Protein expression profiles in yeast cells, in response to salinity stress, were determined using the cleavable isotope-coded affinity tag (cICAT) labeling strategy. The analysis included separation of the mixed protein samples by SDS-PAGE, followed by excision of the entire gel lane, and division of the lane into 14 gel regions. Regions were subjected to in-gel digestion, biotin affinity chromatography, and analysis by nano-scale microcapillary liquid chromatography and analysis by tandem mass spectrometry. The novel 13C-labeled ICAT reagents have identical elution profiles for labeled peptide pairs and broadly spread the distribution of labeled peptides during reversed-phase chromatography. A total of 560 proteins were identified and quantified, with 51 displaying more than 2-fold expression differences. In addition to some known proteins involved in salt stress, four RNA-binding proteins were found to be up-regulated by high salinity, suggesting that selective RNA export from the nucleus is important for the salt-stress response. Some proteins involved in amino acid synthesis, which have been observed to be up-regulated by amino acid starvation, were also found to increase their abundance on salt stress. These results indicate that salt stress and amino acid starvation cause overlapping cellular responses and are likely to be physiologically linked. Molecular & Cellular Proteomics 2: 1198–1204, 2003.

Response and adaptation to high extracellular salinity is a critical event for cell survival. High salinity results in numerous cellular responses, including increased sodium pumping activity to avoid toxic concentrations of cellular sodium ion, synthesis of compatible solutes to counteract dehydration and to stabilize macromolecules, enhanced free-radical scavenging, and changes in redox control (1). Many of the responses to salinity involve changes in gene expression and are mediated by signaling pathways whose functions are to regulate transcription factors (1).

In Saccharomyces cerevisiae, the mitogen-activated protein kinase high-osmolarity glycerol (HOG)1p mediates the osmotic induction of many stress-responsive genes (2). Activation of HOG1p constitutes an early phase of the salinity stress response, which then appears to diverge into different pathways. One pathway is mediated by the transcription factors Msn2p/Msn4p binding to stress-response elements and leads to the transcription of many stress-responsive genes (3). The HOG pathway controls expression of genes encoding enzymes in glycerol production. Glycerol is the main yeast osmolyte, and its production is essential for growth in a high-osmolarity medium (2).

To obtain a more complete understanding of how yeast cells respond to salt stress at the molecular level, a systematic and quantitative analysis of mRNA as well as protein expression is necessary. Although several DNA microarray-based analyses have been carried out to study the transcriptional response of yeast cells to high salinity (4–7), a large-scale analysis of protein expression profiles has not been performed.

In theory, post-harvest labeling with stable isotopes can be used for protein quantitation in cells and tissues from any organism. The isotope-coded affinity tags (ICAT) strategy is a leading technology for relative protein quantification, relying on post-harvested, stable isotope labeling (8). The ICAT reagent consists of three components: (i) a reactive group that reacts with the free thiol functionality of cysteine residues; (ii) a linker in which stable isotopes have been incorporated; and (iii) a biotin tag that makes possible affinity isolation and detection of peptides labeled with either the heavy or light versions of the ICAT reagent. ICAT-labeled peptides elute as pairs from a reverse-phase column. By calculating the ratio of the areas under the curve for identical peptide peaks labeled with the light and heavy ICAT reagent, the relative abundance of that peptide in each sample can be determined, which is directly related to the abundance of the corresponding protein. In addition, because the ICAT reagents are specific for cysteiny1 residues, the complexity of the original peptide mixture is greatly reduced. The original ICAT reagents featured either eight deuterium or hydrogen atoms at particular positions in the linker. However, 2H- and 1H-labeled peptides show slightly different elution profiles during reversed-phase LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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The abbreviations used are: HOG, high-osmolarity glycerol; cICAT, cleavable isotope-coded affinity tags; SCX, strong cation exchange; LC-MS/MS, liquid chromatography-tandem mass spectrometry.
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chromatography, which makes it difficult to quantitatively compare a single moment in time (9). In addition, the rather hydrophobic biotin affinity tag causes peptides to elute in a relatively narrow time window.

