM TEA-Cl (A, B, C, D), 140 mM TEA-Cl (C, D), or 14 mM TEA acetate as bath medium (D). E, I/V relation of whole cell currents recorded as in A and B for mock (square) and AtDHAR1:GFP (open circles) transfected cells (mean ± S.D. of n = 8 cells). F, I/V relation of instantaneous current from B–D; arrows in B–D indicate the point of data collection. Symbols in F cross-reference to those in B–D. G, I of exemplary HEK293 cell-expressing AtDHAR1:GFP. I was obtained by repeatedly clamping cell for 2 s to −100 mV before stepping membrane to test voltages between −70 and +30 mV. The current difference between the beginning and the end of tail pulse is plotted as function of V for recording in 140 mM TEA-Cl (closed symbols) or 14 mM TEA acetate (open symbols). Inset, example for current response of HEK293 cell with AtDHAR1:GFP-induced conductance in 140 mM TEA-Cl to tail voltage protocol.
sional voltages at about 0 mV for all three conditions implies that the AtDHAR1-induced conductance is not selective.

Because the membrane voltage in whole cell recordings is better defined than in cell-attached measurements we used the former configuration for a more extensive characterization of the AtDHAR1-induced conductance. The current responses in Fig. 6 show that the time-dependent component of the AtDHAR1 related current develops in a voltage- and time-dependent manner. To estimate the voltage dependence of the process we determined its activation curve. For this purpose we collected the current response from the recording in Fig. 6 immediately after stepping from the test voltages to the post voltage at +10 mV. Exemplary current responses for step to post-voltage (boxed) are magnified on the right. The activation curve in B reports the initial current at the post voltage as a function of the conditioning voltage. Data are fitted by Boltzmann function (see Equation 1 under “Results”).

![Figure 6](image)

The time-dependent component of AtDHAR1-induced conductance is voltage-dependent. Activation curve for time-dependent component of AtDHAR1::GFP-generated conductance in HEK293 cells. A, currents for activation curve were recorded in whole cell configuration (140 mM TEA-Cl in pipette and 14 mM TEA-Cl in bath medium) by stepping membrane to a range of conditioning voltages between +30 and −90 mV and to a post-voltage at +10 mV. Exemplary current responses for step to post-voltage (boxed) are magnified on the right. The activation curve B reports the initial current at the post voltage as a function of the conditioning voltage. Data are fitted by Boltzmann function (see Equation 1 under “Results”).

DISCUSSION

The present experiments show that a plant protein from the GST superfamily is homologous to members of the channel-forming animal CLICs. As for the CLICs also the plant protein frequently promotes membrane conductance when expressed in heterologous systems. From the data it is not entirely clear whether the protein has by itself channel-forming activity. Because of the multiple functions found in association with animal CLICs it cannot be excluded that the increase in AtDHAR1-generated conductance is of secondary nature. In analogy to animal CLICs the present data could also be interpreted in the context of a CLIC-mediated release of intracellular Ca^{2+} (22) or in the context of AtDHAR1 binding to the cortical actin cytoskeleton (23–25). Nonetheless the present findings support the view of a direct channel function of AtDHAR1: the conductance induced by AtDHAR1 has similar kinetic and rectification in HEK293 cells as in CHO cells. A regulation of endogenous channels would have been expected to result in different currents depending on the difference of endogenous currents in the two cell types.

Worth noting is that the respective conductance has not been observed in all cells expressing AtDHAR1. The reason for this result is not clear. But a reasonable explanation for the failure of recording a AtDHAR1-induced conductance in some cells is that the respective conductance behaves to a large extent like an ohmic resistor with only a few kinetic features. Such a conductance is easily overseen on the background of the leak conductance in case that the number of the protein in the membrane is low.

Other than expected from the similarity to animal CLICs, the AtDHAR1 protein does not result in an anion-selective conductance but in a non-selective conductance. The discrepancy in selectivity might be related to structural differences in the proposed pore region between the proteins (17). However,
Arabidopsis CLIC-like Channel

recent data from planar lipid bilayer studies show that anion/cation selectivity of CLIC1 from human is only moderate (10). Hence anion selectivity may not be obligatory for CLICs.

Assuming that At-DHAR1 is indeed a channel, the present findings have implications for the understanding of CLIC evolution. Currently it is not clear whether CLICs and GSTs originate from a common ancestor. Theoretical considerations already argued for such a common ancestor on the basis of structural motives, which are shared by all members of the GST superfamily (4). The phylogenetic relation between the plant At-DHARs with the CLICs and the apparent channel activity of At-DHAR strongly suggests a common ancestor of the proteins. The plant protein with its dual function, i.e. dehydroascorbate reduction (3) and channel function, might be a primordial version of the protein. The animal proteins may have further specialized to either selectively conduct currents or act as dehydroascorbate reductase. It is notable that the animal omega class GST, GSTO 1-1, which shares structural similarity with AtDHAR1 does not exhibit channel-forming activity (22).