Strong cation exchange (SCX) chromatography is often used as a primary separation tool prior to peptide sequence analysis by reversed-phase liquid chromatography-tandem mass spectrometry (LC-MS/MS). Indeed, the ICAT strategy incorporates SCX chromatography prior to affinity purification of ICAT-labeled peptides (8). However, other separation strategies are also possible. For example, Aebersold and colleagues (10) used two-dimensional gel electrophoresis to separate ICAT-labeled proteins prior to analysis of gel spots by LC-MS/MS. Finally, the use of SDS-PAGE as a primary separation strategy followed by excising the entire gel lane into a manageable number of samples has been successfully implemented as an alternative proteome analysis technology, with hundreds to thousands of proteins identified (11–15). This whole-gel lane analysis strategy is termed “gelLC-MS” to denote the use of multiple dimensions of separation (SDS-PAGE and reversed-phase chromatography).

This study presents the use of cleavable (c)ICAT reagents for the large-scale quantitative profiling of protein expression after salinity stress. In this article, we have tried to highlight novel features of the new reagents, provide their use for the study of salinity stress in yeast, and describe the use of SDS-PAGE as a substitute for SCX chromatography in a multidimensional separation strategy. A total of 560 proteins were identified and quantified, and of these 51 displayed more than 2-fold expression differences in response to salinity stress. Twenty-seven of the proteins with abundance changes had not been previously reported to be involved in salinity stress.

EXPERIMENTAL PROCEDURES

Preparation of Proteins for ICAT Labeling—Yeast cells (BJ5459, MATa ura3–52 trp1 lys2–801 leu2 1 pep4–His3 prb11.6R can1–10 mg/ml, using Protein Assay kit (Bio-Rad, Hercules, CA). An Agilent 1100 high-performance liquid chromatography system (Agilent Technologies, Palo Alto, CA) was used to deliver a gradient across a flow splitter to the column (16). Eluting peptides from the column were ionized by electrospray ionization and analyzed using an API QSTAR Pulsar mass spectrometer (Applied Biosystems). The most intense peptide ions were dynamically selected by the operating software for fragmentation. The ProID and ProCat software packages (Applied Biosystems) were used for the identification and quantitation of proteins based on the peptides analyzed during the LC-MS/MS experiment. ProID provides a peptide match by correlating acquired-tandem mass spectra with computer-predicted spectra. The best matching peptides are returned to the investigator in a database format (Microsoft Access; Microsoft, Redmond, WA). ProCat is then used to calculate an expression ratio for each ICAT-labeled peptide identified by ProID. The data were searched against the yeast protein database followed by manual interpretation of all MS/MS spectra from proteins with changes greater than 2-fold.

RESULTS AND DISCUSSION

cICAT reagents differ in two ways from the original reagents. First, the polyethylene glycol linker has been replaced by an acid-cleavable linker. Second, this new linker contains nine 13C atoms in the heavy version of the reagent instead of...
eight deuterium atoms. Two studies have already appeared using these new reagents (17, 18). In this report, we highlight the novel features of these new reagents in the context of an analysis of protein expression profiles of yeast under salt-stress conditions.

The original ICAT protocol uses ion exchange chromatography after the ICAT labeling and mixing of the two samples to remove excess derived reagents (8). We, however, opted against ion exchange chromatography and used SDS-PAGE instead, for several reasons. First, using gel electrophoresis on the ICAT samples efficiently removes excess reagents, salts, and detergents, and allows easy buffer changes for the following digestion step. Second, gel-separated proteins are highly denatured and, therefore, easily accessible for digestion. Finally, proteins are pre-fractionated according to molecular weight. This reduces complexity of the sample, thereby increasing the number of proteins that can be identified, while increasing the confidence of protein identifications because the molecular weight can be used as an additional criterion for the evaluation of protein identifications. The workflow is outlined in Fig. 1.

Our studies showed that the new generation ICAT reagents and the modified protocol provided improved performance. For instance, the differences in the elution behavior of the \(^{2}\text{H}\)-versus \(^{13}\text{C}\)-ICAT reagents were clearly observed in experiments testing the properties of both ICAT reagents. Fig. 3A shows the base peak chromatogram of a yeast sample labeled with the \(^{2}\text{H}\)-ICAT reagent. The biotin moiety has masked the differences in hydrophobicity of the peptides such that the majority of the peptides eluted in a time window of approximately one-half the size as the smaller \(^{13}\text{C}\)-ICAT-labeled peptides (Fig. 3B).

After growing in either normal or high-salt media (0.7 M NaCl), yeast cells from both samples were separately harvested and lysed (see “Experimental Procedures”). Following ICAT labeling, the samples were combined and pre-fractionated by SDS-PAGE. Subsequently, the entire SDS-PAGE lane with the ICAT sample was cut into 14 bands of approximately equal size. Each gel band was separately digested and ana-
More than 560 proteins (Table I) were identified in 14 1-h LC-MS experiments using the most stringent identification criteria (score, 200), as provided by the ProID software package, which considers the score and the distance to the next best scoring peptide in dependence of the score. Approximately one-third of these proteins (201) were identified based on two or more distinct peptides, thereby increasing the confidence in the identification and quantification. One-tenth (51) of all identified proteins showed significant differences in abundance where changes of the heavy-to-light ratio of 2-fold or greater were considered as significant. As an example, Fig. 4 shows the identification and quantification of Pgk1, which was increased in its expression ratio by 2.3-fold. Because an antibody against Pgk1 was readily available, a Western blot was performed for this protein, confirming the up-regulation (Fig. 4C).

To avoid false-positives in this category, MS/MS spectra used in the identification of all proteins with significant abundance differences were manually confirmed for mass accuracy, expected fragment ions, and expected intensities of ions. Many more proteins showed an increase rather than a decrease in their abundance (43 versus 8; see Table I and II) in response to a 45-min treatment with 0.7 M sodium chloride. Among the 43 proteins with abundance increases in response to salinity, most were involved in glycerol production (Gpd1 and Gpp1), trehalose metabolism (Pgm2, and Tps2), detoxification (Ctt1, Dak1, and Glo1), oxidoreduction (Ald3, Gre3, and Trx2), amino acid catabolism (Aro9, and Car2), and protein folding (Cpr1, and Ssa1), which have all been reported to increase in their mRNA levels (4–7).

Interestingly, we found 20 proteins with increased protein levels in response to salinity, with no reported changes in mRNA levels, from DNA microarray studies. These proteins are involved in amino acid synthesis (Arg1, Lys9, and YHR070W), RNA binding (Arc1, Nab2, Nup133, and Rrp9), carbohydrate metabolism (Adh1, Gnd1, and Pgk1), protein synthesis (Frs1), pyrimidine biosynthesis (Ura2), GTP binding (Nog2), cell cycle and DNA processing (Sim1, and Uth1), cytokinesis (Scw11), as well as hypothetical proteins (YCL033C, YCR090C, and YOR243C) (see Table I).

The eight proteins found to be decreased in abundance in response to 0.7 M-NaCl treatment were involved in phospholipid transport (Sfh5), protein synthesis (Grp1), carbohydrate metabolism (Ade17, and Ade5,7), cell wall architecture (Ecm33), and vesicular transport (Sec21), as well as hypothetical proteins (YNL132W and YOR252W) (Table II). However, the transcript levels of only one gene (YNL132W) have been reported to be down-regulated in response to salinity (4).

Inconsistencies between mRNA levels and their corresponding protein levels have been reported in a number of studies (19–22). In fact, an excellent correlation between mRNA levels and protein changes of steady state would only be expected for transcriptionally regulated genes. Several possible explanations for the discrepancies include differential selection of mRNAs for translation, sequestration of mRNA from translation, and/or regulated protein degradation.

Yeast cells respond to amino acid starvation by increasing...
transcription of amino acid biosynthetic genes (23). High salinity inhibits amino acid uptake in yeast (24), which can cause amino acid starvation. Our finding that salt stress increased the protein abundance of amino acid synthesis genes (Table I) suggests that salt stress and amino acid starvation are physiologically connected.