At present our information does not provide any direct information on the relevance of an At-DHAR1-related conductance in the physiology of plants. Some interesting information however occurs from the analysis of Arabidopsis micro-array data. These data reveal that the protein is abundantly expressed in most organs of Arabidopsis; its expression is increased by stress, in the physiology of plants. Some interesting information how ever occurs from the analysis of Arabidopsis micro-array data. These data reveal that the protein is abundantly expressed in most organs of Arabidopsis; its expression is increased by stress, but osmotic and salt stresses are more effective than UV-light or oxidative stresses. Such a behavior would be more consistent with a function of the protein as a channel rather than a GST.

Acknowledgments—We thank Michele Mazzanti (Rome, Italy) and Paul Curmi (Sydney, Australia) for many valuable suggestions on the project. We are especially grateful to Ralf Kaldenhoff (Darmstadt, Germany) for his help to continue the project.

REFERENCES
1. Barbier-Brygoo, H., Vinauger, M., Colcombet, J., Ephritikhine, G., Frachisse, J., and Maurel, C. (2000) Biochim. Biophys. Acta 1465, 199–218
2. Hechenberger, M., Schwappach, B., Fischer, W. N., Frommer, W. B., Jentsch, T. J., and Steinmayer, K. (1996) J. Biol. Chem. 271, 33632–33638
3. Dixon, D. P., Davis, B. G., and Edwards, R. (2002) J. Biol. Chem. 277, 30859–30869
4. Cromer, B. A., Morton, C. J., Board, P. G., and Parker, M. W. (2002) Eur. Biophys. J. 31, 356–364
5. Ashley, R. H. (2003) Mol. Membr. Biol. 20, 1–11
6. Berry, K. L., Bulow, H. E., Hall, D. H., and Hobert, O. (2003) Science 203, 2134–2137
7. Warton, K., Tonini, R., Fairlie, W. D., Matthews, J. M., Valenzuela, S. M., Qiu, M. R., Wu, W. M., Pankhurst, S., Bauskin, A. R., Harrop, S. J., Campbell, T. J., Curmi, P. M., Breit, S. N., and Mazzanti, M. (2002) J. Biol. Chem. 277, 26003–26011
8. Tonini, R., Ferroni, A., Valenzuela, S. M., Warton, K., Campbell, T. J., Breit, S. N., and Mazzanti, M. (2000) FASEB J. 14, 1171–1178
9. Tulk, B. M., Kapadia, S., and Edwards, J. C. (2002) Am. J. Physiol. 282, C1103–C1112
10. Singh, H., and Ashley, R. H. (2006) Biophys. J. 90, 1628–1638
11. Littler, D. R., Harrop, S. J., Fairlie, W. D., Brown, L. J., Pankhurst, G. J., Pankhurst, S., DeMaere, M. Z., Campbell, T. J., Bauskin, A. R., Mazzanti, M., Breit, S. N., and Curmi, P. M. (2004) J. Biol. Chem. 279, 9298–9305
12. Gouaux, E. (1997) Curr. Opin. Struct. Biol. 7, 566–573
13. Edwards, R., Dixon, D. P., and Walbot, V. (2000) Trends Plant Sci. 5, 193–198
14. Dixon, D. P., Lapthorn, A., and Edwards, R. (2002a) Genome Biol. 3, 30004.1–3004.10
15. Moroni, A., Viscomi, C., Sangiorgio, V., Pagliuca, C., Meckel, T., Horvath, F., Gazzarrini, S., Valbuzzi, P., Van Etten, L., DiFrancesco, D., and Thiel, G. (2002) FEBS Lett. 530, 65–69
16. Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) Pfluegers Arch. 391, 85–100
17. Harrop, S. J., DeMaere, M. Z., Fairlie, W. D., Reztsova, T., Valenzuela, S. M., Mazzanti, M., Tonini, R., Qiu, M. R., Jankova, L., Warton, K., Bauskin, A. R., Wu, W. M., Pankhurst, S., Campbell, T. J., Curmi, P. M. (2001) J. Biol. Chem. 276, 44993–50000
18. Berry, K. L., and Hobert, O. (2006) J. Mol. Biol. 359, 1316–1333
19. Valenzuela, S. M., Martin, D. K., Por, S. B., Robbins, J. M., Warton, K., Bootcov, M. R., Schofield, P. R., Campbell, T. J., and Breit, S. N. (1997) J. Biol. Chem. 272, 12575–12582
20. Sakmann, B., and Neher, E. (1985) Single-channel Recording, pp. 37–51, Plenum Press, New York
21. Puschnig, M., and Neher, E. (1988) Pfluegers Arch. 411, 204–211
22. Dulhunty, A., Gage, P., Curtis, S., Chevlianayagam, G., and Board, P. (2001) J. Biol. Chem. 276, 3319–3323
23. Berryman, M., and Bretscher, A. (2000) Mol. Biol. Cell 11, 1509–1521
24. Berryman, M., Bruno, J., Price, J., and Edwards, J. C. (2004) J. Biol. Chem. 279, 3494–34801
25. Suginta, W., Karoulias, N., Aitken, A., and Ashley, R. H. (2001) Biochem. J. 359, 55–64