Interestingly, we found that four RNA-binding proteins (Arc1, Nab2, Nup133, and Rrp9) increased their abundance in

| Category                        | Gene name | Protein name                                                                 | Fold increase in protein level ± SD | mRNA increased by salt stress |
|---------------------------------|-----------|-------------------------------------------------------------------------------|-------------------------------------|-------------------------------|
| Amino acid synthesis            | ARG1      | Argininosuccinate synthetase                                                   | 2.0 (1)                             | Yes                           |
|                                 | LYS9      | Saccharopine dehydrogenase                                                     | 2.0 (1)                             |                               |
|                                 | YFL030W   | Alanine glyoxylate aminotransferase                                            | 2.6 (1)                             | Yes                           |
|                                 | YHR070W   | Strong similarity to Neurospora crassa met-10⁺ protein                         | 15.6 (1)                            |                               |
| Amino acid catabolism           | ARO9      | Aromatic amino acid aminotransferase II                                       | 2.5 (1)                             | Yes                           |
|                                 | CAR2      | Ornithine aminotransferase                                                     | 2.0 (1)                             | Yes                           |
| Carbohydrate metabolism         | ADH1      | Alcohol dehydrogenase 1                                                        | 2.2 ± 0.2 (6)                       |                               |
|                                 | GND1      | 6-phosphogluconate dehydrogenase                                               | 4.1 (1)                             |                               |
|                                 | PGK1      | Phosphoglycerate kinase                                                        | 2.3 (1)                             |                               |
|                                 | SOL4      | 6-phosphogluconolactonase                                                       | 5.0 (1)pb                           | Yes                           |
|                                 | GLD2      | Glyceraldehyde 3-phosphatedehydrdogenase 2                                    | 2.0 (1)                             | Yes                           |
|                                 | TKL2      | Transketolase 2                                                                | >40.0 (2)b                         | Yes                           |
|                                 | UGP1      | UTP-glucose-1-phosphate uridylyltransferase                                    | 2.3 (1)                             | Yes                           |
| Cell cycle and DNA processing   | SIM1      | SIM1 protein                                                                   | 2.1 (1)                             |                               |
|                                 | UTH1      | UTH1 protein                                                                  | 3.0 (1)                             |                               |
| Cytokinesis                     | SCW11     | Glucan 1,3-beta-glucosidase                                                    | 2.0 (1)                             |                               |
| Detoxification                  | CTT1      | Cytoplasmic catalase T                                                          | 18.1 ± 4.6 (3)                      | Yes                           |
|                                 | DAK1      | Dihydroxyacetone kinase I                                                       | 2.3 (1)                             | Yes                           |
|                                 | GLO1      | Glyoxalase I                                                                   | 3.3 ± 0.7 (2)                       | Yes                           |
| Extracellular secretion         | NCE3      | Non-classical export protein 3                                                 | 3.6 ± 0.6 (3)                       | Yes                           |
|                                 | SSR1      | Secretory stress-response protein 1                                            | 2.7 ± 0.02 (2)                      |                               |
| Glycerol production             | GPD1      | Glycerol-3-phosphate dehydrogenase                                             | 7.1 (1)                             | Yes                           |
|                                 | GPP1      | Glycerol-3-phosphatase                                                         | 2.1 (1)                             | Yes                           |
| GTP-binding protein             | NOG2      | Nuclear/nucleolar GTP-binding protein                                           | 2.0 (1)                             |                               |
| Hypothetical proteins           | YCL033C   |                                                                                | >2.00 (1)                           |                               |
|                                 | YCR090C   |                                                                                | >2.00 (1)                           |                               |
|                                 | YMR090W   |                                                                                | 6.0 ± 2.0 (2)                       | Yes                           |
|                                 | YOR243C   |                                                                                | >3.00 (1)                           |                               |
| Nitrogen utilization            | UGA1      | 4-aminobutyrate aminotransferase                                               | 2.3 (1)                             | Yes                           |
| Oxidoreduction                  | ALD3      | Aldehyde dehydrogenase[NAD(P)]⁻¹                                                | >10.00 (2)                          | Yes                           |
|                                 | GRE3      | Aldose reductase                                                               | 3.0 ± 0.6 (2)                       | Yes                           |
|                                 | TRX2      | Thioredoxin II                                                                | 2.2 (1)                             | Yes                           |
| Protein folding                 | CPR1      | Peptidyl-proly cis-trans isomerase                                             | 2.1 ± 0.1 (2)                       | Yes                           |
|                                 | SSA1      | Heat-shock protein YG100                                                        | 2.0 ± 0.02 (2)                      | Yes                           |
| Protein synthesis               | FRS1      | Phenylalanyl-tRNA synthetase, alpha subunit                                    | 2.0 (1)                             |                               |
|                                 | MES1      | Methionyl-tRNA synthetase                                                       | 2.0 (1)                             |                               |
| Pyrimidine biosynthesis         | URA2      | Aspartate carbamoyltransferase                                                  | 2.6 (1)                             |                               |
| RNA-binding protein             | ARC1      | ARC1 protein                                                                   | 2.0 (1)                             |                               |
|                                 | NAB2      | Nuclear polyadenylated RNA-binding protein                                      | 2.4 ± 0.2 (2)                       |                               |
|                                 | NUP133    | Nucleoporin NUP133                                                             | >2.00 (1)                           |                               |
|                                 | RRP9      | RRP9 protein                                                                   | 2.0 (1)                             |                               |
| Trehalose metabolism            | PGM2      | Phosphoglucomutase                                                             | 5.5 (1)                             | Yes                           |
|                                 | TPS2      | Trehalose 6-phosphate phosphatase                                              | 3.4 (1)                             | Yes                           |

*a* Number of peptides identified is shown in parentheses.

*b* Precise quantification limited by signal-to-noise ratio.
response to salt stress (Table I). It is known that yeast cells respond to heat stress by selectively exporting mRNAs encoding heat-shock proteins (25), and this export requires certain RNA-binding proteins (26). Whether the RNA-binding proteins we found play a role in exporting and/or stabilizing RNAs important for salt stress will be the subject of future studies.

It is critical to detect regulated alterations in protein levels occurring during cellular processes and in response to environmental stimuli such as salt stress. Accurate determination of differentially expressed protein levels in a biological process can provide a more complete understanding of the molecular mechanism. The cICAT strategy provides a large-scale and generic strategy for protein quantification in a variety of types of samples, including primary tissues from all organisms.

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† The on-line version of this article (available at http://www.mcponline.org/) contains Table SI.

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**Fig. 4.** cICAT analysis of yeast phosphoglycerate kinase after salt stress. 
A, Mass spectrum of a cICAT peptide ion pair showing a differential expression ratio. B, Product ion spectrum for precursor ion 714.7 m/z. This peptide ion was identified as having the sequence, DVTFLNDC*VGPEVEAAVK, from the protein phosphoglycerate kinase. C, Western blot analysis for phosphoglycerate kinase before and after salt stress.

**TABLE II**

| Category                  | Gene name    | Protein name                                                                 | Fold decrease in protein levels ± SD | mRNA decreased by salt stress |
|---------------------------|--------------|------------------------------------------------------------------------------|--------------------------------------|-------------------------------|
| Cell wall architecture    | ECM33        | Extracellular mutant 33 protein                                              | 0.32 ± 0.04 (2)*                      | Yes                           |
| Hupothetical proteins     | YNL132W      |                                                                              | 0.53 (1)                             |                               |
|                           | YOR252W      |                                                                              | 0.50 (1)                             |                               |
| Phospholipid transport    | SFH5         | Putative phosphatidylinositol transfer protein                              | 0.43 (1)                             |                               |
| Protein synthesis         | GRS1         | Glycine-tRNA ligase                                                          | 0.52 (1)                             |                               |
| Purine metabolism         | ADE17        | 5-aminimidazole-4-carboxamide ribonucleotide transformylase                  | 0.50 ± 0.1 (3)                       |                               |
|                           | ADE5,7       | Glyceramide ribonucleotide synthetase and aminimidazoleribonucleotide synthetase | 0.53 ± 0.06 (2)                      |                               |
| Vesicular transport       | SEC21        | PEST sequence-containing protein                                            | 0.29 (1)                             |                               |

* Number of peptides identified is shown in parentheses.
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